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FINAL REPORT

STUDY TITLE

ACETAMIDE: 7-DAY REPEAT DOSE ORAL TOXICITY STUDY IN WISTAR RATS WITH TOXICOGENOMICS

STUDY No.: G16084

STUDY DIRECTOR: D.P. SANTHOSH KUMAR, M.Sc., Ph.D., ERT.

STUDY COMPLETED ON: 24 December 2019

SPONSOR

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(TOXICOGENOMICS)

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PLOT NO. 2, 3, 4 & 5, PHASE IV
BIOCON PARK
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STATEMENT OF CONFIDENTIALITY

This report contains confidential and proprietary information of Michigan State University, College of Engineering, Michigan State University, East Lansing, Michigan 48824, which will not be disclosed to anyone, except the employees of this company or to persons authorised by law or judicial judgement, without an expressed or a written approval of Michigan State University, College of Engineering, Michigan State University, East Lansing, Michigan 48824.

STATEMENT OF GLP COMPLIANCE

The study was performed in compliance with the OECD Principles of Good Laboratory Practice [C (97) 186/Final] and US FDA Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58). This study was conducted in accordance with the Standard Operating Procedures of Eurofins Advinus Limited and the mutually agreed Study Plan.

The Study Plan and Amendments were approved by Monitoring Scientist and signed by Study Director on the following dates:

Study Plan and Amendments	Approved by Monitoring Scientist on	Signed by Study Director on
Study plan	13 January 2018	19 January 2018
Amendment No. 1	27 January 2018	29 January 2018
Amendment No. 2	26 March 2018	29 March 2018
Amendment No. 3	02 May 2018	03 May 2018
Amendment No. 4	04 September 2018	18 September 2018
Amendment No. 5	17 November 2019	22 November 2019

The acetamide concentration in diet, water and urine samples and analyses for the presence of N-hydroxyacetamide (Acetohydroxamic Acid, a putative metabolite of acetamide) in plasma were performed at Department of DMPK & CP, Eurofins Advinus Limited in a Non-GLP environment.

Toxicogenomics analysis was performed at Syngene International Ltd., SEZ-16 UNIT-IX, Plot No. 2, 3, 4 & 5, Phase IV, Biocon Park, Jigani Link Road, Bengaluru-560 099, India. This test site is non-GLP laboratory.

DECLARATION

This is to declare that the work was performed under the supervision of study director and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results except for toxicogenomics phase which is the responsibility of Principal Investigator at test site.

D.P. Santhosh Kumar, M.Sc., Ph.D., ERT
Study Director

24 December 2019
Date

QUALITY ASSURANCE STATEMENT

Study No.: G16084, entitled "Acetamide: 7-day repeat dose oral toxicity study in Wistar rats with toxicogenomics" has been inspected in accordance with the OECD Principles of Good Laboratory Practice [C (97) 186/Final] and US FDA Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58).

This study was inspected and findings reported to the Management and Study Director on the dates shown below:

INSPECTION DATE	PHASE	REPORTING DATE
	INITIATION PHASE (Study plan and amendment review dates)	
18 January 2018	Review of Study plan	18 January 2018
29 January 2018	Review of Amendment No. 1 to Study plan	29 January 2018
29 March 2018	Review of Amendment No. 2 to Study plan	29 March 2018
25 April 2018	Review of Amendment No. 3 to Study plan	25 April 2018
12 July 2018	Review of Amendment No. 4 to Study plan	12 July 2018
13 November 2019	Review of Amendment No. 5 to Study plan	13November2019
	IN-LIFE PHASE	
29 January 2018	Body weight measurement and grouping	05 February 2018
30 January 2018	Dose formulation administration by oral gavage - day 1 Dose formulation analysis: Sample preparation and processing	05 February 2018
06 February 2018	Necropsy	06 February 2018
09 March 2018	Bio analysis of study samples	12 March 2018

QUALITY ASSURANCE STATEMENT contd.

INSPECTION DATE	PHASE	REPORTING DATE
	REPORTING PHASE	
11 June 2018 to 14 June 2018	Draft report review (Pathology)	14 June 2018
20 June 2018 to 21 June 2018	Draft report review (Toxicogenomic phase report)	21 June 2018
20 June 2018 to 27 June 2018	Draft report review (Bioanalytical)	27 June 2018
20 June 2018 to 21 June 2018	Draft report review (Dose formulation analysis)	21 June 2018
22 June 2018 to 25 June 2018	Draft report review (Toxicology)	25 June 2018
13 December 2019	Final report review	13 December 2019

The Quality Assurance statement for the Toxicogenomic Phase is furnished in Page No. 518/649 of this final report.

Inspections were performed according to the Standard Operating Procedures of the test facility's Quality Assurance Unit. The report was inspected against the approved study plan and pertinent raw data and accurately reflects the raw data.

23rd December 2019
Date:

Muktha Bhagavan
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Deputy Head
Quality Assurance Unit-GLP
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LIST OF ABBREVIATIONS AND SYMBOLS USED IN THIS REPORT

Alb	Albumin
A/G	Albumin/Globulin
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
ANOV	Analysis of variance
APTT	Activated Partial Thromboplastin Time
AST	Aspartate aminotransferase
Baso	Basophil
BLQ	Below Limit of Quantification
BUN	Blood Urea Nitrogen
CC	Calibration Curve
Ca	Calcium
Cl	Chloride
Contd.	Continued
Creat	Creatinine
cm	Centimetre
C_{max}	Peak plasma concentration
CoA	Certificate of Analysis
CV	Coefficient of Variation
D.Bil	Direct Bilirubin
DLC	Differential Leucocyte Count
dL	Decilitre
DMPK	Drug Metabolism and Pharmacokinetics
DQC	Dilution Quality Control
Eosi	Eosinophil
fL	Femtolitre
g	Gram
G	Giga (10^9)
GGT	Gamma Glutamyl Transpeptidase
GLP	Good Laboratory Practice
Glu	Glucose
Glob	Globulin
h	Hour
Hb	Haemoglobin
Hct	Haematocrit
HDW	Haemoglobin Distribution Width
HQC	High Quality Control
Hyper	Hyperchromic cells
Hypo	Hypochromic cells

LIST OF ABBREVIATIONS AND SYMBOLS USED IN THIS REPORT Contd.

Ind.Bil	Indirect Bilirubin
i.d.	Internal diameter
IS	Internal Standard
K	Potassium
K_2EDTA	Ethylene diamine tetra acetic acid di potassium salt
kg	Kilogram
L	Litre
LDH	Lactate dehydrogenase
LC/MS/MS	Liquid Chromatography and Tandem Mass Spectrometry
LLOQ	Lower Limit of Quantification
LQC	Low Quality Control
Lymp	Lymphocyte
m/M	Male
Macro	Macrocytes
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
mEq	Milliequivalent
Micro	Microcytes
mg	Milligram
min	Minute
mL	Milliliter
mmol	Millimole
mm	Millimetre
Mono	Monocyte
MPV	Mean Platelet Volume
MQC	Medium Quality Control
NA	Not Applicable
Na	Sodium
NAD	No Abnormality Detected
Neut	Neutrophil
ng	Nanogram
nm	Nanometer
No./N	Number
NC	Number of cages
pg	Picogram
Pi	Inorganic Phosphorous
Plat	Platelets
PT	Prothrombin Time
QC	Quality Control
RDW	Red Cell Distribution Width

LIST OF ABBREVIATIONS AND SYMBOLS USED IN THIS REPORT Contd.

RBC	Red Blood Corpuscles
RBC Frag	RBC Fragments
RBC Gh	RBC Ghosts
rpm	Revolutions per Minute
Retic	Reticulocyte Counts
SD/S.D.	Standard Deviation
T	Tera (10^{12})
T.Bil	Total Bilirubin
T.Chol	Total Cholesterol
T_{max}	Time to reach the Peak Plasma Concentration
TK	Toxicokinetics
T.Pro	Total plasma Protein
Trig	Triglycerides
$T_{1/2}$	Elimination Half Life
U	Units
ULOQ	Upper Limit of Quantification
UV	Ultra Violet
Ver	Version
Vesi/ves	Vesicles
v/v	Volume/Volume
WBC	White Blood Corpuscles
WSTD	Working Standard
µmole	Micromole
°C	Degree Celsius
µ	Microns
µg/mL	Microgram per milliliter
ng/mL	Nanogram per milliliter
%	per cent

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1 STUDY DETAILS

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Study Schedule

Study Initiation Date	:	19 January 2018
Experiment Start Date	:	19 January 2018
Acclimatization	:	19 January 2018 to 29 January 2018
Treatment	:	30 January 2018 to 05 February 2018
Sacrifice	:	06 February 2018
Experiment Completion	:	22 May 2018
Draft Report Date	:	11 June 2018
Study Completion Date	:	24 December 2019

2 LIST OF SCIENTISTS / PROFESSIONALS

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3 SUMMARY

The objective of this study was to assess the toxicological and toxicogenomic profile of the test item acetamide when administered by oral gavage to Wistar rats for a period of 7 consecutive days. The study was also designed, to provide information on major toxic effects, target organs and an estimate of the No Observed Effect Level (NOEL) / No Observed Adverse Effect Level (NOAEL).

The test item, acetamide was mixed in vehicle (Milli Q water). The test item solutions were administered to Wistar rats daily at the doses of 30 (low - G2A and G2B), 100 (mid - G3A and G3B), 300 (Intermediate - G4A and G4B) and 1000 (high - G5A and G5B) mg/kg/day for 7 consecutive days. The rats in vehicle control groups (G1A and G1B) received the vehicle alone. The dose volume administered was at an equivolume of 10 mL/kg/day for all groups.

Additionally, G1B to G5B group rats received 5-bromo-2'-deoxyuridine (BrdU) in drinking water BrdU: 0.8 mg/ml; 10 mg/ml glucose) for 7 consecutive days. All groups consisted of 6 male rats/group.

The identity of the test item was provided by the Tokyo Chemical Industry by a certificate of analysis. The results of the stability of the test item in the vehicle at the concentrations of 1.00 and 150.0 mg/mL were found stable in dose formulations for 24 hours at room temperature (25 ± 5 ºC). The overall mean concentration of acetamide in dose formulations samples prepared on Day 1 were found within ± 15% of the nominal concentration during the conduct of the study.

The parameters evaluated included clinical signs, mortalities, body weight, food consumption, water consumption, haematology, coagulation, clinical chemistry, urinalysis, gross pathology and organ weights. The histopathological evaluation was carried out for liver from all rats and femur bone with distal joint, sternum and spleen for control and high dose rats. The blood samples and liver were collected on Day 8 to determine acetamide concentration. In addition, a section of liver was collected and shipped to Syngene for toxicogenomics analysis.

The salient findings of the study are as below:

- **Clinical Signs and Mortalities:** There were no clinical signs or mortalities in the vehicle control group and in all the treated groups.

- **Ophthalmological Examination:** No ocular abnormalities were observed.

- **Body Weights and Body Weight Gains:** There were no test item-related changes observed in body weights and body weight gains at 30, 100, 300 and 1000 mg/kg/day dose groups.

- **Food Consumption:** There were no test item-related variations in the food consumption in all tested dose groups.

- **Water Consumption:** There were no test item-related variations in the water consumption in all tested dose groups.

- **Plasma and Liver Acetamide Concentration:** The plasma and liver concentrations after repeated 7 day oral gavage administration of acetamide alone and acetamide in presence of BrdU are presented below:

Dose (mg/kg/day)	Day	Acetamide groups		Acetamide + BrdU groups	
		Mean Plasma Concentration (µg/mL)	Mean Liver Concentration (µg/g)	Mean Plasma Concentration (µg/mL)	Mean Liver Concentration (µg/g)
0	8	0.350±0.116	2.85±0.233	0.310±0.026	3.51±2.030
30	8	25.8±3.53	19.5±1.62	29.7±3.96	26.7±2.94
100	8	80.5±14.3	57.1±14.7	112±11.8	78.6±10.3
300	8	222±22.9	126±25.3	314±51.1	179±35.9
1000	8	513±48.4	297±64.5	849±85.7	506±42.3

The results suggested that there was dose-related increase in plasma and liver concentrations.

- **N-hydroxyacetamide (Acetohydroxamic Acid) in Plasma:** N-hydroxyacetamide (putative metabolite of Acetamide) levels were estimated from the plasma samples collected on day 8 using fit-for-purpose colorimetric method and the observed concentrations are as below:

Dose (mg/kg/day)	Individual N-hydroxyacetamide concentration (µg/mL) in plasma on Day 8						Mean concentration ± SD (µg/mL)	n
0	14.3	5.32	6.11	BLQ	BLQ	BLQ	8.59 ± 5.0	3
30	BLQ	BLQ	BLQ	BLQ	6.61	BLQ	6.61[a]	1
100	BLQ	6.71	9.68	5.02	BLQ	BLQ	7.14 ± 2.4	3
300	BLQ	9.19	BLQ	BLQ	9.48	BLQ	9.34[b]	2
1000	21.5	BLQ	39	7.3	BLQ	35.5	25.8 ± 14	4
[a] n=1 animal data; [b] n=2 animal data, values were below quantification limit for other animals in particular group								

No consistent dose-dependent increase in N-hydroxyacetamide was observed. The plasma N-hydroxyacetamide concentrations were comparable in 0, 30, 100, 300 mg/kg/day group except 1000 mg/kg group. At the highest dose (1000 mg/kg/day), 4/6 animals showed higher N-hydroxyacetamide levels whereas the other 2 were not distinguishable from vehicle controls or below limit of quantification. The results suggest that no significant presence of N-hydroxyacetamide in plasma following Acetamide administration except 1000 mg/kg/day dose group.

Dose (mg/kg/day)	Acetohydroxamic acid conc. (µg/mL) in plasma
0	8.59 ± 5.0
30	6.61[a]
100	7.14 ± 2.4
300	9.34[b]
1000	25.8 ± 14
[a]n=1 animal data; [b]n=2 animal data, values were below quantification limit for other animals in particular group	

- **Acetamide Concentration in Urine:** Acetamide levels were estimated from the pooled urine collected on Day 7 and the observed concentrations are as below:

Dose (mg/kg/day)	Day Nominal	Mean Urine Concentration in Acetamide group (µg/mL)
0	7	4.71 ± 1.14
30	7	833 ± 358
100	7	2482 ± 817
300	7	5647 ± 1313
1000	7	15150 ± 2616

- **Acetamide Concentration in Feed:** A sample of feed was collected during the study which was monitored for Acetamide levels using fit-for-purpose GC-MS method and concentration was found to be 1.15 µg/g of feed.

- **Acetamide Concentration in Water:** A sample of water was collected from the Aquaguard during the study which was monitored for Acetamide levels using fit-for-purpose GC-MS method and concentration was below the limit of quantification (LLOQ 0.1 µg/mL) suggesting absence of significant level of Acetamide.

- **Haematology:** Increase in RBC counts, haemoglobin concentration, haematocrit and decrease reticulocytes in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered a test item-related non-adverse change as it was not associated with histopathological changes in haemopoietic organs (bone marrow and spleen).

  Clinical Chemistry: Increase in ALT, AST and ALP activity in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related adverse change as it was associated with single cell necrosis of hepatocytes in liver. Decreased triglycerides concentration in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change and this change was associated with hepatocytes vacuolation in liver. Increased total bilirubin concentration in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change.

- **Urinalysis:** There were no test item-related variations in the urinalysis parameters in all tested dose groups.

- **Organ Weights:** Decrease absolute and relative weight of liver in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered as test item-related change.

- **Gross Pathology:** There were no test item-related gross changes in all treated groups.

- **Histopathology:** Increased mitotic activity, single cell necrosis and vacuolation of hepatocytes in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change. Increased mitotic activity at 1000 mg/kg was associated with high Ki67 proliferation index.

- **Mitotic Index:** High hepatocytes mitotic index in 1000 mg/kg/day acetamide was considered test item-related change. The mitotic index at <1000 mg/kg/day doses was comparable to vehicle control group.

- **Immunohistochemistry:** Immuno-histochemical staining was performed for Ki 67 proliferation antigen and BrdU. The optimum immunostaining of Ki 67 proliferation antigen was observed in positive control tissue (duodenum) and Ki 67 proliferation index was calculated for all the groups. Suboptimal immunostaining of BrdU was noted in (duodenum) positive control. BrdU index of hepatocytes was not calculated by counting BrdU immuno-stained hepatocytes as optimal staining was not observed in positive control tissue (crypt epithelium of duodenum) and BrdU index thus obtained was not considered to be an accurate representations of the cell proliferation activity.

  Ki67 proliferation index: High Ki 67 proliferation index in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change.

  Toxicogenomics: The response by fold change alone was evident at all treatment concentrations. However, none of these changes met the standard statistical False Discovery Rate (FDR) ≤ 5% for the two lowest concentrations of 30 and 100 mg/kg/day, and only a single gene was statistically significant for the 300 mg/kg/day treated group. At 1000 mg/kg/day, there were numerous genes that were either up- or down-regulated by at least 1.5 fold relative to vehicle, and were statistically significantly altered (FDR<0.05). These genes, significant by both FDR<0.05 & |FC|>1.5 fold) that were used to investigate ontology enrichment with the Reactome Ontology. The metabolic processes appear enriched for the down-regulated genes and cell cycle, signaling and immune system responses are enriched with up-regulated genes. The PPARa- and RXR-associated genes overrepresented among the down-regulated gene lists, a finding consistent with the observed Reactome

enrichment in fatty acid metabolic processes. The up-regulated genes had enrichment for various FOX controlled pathways and regulated elements. FOX proteins are key transcription factors in cell growth and proliferation and are among some of the most conserved transcription factors in mammals. A Reactome pathway analysis of BenchMark Dose for the 11,373 genes for which all 25 animals in the study had a feature count of at least one was performed. Hill models where the shape (or slope) parameter, k, was less than 1/3rd of the maximum concentration were rejected from consideration as these tend to have extreme inflections in the shape of the curve leading to biologically non-sensical (e.g., vanishingly small) BMD estimates. Genes where a best fitting model was found that had both a gene-based BMD estimate less than the maximum treatment concentration of 1000 mg/kg/day (to avoid extrapolation of estimates) and which also had a goodness of fit p-value >0.1 were then used in an enrichment analysis using Reactome. Pathway BMDs were estimated for pathways with 10 or more such genes as the median of the BMD of the genes which matched ontology elements in that pathway. Collectively, pathway median BMDs were estimated between 400 and 1000 mg/kg/day.

In conclusion, the 7-day repeated dose oral gavage administration of acetamide at the tested doses of 30, 100 and 300 mg/kg/day to Wistar rats did not cause any toxic effects. Hence, the No Observed Effect Level (NOEL) is defined at 300 mg/kg body weight/day for test item acetamide under the conditions of this study.

4 OBJECTIVES

The objectives of the study were:

- To assess the toxicological and toxicogenomic profile of the test item acetamide when administered by oral gavage to Wistar rats for a period of 7 consecutive days.
- To provide information on major toxic effects, target organs and an estimate of the No Observed Effect Level (NOEL) / No Observed Adverse Effect Level (NOAEL).

5 ANIMAL WELFARE

Use of live animals is inevitable to accomplish the purpose of the study. This study was designed to use minimum number of animals to meet the scientific objectives, the goals of the sponsor and considering applicable regulatory requirements.

This study was performed in an AAALAC approved laboratory. All procedures were in compliance with the guidelines provided by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India. This study plan has been approved by the Institutional Ethics Committee (IAEC) of Eurofins Advinus Limited (Approval No. 024 September 2017).

6 STUDY GUIDELINES

This study was conducted in accordance with the following guideline:

- OECD Guideline No. 407 for Testing of Chemicals, "Repeated Dose 28-Day Oral Toxicity Study in Rodents'' adopted by the Council on 3rd October, 2008 with exception to the duration of treatment period. In this study, only males were used and age of rats was as per the study plan.

7 JUSTIFICATION FOR THE SELECTION OF TEST SITE

The test site has already developed the analytical methods for toxicogenomics analysis. The test site QA shall perform the QA functions for these phase.

8 MATERIALS AND METHODS

8.1 Materials

8.1.1 Test Item Information

(Reference: TIDS and / or COA)

Test Item	:	Acetamide
Lot No.	:	XHH4A
Batch Manufactured by (Name and Address)	:	Tokyo Chemical Industry Co., Ltd. 4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan
Batch Supplied by (Name and Address)	:	Tokyo Chemical Industry Co.,Ltd. 4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan
Manufactured Date	:	7 December 2017
Retest Date	:	7 December 2018
Purity as per Certificate of Analysis	:	100.0 %
Physical Appearance	:	Crystal- Powder
Recommended Storage Condition	:	Ambient (+15 to +25ºC)

Notes

1. Date of receipt of test item at test facility: 19 December 2017
2. Test item code by test facility: M033-01

8.1.2 Identity of the Test Item

Refer Appendix 11

The identity of the test item has been provided by the Tokyo Chemical Industry by a Certificate of Analysis. The test item was not authenticated at the test facility. The Certificate of Analysis is enclosed in Appendix 11.

8.2 Test System

Species	:	Rats (males)
Strain	:	HsdHan™: WIST Rats
Source	:	Envigo Laboratories Envigo RMS (Israel) Ltd, P.O. Box 12085, Jerusalem, Israel
Justification for the selection of species	:	Rat is one of the standard rodent species recommended by various regulatory authorities for toxicity assessment.
Justification for route of administration	:	The oral route was chosen because it is a potential route of human exposure.
No. of groups	:	10 Toxicity groups a. Acetamide groups: G1A - Vehicle control G2A - Low dose G3A - Mid dose G4A - Intermediate G5A - High dose b. Acetamide + BrdU groups: G1B - Vehicle control G2B - Low dose G3B - Mid dose G4B - Intermediate G5B - High dose
No. of rats/group	:	6 males Total number of rats: 60 males
Age at the start of treatment	:	9 - 10 weeks

Body weight range	:	At the commencement of treatment, the weight variation of rats used did not exceed ± 20 % of the mean body weight in each sex and group.
Identification	:	Temporary Identification: The temporary identification of animals during acclimatization period was done with crystal violet body marking and cage cards Permanent Identification: After grouping all animals were identified using rat accession number, cage cards, body marking with turmeric solution
Acclimatization	:	After detailed clinical examination, for good health and the suitability for the study, the rats were acclimatized for 11 days before start of the treatment.

8.3 Methods

8.3.1 Husbandry Conditions

Rats were housed in an environment controlled room at 20-24°C and relative humidity of 58 to 67 percent. The photoperiod was 12 hours light and 12 hours dark. Adequate fresh air supply of 12 - 15 air changes/hour was maintained in the experimental room.

The maximum and minimum temperature and relative humidity in the experimental rooms was recorded once daily. The relative humidity in the experimental room was calculated daily from dry and wet bulb temperature recordings.

8.3.2 Housing

Two rats per sex per cage was housed in sterilized standard polysulfone cages with stainless steel top grill having facilities for pelletted food and drinking water in polycarbonate bottle with stainless steel sipper tube. Cage size was approximately 425 mm (length) x 266 mm (breadth) x 185 mm (height). Additionally, polycarbonate rat huts were placed inside the cage as environmental enrichment objects. These enrichment objects were replaced at least once a week. During the experimental period, animals were housed in experimental room of barrier area.

8.3.3 Bedding

Steam sterilized corn cob were used as bedding and changed along with the cage at least once a week.

Contaminant analysis report for bedding material (corn cob) is presented as Annexure 1.

8.3.4 Diet

Teklad Certified (2014C) Global 14% Protein Rodent Maintenance Diet – pellet (Certified) manufactured by Envigo, PO Box 44220, Madison, WI 53744-4220, was provided *ad libitum* to rats.

Feed analysis and contaminant analysis report of Teklad Certified (2014C) Global 14 % Protein Rodent Maintenance Diet – pellet are given in Annexure 2.

8.3.5 Water

Deep bore-well water passed through activated charcoal filter and exposed to UV rays in Aquaguard on-line water filter-cum-purifier manufactured by Eureka Forbes Ltd., Mumbai 400 001, India, was provided *ad libitum* to rats in polycarbonate bottles with stainless steel sipper tubes.

Analysis and contaminant analysis reports of water sample are given in Annexure 3 and 4, respectively.

Based on the latest analytical certificate/s available, there are no known contaminants in the feed, water and bedding that are expected to interfere with the results of this study.

About 200 grams of the diet and 200 mL of drinking water was stored under -50°C. This analyses was done to confirm that rats could not have been exposed to acetamide concentration either through drinking water or feed diet used for this study. The analysis was done on a Non-GLP basis using a fit-for-purpose GC-MS method and results were included as Appendix 8 to the report.

8.3.6 Dose Levels and Dose Justification

The dose levels of 30, 100, 300 and 1000 mg/kg/day were selected based on the available literature and in consultation with the Sponsor.

The selection of low dose (30 mg/kg/day) was based upon acetamide levels anticipated in foods when certain ammoniation feed-production technologies are widely adopted. Selection of high dose (1000 mg/kg/day) was based upon acetamide levels known to cause rodent cancers in historical studies. The mid (100 mg/kg/day) and intermediate (300 mg/kg/day) doses were selected to bridge the low and high doses and to provide a dose-response assessment for acetamide toxicity.

8.3.7 Grouping

Rats were randomly distributed to different groups by the body weight stratification method using Provantis™ software (10.1.0.1, Instem LSS, Staffordshire ST15OSD, United Kingdom). Rats with extreme body weights were excluded from the study. Grouping was done during one day prior to initiation of the treatment.

8.3.8 Experimental Design, Group Allocation and Number of Rats

The selected rats were assigned to vehicle control and different treatment groups as shown below:

Toxicity Groups

a. Acetamide groups:

Group No.	Group	Colour of cage card	Dose (mg/kg /day)	Concent-ration (mg/mL)	Dose volume (mL/kg)	No. of Rats	Sex	Rat Numbers	
								From	To
G1A	Vehicle control	White	0	0	10	6	Male	Ru5681	Ru5686
G2A	Low dose	Yellow	30	3	10	6	Male	Ru5687	Ru5692
G3A	Mid dose	Green	100	10	10	6	Male	Ru5693	Ru5698
G4A	Intermediate dose	Blue	300	30	10	6	Male	Ru5699	Ru5704
G5A	High dose	Pink	1000	100	10	6	Male	Ru5705	Ru5710

b. Acetamide + BrdU groups:

Group No.	Group	Colour of cage card	Dose (mg/kg/ day)	Concent-ration (mg/mL)	Dose volume (mL/kg)	No. of Rats	Sex	Rat Numbers	
								From	To
G1B	Vehicle control	White	0	0	10	6	Male	Ru5711	Ru5716
G2B	Low dose	Yellow	30	3	10	6	Male	Ru5717	Ru5722
G3B	Mid dose	Green	100	10	10	6	Male	Ru5723	Ru5728
G4B	Intermediate dose	Blue	300	30	10	6	Male	Ru5729	Ru5734
G5B	High dose	Pink	1000	100	10	6	Male	Ru5735	Ru5740

8.3.9 Justification for Selection of vehicle

As per the information provided by the Sponsor, the test item acetamide was soluble and formed solution in Milli Q water. Hence, Milli Q water was selected as vehicle for this study.

8.3.10 Stability of the Test Item in the vehicle

The stability testing of the test item in the vehicle was determined separately under Eurofins Advinus Study No: G16081 at the concentrations of 1.00 and 150.0 mg/mL. The test item was found stable in dose formulations for 24 hours at room temperature (25 ± 5 ºC).

8.3.11 Dose Formulation Preparation

The dose formulations were prepared daily prior to administration of the test item. The prepared dose formulations were used within the prescribed stability period.

The required quantities of test item for each dose was weighed and taken in a beaker. Then small volume of vehicle was added and mixed to form a solution, the same was transferred to the measuring cylinder. The beaker was rinsed and the contents were transferred to the measuring cylinder. Required volume was made up with vehicle to get the desired concentration.

Day 1 formulation preparation was as in the following table:

Groups	G1A/G1B	G2A/G2B	G3A/G3B	G4A/G4B	G5A/G5B
Dose (mg/kg/day)	0	30	100	300	1000
Dose volume (mL/kg)	10	10	10	10	10
Test item weight (mg)	-	150	500	1500	5000
Final volume made up with vehicle (mL)	50	50	50	50	50
Concentration (mg/mL)	0	3	10	30	100

8.3.12 Dose Formulation Analysis

Dose formulations of acetamide were analyzed for active ingredient concentration (a.i.) from the first preparation.

The prepared formulations were sampled in duplicate sets wherein one set was used for analysis and another as back up set which was stored at ambient. The composite sample was drawn from the prepared formulations at all the groups and was sent to Department of DMPK & CP, for analysis. The analyses was done as per the analytical method validated under Advinus Study No.: G16081. The dose formulation analysis was performed within the stability period.

Dose formulations were considered acceptable as the overall mean result was within ± 15% of the nominal concentration and the relative standard deviation (%RSD, calculated using all the replicate values) of assay of all layers was less than or equal to 15%. Hence, back up samples were disposed.

Dose formulation analysis report is included in the Appendix 7 of this report.

8.3.13 BrdU Preparation in Drinking Water

Materials: BrdU (5-Bromo-2'-deoxyuridine) from Sigma, Cat# B5002-5G and Glucose from Sigma, Cat# G8270-1KG

Procedure: BrdU (0.8 mg/ml + 1% glucose) in drinking water was prepared freshly in a fume hood and protected from light. The bottles containing drinking water was covered with aluminum foil. Rats assigned to BrdU groups (G1B to G5B) were given BrdU containing drinking water continuously from Day 1 of treatment until sacrifice in order to stain the accumulated nuclear antigen (BrdU) over a period of 7 days.

The BrdU-containing drinking water was changed twice a week. Excess remaining water was diluted with 10% bleach, disposed into sink, flushed and the bottle was rinsed thoroughly.

The details of 5-Bromo-2'-deoxyuridine and Glucose is provided below:

Product Name	:	5-Bromo-2'-deoxyuridine
Lot No.	:	HMBG2687V
Date of receipt	:	29 December 2017
Date of expiry	:	05 January 2021
Manufactured by	:	Sigma-Aldrich Biochemie Gmbh, Germany

Product Name	:	Glucose
Batch No.	:	SLBT7079
Date of receipt	:	26 December 2017
Retest date	:	March 2023
Manufactured by	:	Sigma Aldrich, USA

8.3.14 Treatment

Acetamide dose formulations and vehicle were administered by oral gavage to rats in specific treatment or vehicle control groups once daily at approximately the same time each day (varied by ± 1 hours) for a period of 7 consecutive days.

The dose volume administered to all the rats were as specified in the 'Experimental design' table. The dose volume was calculated for individual animal on the first day of the treatment and was adjusted according to the recent body weights recorded at different intervals of the study.

The unused dose formulations were stored and sent for disposal.

In addition, BrdU formulations were provided through drinking water to G1B to G5B groups rats from Day 1 until sacrifice.

9 OBSERVATIONS

9.1 Clinical Signs and Mortality

Each rat was observed twice daily for mortality and morbidity. Routine observations for checking general clinical signs was performed twice a day (pre-dose and post-dose) during treatment days.

As there were no clinical signs of concern, the observation for morbidity and mortality was carried out once during weekends and public holidays.

9.2 Detailed Clinical Examinations

Detailed clinical examination was done prior to start of treatment and on Day 7. On days of detailed clinical examination, these observations replaced the daily clinical sign (post-dose) observations except for Day 1.

During detailed clinical examination, all rats were observed for changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern), changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling and bizarre behaviour (e.g. self-mutilation, walking backwards).

9.3 Ophthalmological Examination

Ophthalmological examination of all animals were performed with an ophthalmoscope prior to start of treatment and at the end of the treatment period. Prior to examination, mydriasis was induced using a 1 % solution of Tropicamide.

9.4 Body Weights

Individual body weights (g) were recorded prior to test item administration on Day 1 and on a daily basis during the treatment period. Fasting body weight was recorded prior to sacrifice for all rats.

9.5 Food Consumption

The food consumption was measured on days 1-2, 2-5 and 5-7. The cage wise average food consumption (g/rat/day) was calculated and presented in the report. There was no spillage recorded.

9.6 Water Consumption

The water consumption was measured twice a week (Days 1-5 and 5-7) during the treatment Period.

The cage wise water consumption was estimated using the water consumed at each measuring interval per cage and dividing by the number of animals per cage and the number of days in the intervening period to determine the water consumption/rat/day.

9.7 Bioanalysis

The objective of bioanalysis study was to determine the plasma exposures of acetamide following once daily oral gavage administration of acetamide dose formulation to Wistar rats for 7 days. The blood samples were collected once on Day 8 from all rats for bioanalysis.

About 0.5 mL of blood was collected from retro orbital plexus into tubes containing K_2EDTA as an anticoagulant. Plasma was separated by centrifuging at 5000 rpm, 2 to 8 ºC for 5 minutes within 30 minutes of blood collection. Plasma samples were stored at ≤-70 ºC until analysis.

The concentration of acetamide, in rat plasma was determined using GC-MS method in the Bioanalytical section, Drug Metabolism, Pharmacokinetics and Clinical Pharmacology department, Eurofins Advinus Limited, Bengaluru as per Advinus Study No. G16082. The observed plasma concentration was reported.

In addition, the left over plasma samples (after bioanalysis) from Acetamide groups (G1A, G2A, G3A, G4A and G5A) were analysed for presence of N-hydroxyacetamide (Acetohydroxamic Acid, a putative metabolite of acetamide) on a Non-GLP basis using a fit-for-purpose colorimetric method and the results are included as Appendix 8 to the report.

Tissue Sample

At necropsy, right caudal liver lobe was collected from all rats after completion of 7-days treatment. The liver tissue collected for drug concentration analysis was flash frozen with liquid Nitrogen and was stored at -70° C. These samples were sent to Department of DMPK & CP, Eurofins Advinus Limited for bioanalysis. These samples were transferred along with labelled tubes/containers, and stored at ~ -70 ºC until bioanalysis for acetamide.

The liver tissue concentration of acetamide was determined using a validated GC-MS method as per Advinus Study No. G16083.

Bioanalytical report is included in Appendix 8.

9.8 Clinical Pathology Investigations for Toxicity Groups

9.8.1 Blood Collection

Blood was collected at the end of the treatment period (prior to necropsy on Day 8) from all rats. All rats were fasted overnight (water allowed) and approximately 3 mL of blood was collected from each rat (as indicated in the Table below) under isoflurane anaesthesia, with the help of a fine capillary tube, by retro-orbital plexus puncture.

Parameter	Approximate volume (mL)	Anticoagulant	
		Name	Concentration per mL of blood
Hematology	0.7	K_2 EDTA	1.6 mg
Clinical chemistry	1.8	Lithium heparin	10 Unit
Coagulation	0.5	Trisodium citrate	3.2 mg

After analysis and data review by the analyst, the residual haematology samples were discarded after data review. The plasma samples used for clinical chemistry analysis were stored frozen. These samples were discarded at the time of final report preparation.

9.8.2 Haematology

The following haematological parameters were determined using ADVIA 2120i haematology system (Siemens Healthcare Diagnostics Inc., NY, USA).

Sl. No.	Parameter	Abbreviations	Units[2]
1	Red Blood Corpuscles	RBC	10^{12}/L
2	Haemoglobin	Hb	g/L
3	Haematocrit	Hct	L/L
4	Mean Corpuscular Volume	MCV	fL
5	Mean Corpuscular Haemoglobin	MCH	pg
6	Mean Corpuscular Haemoglobin Concentration	MCHC	g/L
7	Reticulocytes count	Retic	10^{12}/L
8	White Blood Corpuscles	WBC	10^{9}/L
9	Differential leukocyte count[1]	DLC	10^{9}/L
10	Platelets	Plat	10^{9}/L
11	Red Blood Cell Morphology		
	a. Red Cell Distribution Width	RDW	%
	b. Haemoglobin Distribution Width	HDW	g/L
	c. Hyperchromic cells	Hyper	%
	d. Hypochromic cells	Hypo	%
	e. Macrocytes	Macro	%
	f. Microcytes	Micro	%
	g. RBC Fragments	RBC Frag	10^{12}/L
	h. RBC Ghosts	RBC Gh	10^{12}/L

[1]: Differential Leukocyte parameters and their respective abbreviations are: Neutrophils (Neut), Lymphocytes (Lymp), Monocytes (Mono), Eosinophils (Eosi) and Basophils (Baso).
[2]: Expansion of units are as follows: L:Litre, g: Gram, pg: Picogram, fL:Femtolitre,

9.8.3 Coagulation

Blood samples collected for coagulation analysis were centrifuged at 2500 times gravity (x*g*) for 10 minutes for separation of plasma and analysed for the following parameters in plasma sample using STart-4 coagulation analyzer (Diagnostica stago, 92600 Asnieres, France):

Sl. No.	Parameter	Abbreviations	Units
1	Prothrombin Time	PT	Sec
2	Activated Partial Thromboplastin Time	APTT	Sec

9.8.4 Clinical Chemistry

Plasma was separated in a refrigerated centrifuge at 5000 rpm, 4° C for 5 minutes and analysed using Dimension RxL MaX Clinical Chemistry System (Dade Behring Inc. Newark, DE 19714, USA) Automatic Analyser for the following parameters:

Serial Number	Parameters	Abbreviations	Units [b]
1	Alkaline Phosphatase	ALP	U/L
2	Alanine aminotransferase	ALT	U/L
3	Albumin	Alb	g/L
4	Albumin/Globulin ratio (calculated)	A/G	-
5	Aspartate aminotransferase	AST	U/L
6	Blood Urea Nitrogen	BUN	mmol/L
7	Calcium	Ca	mmol/L
8	Chloride	Cl	mEq/L
9	Creatinine	Creat	µmol/L
10	Direct Bilirubin [a]	D.Bil	µmol/L
11	Gamma Glutamyl Transpeptidase	GGT	U/L
12	Globulin (calculated)	Glob	g/L
13	Glucose	Glu	mmol/L
14	Indirect Bilirubin [a] (calculated)	Ind. Bil	µmol/L
15	Inorganic phosphorous	Pi	mmol/L
16	Lactate dehydrogenase	LDH	U/L
17	Potassium	K	mEq/L
18	Sodium	Na	mEq/L
19	Total Cholesterol	T.Chol	mmol/L
20	Total Protein	T.Pro	g/L
21	Total Bilirubin	T.Bil	µmol/L
22	Triglycerides	Trig	mmol/L

Notes:
[a]: Expansion of units are as follows: U:Unit, L:Litre, g: Gram, mmol: Millimole, mEq: Milli equivalent, µmol: Micromole

9.8.5 Urinalysis

Urine was collected in urine collection tubes from all main group rats on Day 7.

Each rat was placed in a specially fabricated cage (water allowed) for about 3-4 hours and the collected urine was sent for analysis.

Urine was analyzed for the following parameters:

Sl. No.	Parameters
1	Specific gravity[1]
2	Nitrite[2]
3	pH[2]
4	Proteins[2]
5	Glucose[2]
6	Ketone bodies[2]
7	Urobilinogen[2]
8	Bilirubin[2]
9	Appearance (colour and clarity)[3]
10	Volume (approximate)[3]

[1]: Measured using Refractometry method (Refractometer-PAL-10S. Atago Co., Ltd., Japan).
[2]: Analyzed using Multistix 10 SG strips, read with Clinitek status analyzer (Bayer Healthcare LLC, UK)
[3]: Recorded manually.

Urine was also subjected to microscopic examination for sediments such as crystals, epithelial cells, erythrocytes, leukocytes and casts.

In addition, the urine samples (after urinalysis) from Acetamide groups (G1A, G2A, G3A, G4A and G5A) were analysed for presence of Acetamide or to determine how much Acetamide was present in urine samples to provide an insight into clearance rates. The analysis was performed on a Non-GLP basis using a fit-for-purpose GC-MS method and the results are included as Appendix 8 to the report.

9.9 Anatomic Pathology

Toxicity groups (main and recovery)

9.9.1 Necropsy

All rats in the study were subjected to detailed necropsy (examination of external surfaces of the body, all orifices, cranial, thoracic and abdominal cavities and their contents) and findings were recorded. All rats were subjected to necropsy after overnight fasting (water allowed) at the end of treatment period. Terminal body weights were recorded for all animals immediately prior to sacrifice. Rats sacrificed were euthanized by exsanguination under isoflurane anaesthesia. The terminal necropsy was done as per the random numbers generated. Between the different groups and the rats in each group was randomized for each round of sacrifice, one animal in each group were necropsied. To begin with, in the total of 10 groups with 6 rats each, the randomization was done among the 10 groups and among the 6 rats in each group. For the following first round, 10 groups were randomized with the randomization among the remaining 5 rats in each group. This procedure was followed till the last round of rats subjected for terminal necropsy.

9.9.2 Tissue Collection, Weighing and Preservation

On completion of the gross pathology examination, the tissues and organs noted below were collected from all rats. The tissues were weighed from all rats. The organ weight ratios (organ to terminal fasting body weight and organ to brain weight) was determined and presented in the report. Paired organs were weighed together and combined weight was presented. Tissues were preserved in 10% Neutral Buffered Formalin (NBF) unless noted otherwise.

Sl. No.	Tissue	Organ weight	Collection and preservation
1	Adrenals	X	X
2	All gross lesions		X
3	Aorta		X
4	Bone marrow smear		X
5	Brain including cerebrum, cerebellum and medulla/pons	X	X
6	Cecum		X
7	Colon		X
8	Duodenum		X
9	Epididymides	X	X
10	Eyes with optic nerves[1]		X
11	Femur bone with distal joint[6]		X
12	Heart	X	X
13	Ileum (with Peyer's patches)		X
14	Jejunum		X

Sl. No.	Tissue	Organ weight	Collection and preservation
15	Kidneys	X	X
16	Liver	X	X
17	Larynx		X
18	Lungs[2]	X	X
19	Mandibular lymph nodes		X
20	Mesenteric lymph nodes		X
21	Nose		X
22	Oesophagus		X
23	Pancreas		X
24	Pituitary[3]	X	X
25	Prostate[5]	X	X
26	Rectum		X
27	Salivary glands		X
28	Sciatic nerves		X
29	Seminal vesicles and coagulating glands [5]	X	X
30	Skeletal muscle		X
31	Skin		X
32	Spinal cord at 3 levels (cervical, mid thoracic and lumber)		X
33	Spleen	X	X
34	Sternum[6]		X
35	Stomach		X
36	Tongue		X
37	Testes[4]	X	X
38	Thymus	X	X
39	Thyroid with parathyroids[3]	X	X
40	Trachea		X
41	Urinary bladder		X
42	Ureters		X

[1]: Collected in Davidson's fluid
[2]: Inflated with 10 % neutral buffered formalin and then immersed in formalin
[3]: Weighed after formalin fixation
[4]: Collected in modified Davidson's fluid
[5]: Prostate + Seminal vesicles with coagulating glands were weighed as a whole; subsequently prostate was separated and weighed. The derived weight was presented for the seminal vesicles and coagulating glands.
[6]: Decalcified prior to sectioning
X: Organs weighed, collected and preserved

Note 1: A section of right caudal liver lobe was preserved for determining the tissue concentration of acetamide.

Note 2: A section of liver specimen from left lobe was snap frozen and shipped to Syngene for toxicogenomics analysis adopting the following procedure:

- To prevent microbial contamination (and subsequent RNAse contamination) disposable gloves was used and good sterile technique was practiced when handling samples and reagents.

- Before starting isolation, work surface area was wiped out with 10% sodium hypochloride followed by 70% ethanol and then by RNAse ZAP RNAse ZAP™ to ensure an RNAse free environment.

- All surgical instruments which were used for dissection were autoclaved. After tissue collection from each animal, surgical instruments were cleaned with RNase Zap.

- Aluminum foil was wiped with RNAse ZAP before placing in balance for weighing.

- 20 to 30 mg of liver was placed into individually labelled sterile / RNAse / DNAse free cryo vials, and was snap frozen in liquid nitrogen container as soon as possible.

- RNaseZAP agent was used to wipe surfaces between samples, as well as cryo vials after labeling.

- Work surface area was wiped with RNAse ZAP™ after each collection.

- These samples were stored -70° C deep freezer and stored until shipment. The samples were shipped in frozen condition in dry ice to Syngene for toxicogenomics assay.

Address for shipment:

Seetha K, Ph.D
Lead Investigator, Biology
Syngene International Ltd.
SEZ-16 UNIT-IX,
Plot No.2,3,4 & 5, Phase IV,
Biocon Park,
Jigani Link Road
Bengaluru-560099
Telephone: +91 804014 8607
Mobile: +91 99005 80779
Email: seetha.krishnan@syngeneintl.com

The above mentioned samples were collected in duplicate and one set of samples were stored at Eurofins Advinus Limited. The stored second set of samples were discarded prior to finalization of the report as per the request of the Sponsor.

Note 2: A small quantity of liver sample was also collected and stored. The stored liver sample was discarded prior to finalization of the report as per the request of the Sponsor.

In addition, the RNA-sequencing data generated at the Syngene test site by Seetha K, Ph.D (Lead Investigator, Biology, Syngene International Ltd.) was shared with;

Michael B. Black, Ph.D.
Scientist IV
ScitoVation
6 Davis Drive
Research Triangle Park, NC 27709
Phone: (919)-558-1338
Email: mblack@scitovation.com
Website: www.scitovation.com

The interpretation of data was done by ScitoVation based upon the RNA sequencing data generated by Syngene. The interpretation data from ScitoVation are included in the Toxicogenomics Phase Report prepared by Seetha K, Ph.D., Syngene International Ltd. The toxicogenomics report is included as in Appendix 10.

9.9.3 Histopathology

Histopathological examination was carried out only for liver of all rats. A section of left lobe of liver was used.

Additionally, histopathological examination was carried out for femur bone with distal joint, sternum and spleen for control and high dose rats as there were changes observed in haematological parameters (increase in RBC counts, haemoglobin concentration, haematocrit values and decrease in reticulocytes). These tissues were not examined in lower dose groups as treatment-related changes were not present in the above tissues.

Tissues were processed for routine paraffin embedding and 4-5 micron sections were stained with Mayer's Haematoxylin and Eosin stain. Unused tissues were archived.

Mitotic Index:

Calculation of Mitotic Index of Hepatocytes: This was done only for G1A-G5A groups (BrdU minus groups).

Procedure for mitotic index scoring:

1. Haematoxylin and Eosin stained liver slides were placed under a microscope.
2. First the slide was viewed under low power, 10X. Then the slide was viewed under 40X so as to distinguish each cells from one another.
3. Total number of hepatocytes in mitosis and not in mitosis contained within one high power (40X) field was counted.
4. The slide stage was randomly moved around the tissue and 10 different counts of this nature was recorded.
5. 10 counts/tissue slice and >2000 hepatocyte per slide were recorded.
6. Mitotic index of the slide was calculated by dividing the total number of hepatocytes in mitosis by the total number of hepatocytes counted (see below).

$$\text{Mitotic Index \%} = \frac{\text{Total number of hepatocyte in mitosis} \times 100}{\text{Total number of hepatocytes counted}}.$$

9.9.4 Immunohistochemistry

From all rats, paraffin embedded liver sections were subjected for Ki67 immunohistochemistry staining. From all rats administered with BrdU, the immunohistochemistry staining for BrdU was performed. The formalin preserved tissues were processed for paraffin embedding during 72 hours post fixation. The procedure for BrdU and Ki67 immunohistochemistry staining are given below

Detection of BrdU in Formalin-Fixed Paraffin-Embedded Rat Tissue

Reagent and Antibody Information

- 1x Wash Buffer (PBS)
- 3% Hydrogen Peroxide
- 1% BSA Diluent
- 1x citrate buffer
- DAB chromogen and substrate. Dako Cytomation

- Hematoxylin solution
- Blocking Solution: Dakocytomation protein block (Ready-to-Use)
- Avidin / Biotin Blocking Kit. Vector Labs.
- Primary Antibody: Mouse Monoclonal BrdU Antibody (Clone BU20a) Dako Cytomation.
- Secondary Antibody: Biotinylated Horse Anti-Mouse IgG (H+L). Vector Labs
- Label complex: R.T.U. Vectastain Elite ABC reagent Vector Labs

Staining Procedure

Positive Control Tissue: Intestine section for each BrdU incorporated rat

Stain localization: Nuclear

1. Slides were deparaffinized and hydrated through the following solutions:

Solution	Repetitions	Time
Xylene	2 times	5 minutes
100% Ethanol	2 times	3 minutes
95% Ethanol	2 times	3 minutes
1X Wash Buffer	2 times	5 minutes

2. Heat-Induced Epitope Retrieval Using the Decloaker :

   500 ml of distilled water was added to the pan inside the decloaker. Full rack of slides were placed into container with 200 ml of 1X citrate buffer. The container was placed stably inside the pan and the slides were decloaked for 15 minutes at 110°C. Pan top was removed and cooled for 10 minutes.

3. Slides were rinsed in 2 changes of distilled water for 3 minutes each time.
4. Endogenous peroxidase was quenched by placing the slides in 3% hydrogen peroxide for 15 minutes.
5. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
6. Non-specific protein binding was blocked with the Dako protein-blocking reagent for 10 minutes at room temperature.
7. Slides were not rinsed and Avidin-Biotin blocking was continued.
8. Avidin block was applied for 15 minutes at room temperature. Slides were quick rinsed in 1X wash buffer. Biotin block was applied for 15 minutes at room temperature. Sections were not rinsed with buffer before adding primary antibody, only excess block was wiped out.

9. Primary antibody was applied at 1:200 dilution and incubated for 1 hour at room temperature.
10. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
11. Horse anti-mouse secondary antibody was applied at a 1:500 dilution and incubated for 30 minutes at room temperature.
12. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each
13. Vectastain R.T.U. Elite Label was applied and incubated for 30 minutes at room temperature.
14. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
15. DAB chromogen was applied and incubated in the dark for 6 minutes at room temperature.
16. Slides were rinsed in tap water 3 minutes.
17. Slides were counterstained with hematoxylin for 20 seconds.
18. Slides were rinsed in running tap water until water was clear.
19. Slides were gently agitated in 1X wash buffer until the tissues turned blue.
20. Slides were dehydrate through the following solutions:

Solutions	Repetitions	Time
95% Ethanol	1 time	3 minutes
100% Ethanol	3 times	3 minutes
Xylene	2 times	5 minutes

21. 1-2 drops of Vecta Mount was applied to each slide and covered with cover glass. Slides were examined under microscope for brown staining of Nucleus.

Detection of Ki 67 proliferation antigen in Formalin-Fixed Paraffin-Embedded Rat Tissue

Reagent and Antibody Information

1. 1X Wash Buffer (PBS)
2. 1X Citrate buffer
3. 3% Hydrogen Peroxide
4 Blocking Solution: Dakocytomation protein block (Ready-to-Use)
5 Anti-Ki67 [SP6] antibody (Abcam – catalog # ab16667)
6 Vectastain Elite ABC Kit Rabbit IgG (Vector Lab – catalog # PK-6101)

a) Blocking goat serum
b) Biotinylated secondary antibody
c) Reagent A (Avidin DH)
d) Reagent B (Biotinylated Horseradish Peroxidase H)

7 DAB Chromogen and substrate buffer (Dako)
8 Hematoxylin solution

Staining Procedure

Positive Control Tissue: Intestine

Stain Localization: Nuclear (proliferating cells, distinctly stains nucleoli)

1. Slides were deparaffinized and hydrated through the following solutions :

Solution	Repetitions	Time
Xylene	2 times	5 minutes
100% Ethanol	2 times	3 minutes
95% Ethanol	2 times	3 minutes
1X Wash Buffer	2 times	5 minutes

2. Heat-Induced Epitope Retrieval Using The Decloaker:
   500 ml of distilled water was added to the pan inside the decloaker. Full rack of slides were placed into container with 200 ml of 1X citrate buffer. The container was placed stably inside the pan and the slides were decloaked for 15 minutes at 110°C. Pan top was removed and cooled for 10 minutes.
3. Slides were rinsed in 2 changes of distilled water for 3 minutes each time.
4. Then Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
5. Endogenous peroxidase was quenched by placing the slides in 3% hydrogen peroxide for 15 minutes.
6. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each
7. Non-specific protein binding was blocked with the Dako protein-blocking reagent for 10 minutes at room temperature.
8. The slides were not rinsed and continued to next step.
9. Avidin block was applied for 15 minutes at room temperature. Slides were quick rinsed in 1X wash buffer. Then biotin block was applied for 15 minutes at room temperature.
10. Before adding primary antibody slides were not rinsed with buffer, only excess block was wiped.
11. Primary antibody was applied at 1:200 dilution and incubated for 2 hour at room temperature.
12. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
13. Biotinylated secondary antibody was applied at a 1:1000 dilution and incubated for 1 hour at room temperature.

14. ABC reagent was prepared 30 minutes before its usage. ABC reagent was prepared by adding 1 drop of reagent A into 5ml of 1X PBS and then add 1drop of B.
15. Slides were rinsed in 3 changes of 1X wash buffer for 5 minutes each
16. Then slides were incubated with ABC reagent for 40 minutes at room temperature.
17. Slides were rinsed in 3 changes of 1X wash buffer for 5 minutes each
18. DAB chromogen was applied and incubated in the dark for 6 minutes at room temperature.
19. Slides were rinsed in tap water 3 minutes.
20. Counterstained with hematoxylin for 20 seconds.
21. Slides were rinsed in running tap water until water is clear.
22. Slides were gently agitated in 1X wash buffer until the tissues turn blue.
23. Slides were dehydrated through the following solutions:

Solutions	Repetitions	Time
95% Ethanol	1 time	3 minutes
100% Ethanol	3 times	3 minutes
Xylene	2 times	5 minutes

24. 1-2 drops of Vecta Mount was applied to each slide and covered with cover glass. Slides were examined under microscope for brown staining of nucleus.

Analysis of BrdU/Ki67 Stained Slides

Procedure for scoring slides:

1 After the preparation of slides by the appropriate staining procedure, slides were placed under microscope.
2 Slides were viewed under low power, 10X. Then slides viewed under 40X so as to distinguish cells from one another.
3 The total number of positively and negatively hepatocytes within one high power (40X) field was counted.
4 Slide stage was randomly moved around the tissue to make 10 different such counts.
5 counts/tissue slice and >2000 nuclei per slide were recorded.
6 Labeling index of the slide was calculated by dividing the total number of positively stained hepatocytes by the total number of hepatocytes counted (see below).

$$\text{BrdU/Ki67 Index \%} = \frac{\text{Total number of BrdU/Ki67 stained cells}}{\text{Total number of hepatocytes counted}} \times 100.$$

Peer review: Haematoxylin and Eosin stained and Ki-67 immuno-stained liver slides of acetamide + BrdU groups were shipped for the Peer review to:

Dr. J. E. Klaunig, Ph.D., Fellow ATS, Fellow IATP (Peer Review Pathologist)
To the following address:

Dr. J. E. Klaunig, Ph.D., Fellow ATS, Fellow IATP
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The findings from the Peer review were discussed by the study pathologist and reviewer. The consensus diagnoses/findings were included in the report. The review process was indicated in the peer review statement, signed peer review statement was included in the main report. All the slides were shipped back to Eurofins Advinus for archiving.

10 STATISTICAL ANALYSES

Data captured using Provantis™: Parameters such as body weight, body weight gains, food consumption, organ weights and their ratios, laboratory Investigations - Haematology (Coagulation tests PT and APTT data was entered retrospectively in Provantis™) and Clinical Chemistry data was analyzed using Provantis built-in statistical tests.

The integrated decision tree of Provantis™:

(i) Test variance homogeneity by Levene's method was tested. When variances are heterogeneous, suitable transformation was performed automatically by the software.

(ii) Further one-way analysis of variance (ANOVA) was performed. When ANOVA is significant, Dunnett's control versus treatment group mean comparisons were performed.

Statistical analysis of the experimental data generated outside Provantis™ (Ki 67 proliferation index and Mitotic index) was carried out using licensed copies of SYSTAT Statistical package Version 12.0. The data was tested for homogeneity of variances (Levene's test) within the group before performing

One-Way Analysis of Variance (ANOVA). If the data was found non-optimal (non-normal or heteroschedastic), ANOVA was done using suitable transformation. Comparison of means between treatment groups and vehicle control group was be done using Dunnett's test, asthe overall treatment 'F' test was found significant.

All analyses and comparisons were evaluated at the 5% ($p< 0.05$) level. Statistically significant differences ($p<0.05$), indicated by the aforementioned tests were designated by the following throughout the report:

+/-: Significantly higher (+) /lower (-) than the vehicle control group

11 DATA COMPILATION

All individual animal data are presented in Appendices and/or summarized and presented in Tables along with results of statistical analyses. All findings are presented in the report as per the standard reporting format.

The Provantis™ system for on-line data capturing in toxicology studies was used for recording the daily clinical sign observations, detailed clinical examination, treatment, body weights, food input and output records, terminal body weights, organ weights, laboratory investigations – Haematology (except coagulation parameters) and Clinical Chemistry, gross pathology and histopathology data.

Printouts from STart-4 coagulation analyzer was the raw data for Coagulation tests. The Clinitek status analyzer printouts was the raw data for the tests analysed using Multistix 10 SG strips in urinalysis, while other urinalysis data was recorded in respective standard SOP formats.

On Day 8 of acclimatization, the clinical observation data could not be captured online due to migration of Provantis version 8.7.3 to Provantis 10.1.0.1. Hence, clinical observation data was recorded in the SOP format. Further entry of this data in Provantis™ was carried out retrospectively.

On Day 5 of the treatment period, the water input and output data could not be captured online due to network failure. Hence, water input and output data on Day 5 was recorded in the SOP format. Further entry of these data in Provantis™ was carried out retrospectively.

However, for Clinical Pathology, the instrument generated printouts were preserved as raw data. Further entry of clinical pathology data in Provantis™ was carried out retrospectively. Other data/ procedures of the study was recorded and documented manually in the respective standard SOP formats

12 RESULTS AND DISCUSSION

Details of Experimental Design, Treatment Schedule, Clinical Pathology Investigations, Pathology and Sacrifice Schedule are as provided in Table 1.

12.1 Dose formulation analysis

The dose formulations were analyzed for concentration of acetamide using a validated analytical method as per G16082. Vehicle control sample was found to be free of any significant presence of test-item. The mean concentration of acetamide prepared at 3, 10, 30 and 100 mg/mL was within ± 15% of the nominal concentration and the relative standard deviation (% RSD) at each dose level was less than 15 %. This indicates that the prepared dose formulations met the acceptance criteria for acetamide concentration.

12.2 In-Life Data

12.2.1 Clinical Signs and Detailed Clinical Examination

Refer to Table 2, Appendix 1

There were no clinical signs in the vehicle control group.

There were no clinical signs throughout the treatment period in all the treated groups during the treatment period.

12.2.2 Mortality

Refer to Table 2, Appendix 1

There were no mortalities or morbidity observed during the treatment in all the treated groups.

12.2.3 Ophthalmological Examination

Refer to Table 3, Appendix 2

Ophthalmological examination performed before the start of treatment and at the end of the treatment period did not reveal any abnormalities in the eyes of the experimental rats.

12.2.4 Body Weights and Body Weight Gains

Refer to Tables 4 and 5, Appendices 3 and 4, and Figure 1

The mean body weights were unaffected during the treatment period in all groups.

A few statistically significant lower body weight gains were observed during treatment period in G5A during days 3-4 and 1-7 (absolute weight gains) and in G5B during days 5-6 and days 1-7(absolute weight gains).

The absolute weight gains and total % weight gain are provided below:

Group No.	Group	Dose (mg/kg/day)	Absolute Weight Gains (g)	Total % Weight gains
G1A	Vehicle control	0	21.97	7.12
G2A	Low dose	30	20.70	6.65
G3A	Mid dose	100	18.36	5.88
G4A	Intermediate dose	300	17.35	5.55
G5A	High dose	1000	2.26 *	0.75
G1B	Vehicle control	0	15.66	5.06
G2B	Low dose	30	17.84	5.83
G3B	Mid dose	100	16.85	5.43
G4B	Intermediate dose	300	11.82	3.73
G5B	High dose	1000	-3.66 -	-1.15
*: Significantly lower (-) than the vehicle control group				

The decrease in body weight gain did not result in differences in terminal body weight and was not associated with changes in food consumption and hence considered as non-adverse finding.

12.2.5 Food Consumption

Refer to Table 6, Appendix 5, and Figures 2

There were no test item-related variations in the food consumption in all tested dose groups during the treatment period.

A statistically significant higher food consumption was observed in G3A (days 1-2), G4A (days 1-2 and 2-5), and G5A (days 1-2) as compared to the vehicle control group. Statistically significant lower food consumption was observed in G5A and G5B (days 5-7) groups as compared to the vehicle control group. These few inconsistent changes without any dose-relation were considered incidental findings and not considered to be treatment related.

12.2.6 Water Consumption

Refer to Table 7, Appendix 6, and Figure 3

The statistically significant higher water consumption in G2A (days 5-7) and G4A (days 5-7) and lower water consumption in G5B (days 5-7) was observed when compared to the respective vehicle control group. These few inconsistent changes were considered incidental findings and not considered to be treatment-related..

12.2.7 Bioanalysis of Plasma and Liver Acetamide Concentration

Refer to Appendix 8

Following repeated daily oral gavage administration of Acetamide alone and Acetamide in presence of BrdU in male wistar rats (n=6) for 7 Days, liver and plasma samples were collected at 24h from last dosing (on Day 8) and the plasma and liver concentrations of Acetamide were determined by validated GC-MS method. The plasma and liver concentrations of Acetamide are presented in table below:

Dose (mg/kg/day)	Day Nominal	Acetamide groups		Acetamide + BrdU groups	
		Mean Plasma Concentration (µg/mL)	Mean Liver Concentration (µg/g)	Mean Plasma Concentration (µg/mL)	Mean Liver Concentration (µg/g)
0	8	0.350±0.116	2.85±0.233	0.310±0.026	3.51±2.030
30	8	25.8±3.53	19.5±1.62	29.7±3.96	26.7±2.94
100	8	80.5±14.3	57.1±14.7	112±11.8	78.6±10.3
300	8	222±22.9	126±25.3	314±51.1	179±35.9
1000	8	513±48.4	297±64.5	849±85.7	506±42.3

The results suggested that there was dose-related increase in plasma and liver concentrations.

12.2.8 N-hydroxyacetamide (Acetohydroxamic Acid) in Plasma

Refer to Appendix 8

N-hydroxyacetamide (putative metabolite of Acetamide) levels were estimated from the plasma samples collected on day 8 using fit-for-purpose colorimetric method and the observed concentrations are as below:

Dose (mg/kg/day)	Individual N-hydroxyacetamide concentration (µg/mL) in plasma on Day 8						Mean concentration ± SD (µg/mL)	n
0	14.3	5.32	6.11	BLQ	BLQ	BLQ	8.59 ± 5.0	3
30	BLQ	BLQ	BLQ	BLQ	6.61	BLQ	6.61[a]	1
100	BLQ	6.71	9.68	5.02	BLQ	BLQ	7.14 ± 2.4	3
300	BLQ	9.19	BLQ	BLQ	9.48	BLQ	9.34[b]	2
1000	21.5	BLQ	39	7.3	BLQ	35.5	25.8 ± 14	4
[a] n=1 animal data; [b] n=2 animal data, values were below quantification limit for other animals in particular group								

No consistent dose-dependent increase in N-hydroxyacetamide was observed. The plasma N-hydroxyacetamide concentrations were comparable in 0, 30, 100, 300 mg/kg/day group except 1000 mg/kg group. At the highest dose (1000 mg/kg/day), 4/6 animals showed higher N-hydroxyacetamide levels whereas the other 2 were not distinguishable from vehicle controls or below limit of quantification. The results suggest that no significant presence of N-hydroxyacetamide in plasma following Acetamide administration except 1000 mg/kg/day dose group.

Dose (mg/kg/day)	Acetohydroxamic acid conc. (µg/mL) in plasma
0	8.59 ± 5.0
30	6.61[a]
100	7.14 ± 2.4
300	9.34[b]
1000	25.8 ± 14
[a]n=1 animal data; [b]n=2 animal data, values were below quantification limit for other animals in particular group	

12.2.9 Acetamide Concentration in Urine

Refer to Appendix 8

Acetamide levels were estimated from the pooled urine collected on Day 7 and the observed concentrations are as below:

Dose (mg/kg/day)	Day Nominal	Mean Urine Concentration in Acetamide group (µg/mL)
0	7	4.71 ± 1.14
30	7	833 ± 358
100	7	2482 ± 817
300	7	5647 ± 1313
1000	7	15150 ± 2616

Following repeated oral gavage administration of acetamide at 30, 100, 300 and 1000 mg/kg/day to Wistar rats for 7-consecutive days, urine samples were collected at 24 h post dose administration, showed dose dependent increase in acetamide concentration in urine samples.

12.2.10 Acetamide Concentration in Feed

Refer to Appendix 8

A sample of feed was collected during the study which was monitored for Acetamide levels using fit-for-purpose GC-MS method and concentration was found to be 1.15 µg/g of feed.

12.2.11 Acetamide Concentration in Water

Refer to Appendix 8

A sample of water was collected from the Aquaguard during the study which was monitored for Acetamide levels using fit-for-purpose GC-MS method and concentration was below the limit of quantification (LLOQ 0.1 µg/mL) suggesting absence of significant level of Acetamide.

12.3 Clinical Pathology Investigations

12.3.1 Haematology

Refer to Appendix 9

The haematology changes associated with test item administration when compared to vehicle control are tabulated below and the details are presented below:

Treatment	**Acetamide groups**				**Acetamide + BrdU groups**			
Group	**G2A**	**G3A**	**G4A**	**G5A**	**G2A**	**G3A**	**G4A**	**G5A**
Dose (mg/kg/day)	**30**	**100**	**300**	**1000**	**30**	**100**	**300**	**1000**
No. of rats	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Red Blood Corpuscles	—	—	—	↑9%*	—	—	—	↑10%*
Haemoglobin	—	—	—	↑9%*	—	—	—	↑12%*
Haematocrit	—	—	—	↑6%*	—	—	—	↑11%*
Reticulocytes- absolute count	—	—	—	↓48%*	—	—	—	↓40%*

↑: Increase ↓: Decrease *: Statistically significant change
Changes are expressed as mean % change from control values
'-'indicates that values are not considered meaningfully different from controls.

Increase in RBC counts, haemoglobin concentration, haematocrit and decrease reticulocytes in 1000 mg/kg/day acetamide and acetamide + BrdU groups were considered test item-related non-adverse change as they were not associated with histopathological changes in haemopoietic organs (bone marrow and spleen).

Decreased hypochromic RBC in 1000 mg/kg/day acetamide dose group was considered toxicologically insignificant as it was not associated with any significant changes in erythrocyte indices.

Increased MCHC in 1000 mg/kg/day acetamide dose group was considered incidental change and was likely due to random biological variation as the magnitude of change was very minimal (<5%).

12.3.2 Coagulation

Refer to Appendix 9

There were no test item-related changes in the coagulation parameters.

Increased prothrombin time in 1000 mg/kg/day acetamide + BrdU dose groups was considered toxicologically insignificant as it was not associated with any significant changes in APTT and platelet counts.

12.3.3 Clinical Chemistry

Refer to Appendix 9

The clinical chemistry changes associated with test item administration when compared to vehicle control are tabulated below and the details are shown in the table provided below:

Treatment	Acetamide groups				Acetamide + BrdU groups			
Group	**G2A**	**G3A**	**G4A**	**G5A**	**G2A**	**G3A**	**G4A**	**G5A**
Dose (mg/kg/day)	**30**	**100**	**300**	**1000**	**30**	**100**	**300**	**1000**
No. of rats	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
AST	—	—	—	↑119%*	—	—	—	↑36%*
ALT	—	—	—	↑101%*	—	—	—	↑25%*
ALP	—	—	—	↑61%*	—	—	—	↑84%*
Triglycerides	—	—	—	↓47%*	—	—	—	↓34%
Total Bilirubin	—	—	—	↑43%	—	—	—	↑91%*

↑: Increase ↓: Decrease *: Statistically significant change
'-'indicates that values are not considered meaningfully different from controls.
Changes are expressed as mean % change from control values

Increase in ALT, AST and ALP activity in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related adverse change as it was associated with single cell necrosis of hepatocytes in liver.

Decreased triglycerides concentration in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change. This change was associated with hepatocytes vacuolation in liver.

Increased total bilirubin concentration in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change.

Decreased creatinine values in all treated acetamide + BrdU groups was considered as toxicologically insignificant as it was not associated with any decreased body weight (muscle mass).

Decreased total bilirubin concentration in 300 mg/kg/day acetamide and acetamide + BrdU groups was considered toxicologically insignificant change as decreased in total bilirubin does not have any biological significance.

Increased total protein and globulins and decreased albumin globulin ratio in 100 and 300 mg/kg/day acetamide dose groups was considered as toxicologically insignificant changes as the change did not show a dose-dependent progression at 1000 mg/kg/day.

All the electrolytes alteration (calcium, phosphorous, sodium and chloride) which reached statistical significance at different acetamide dose levels were likely due to random biological variation as these changes were within historical control range for vehicle treated rats.

12.3.4 Urinalysis

Refer to Appendix 9

There were no test item-related alterations in any of the urinalysis parameters analyzed.

12.4 Pathology

12.4.1 Terminal Fasting Body Weights, Organ Weights and Organ to Body Weight Ratios

Refer to Appendix 9

There were no significant changes in terminal fasting body weights in treated groups when compared to respective controls.

The organ weight changes in liver associated with test item administration when compared to vehicle control are tabulated below:

Treatment	**Acetamide groups**				**Acetamide + BrdU groups**			
Group	**G2A**	**G3A**	**G4A**	**G5A**	**G2A**	**G3A**	**G4A**	**G5A**
Dose (mg/kg/day)	**30**	**100**	**300**	**1000**	**30**	**100**	**300**	**1000**
No. of rats	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Liver – absolute weights	—	—	—	↓25%*	—	—	—	↓15%*
– relative to body weights	—	—	—	↓20%*	—	—	—	↓10%*
– relative to brain weights	—	—	—	↓22%*	—	—	—	↓16%*

↓: Decrease *: Statistically significant change
'-'indicates that values are not considered meaningfully different from controls
Changes are expressed as mean % change from control values

Decreased absolute and relative weight of liver in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change.

Decreased absolute weight and organ-to-brain weight ratios of seminal vesicles and coagulating glands were considered as incidental changes as the values were within historical control range.

12.4.2 Gross Pathology

Refer to Appendix 9

There were no test item-related gross changes in all treated groups.

All the gross changes observed in different dose groups were considered as incidental change of spontaneous origin as they were randomly distributed across the groups.

12.4.3 Histopathology

Refer to Appendix 9

The incidences and severity of test item-related microscopic changes in liver are tabulated below:

Group	G1A	G2A	G3A	G4A	G5A	G1B	G2B	G3B	G4B	G5B
Dose (mg/kg Bwt/day)	**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
No. of rats	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
LIVER;	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
Increased mitotic activity	0	0	0	0	6	0	0	0	0	6
minimal	–	–	–	–	5	–	–	–	–	5
mild	–	–	–	–	1	–	–	–	–	1
Single cell necrosis; hepatocyte	0	0	0	0	5	0	0	0	0	5
minimal	–	–	–	–	3	–	–	–	–	4
mild	–	–	–	–	2	–	–	–	–	1
Vacuolation; hepatocyte	0	0	0	0	5	0	0	0	0	3
minimal	–	–	–	–	5	–	–	–	–	3

Increased mitotic activity, single cell necrosis and vacuolation of hepatocytes in 1000 mg/kg/day acetamide and acetamide + BrdU groups were considered test item-related change. Increased mitotic activity in high dose was associated with high Ki67 proliferation index.

All the microscopic changes observed in other dose groups were considered as incidental change of spontaneous origin as they were randomly distributed across the groups.

12.4.4 Mitotic Index

Refer to Appendix 9

High hepatocytes mitotic index in 1000 mg/kg/day acetamide was considered test item-related change. The mitotic index at <1000 mg/kg/day doses was comparable to vehicle control.

12.4.5 Immunohistochemistry

Refer to Appendix 9

Immuno-histochemical staining was performed for Ki 67 proliferation antigen and BrdU. The optimum immunostaining of Ki 67 proliferation antigen was observed in (duodenum) positive control and Ki 67 proliferation index was calculated for all the groups.

Suboptimal immunostaining of BrdU was noted in the positive control tissue (duodenum). BrdU index of hepatocytes was not calculated by counting BrdU immuno-stained hepatocytes as optimal staining was not observed in positive control tissue (crypt epithelium of duodenum) and BrdU index thus obtained was not considered to be an accurate representations of the cell proliferation activity.

Ki67 proliferation index.

Refer to Appendix 9

High Ki 67 proliferation index in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change.

Low Ki 67 proliferation index in 30 and 100 mg/kg/day acetamide dose groups when compared to control was considered as incidental change as low index in these two groups was due to slightly high index in two controls (Ru5682 and Ru5685) resulting in marginally high Ki 67 index of control group. Hence, low Ki 67 proliferation index in 30 and 100 mg/kg/day acetamide dose groups was not considered to be related to test item treatment.

12.5 Toxicogenomics

Refer to Appendix 10

The following is a brief summary of the findings from whole transcriptomic gene expression analyses in male Wistar rats treated with four doses of acetamide plus a vehicle only control group (0 (vehicle controls), 30, 100, 300 and 1000 mg/kg/day).

Differential Gene Expression: The response by fold change (FC) alone was evident at all treatment concentrations. However, none of these changes met the standard statistical False Discovery Rate (FDR) criterion of ≤ 5% for the two lowest concentrations of 30 and 100 mg/kg/day, and only a single gene was statistically significant for the 300 mg/kg/day treated group. At 1000 mg/kg/day, there were numerous genes that were either up- or down-regulated by at least 1.5 fold relative to vehicle, and were statistically significantly altered (FDR<0.05). These genes in the 1000 mg/kg/day dose group that were significant by both FDR<0.05 & |FC|>1.5 fold) that were used to investigate ontology enrichment with the Reactome Ontology).

Functional Ontology Enrichment: The metabolic processes appear enriched for the down-regulated genes and cell cycle, signalling and immune system responses are enriched with up-regulated genes.

Transcription factor binding predictions: The PPARa- and RXR-associated genes overrepresented among the down-regulated gene lists, a finding consistent with the observed Reactome enrichment in fatty acid metabolic processes. The up-regulated genes had enrichment for various FOX controlled pathways and regulated elements. FOX proteins are key transcription factors in cell growth and proliferation and are among some of the most conserved transcription factors in mammals.

Benchmark dose analyses: A Reactome pathway analysis of BenchMark Dose for the 11,373 genes for which all 25 animals in the study had a feature count of at least one was performed. Any gene was excluded where even a single replicate failed to detect the gene (a feature count = 0) to avoid issues with mathematical curve fitting to missing data or creating a need to impute missing data for parts of the dataset. BMD Express 2.2 software was used to fit five models to every one of the 11,373 genes - power model (power coefficient restricted to >1), the Hill model, a linear model and a 2° polynomial and exponential model. Hill models where the shape (or slope) parameter, k, was less than 1/3rd of the maximum concentration were rejected from consideration as these tend to have extreme inflections the shape of the curve leading to biologically non-sensical (e.g., vanishingly small) BMD estimates. Genes where a best fitting model was found that had both a gene-based BMD estimate less than the maximum treatment concentration of 1000 mg/kg/day (to avoid extrapolation of estimates) and which also had a goodness of fit p-value >0.1 were then used in an enrichment analysis using Reactome. Pathways with 10 or more such genes found had a pathway BMD estimated as the median of the BMD of the genes which matched ontology elements in that pathway. Collectively, pathway median BMDs were estimated between 400 and 1000 mg/kg/day.

13 CONCLUSION

The results of the study indicated that the oral gavage administration of the test item, acetamide (acetamide and acetamide + BrdU) at the doses of 30, 100 and 300 mg/kg/day to male Wistar rats for 7 consecutive days had no effects on clinical signs, mortality, ophthalmological examinations, body weights, food consumption, water consumption, clinical pathology (haematology, clinical chemistry and urinalysis), gross pathology, organ weights, histopathology and Ki 67 proliferation indices of hepatocytes.

At 1000 mg/kg/day (acetamide and acetamide + BrdU) groups, no effects were observed on clinical signs, mortality, ophthalmological examinations, body weights, food consumption, water consumption, urinalysis and gross pathology. However, changes were changes in haematology (increased RBC, haemoglobin and haematocrit and decreased reticulocytes), clinical chemistry (increased ALT, AST and ALP and decreased triglycerides and increased total bilirubin) parameters, decreased liver weights, increased mitotic activity of hepatocytes, single cell necrosis and vacuolation of hepatocytes and high Ki 67 proliferation indices of hepatocytes.

The bioanalysis indicated that, there was dose-related increase in plasma and liver acetamide concentrations at all the tested dose levels.

In conclusion, the 7-day repeated dose oral gavage administration of acetamide at the tested doses of 30, 100 and 300 mg/kg/day to Wistar rats did not cause any toxic effects. Hence, No Observed Effect Level (NOEL) is defined at 300 mg/kg body weight/day for test item acetamide under the conditions of this study. This estimate is consistent with results of the toxicogenomic analysis which showed differential expression only at 1000 mg/kg body weight/day and pathway median BMDs falling between 400 and 1000 mg/kg body weight/day.

14 ARCHIVING

Eurofins Advinus Limited will archive at the test facility the following for 9 years after completion of the study: Study Plan, Study plan amendments, raw data, draft and final reports. The raw data captured through Provantis™ software will be archived in data base server and also raw data available in Provantis™ will be transferred to EDA (Electronic Data Archives) and archived at the archives of test facility. A sample of test item was sent from the test item stores to the archives before the first dispensing of test item to studies. This sample shall be stored for a period of 9 years from the date of this final report. All tissue specimens, blocks and slides will be archived for 9 years.

15 REPORT DISTRIBUTION

Test Facility : One signed final report in original (Copy No. 1/1).

Sponsor : A searchable electronic copy in the PDF Format (eCTD compliant) of Copy No.1/1

16 REFERENCES

- OECD Principles of Good Laboratory Practice [C (97)186/Final].
- US FDA Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58).
- OECD Guideline No. 407 for Testing of Chemicals, "Repeated Dose 28-Day Oral Toxicity Study in Rodents'' adopted by the Council on 3rd October, 2008 with exception to the duration of treatment period.

17 TABLES

TABLE 1. Details of Experimental Design, Treatment, Clinical Pathology Investigations, Pathology and Sacrifice Schedule

Group No.	Dose (mg/kg/day)	No. of rats per group	Dosing period (days)	Clinical pathology investigations			Pathology			Sacrifice on day
		M		Haema-tology	Clinical Chemistry	Urina-lysis	Gross pathology	Organ weights	Histo-pathology	
G1A	0	6	7	+	+	+	+	+	a	8
G2A	30	6	7	+	+	+	+	+	b	8
G3A	100	6	7	+	+	+	+	+	b	8
G4A	300	6	7	+	+	+	+	+	b	8
G5A	1000	6	7	+	+	+	+	+	a	8
G1B	0	6	7	+	+	+	+	+	a	8
G2B	30	6	7	+	+	+	+	+	b	8
G3B	100	6	7	+	+	+	+	+	b	8
G4B	300	6	7	+	+	+	+	+	b	8
G5B	1000	6	7	+	+	+	+	+	a	8

+: Yes; M: Males; NA: Not applicable a: Only Liver, femur bone with distal joint, sternum and spleen or target organs; b: Only Liver or target organs
Note: Additionally, G1B to G5B group rats received BrdU in drinking water (BrdU - 0.8 mg/ml + 1% glucose) for 7 consecutive days.

TABLE 2. Summary of Clinical Signs, Detailed Clinical Examination and Mortality

Refer Appendix 1

Observation Type: All Types From Day 1 (Start Date) to 8 (Start Date)	Male							
	G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day	G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day
Normal	6	6	6	6	6	6	6	6

Values = Number of Animals Affected

TABLE 2 contd. Summary of Clinical Signs, Detailed Clinical Examination and Mortality

Refer Appendix 1

Observation Type: All Types From Day 1 (Start Date) to 8 (Start Date)	Male G4B 300 mg/kg/day	 G5B 1000 mg/kg/day
Normal	6	6

Values = Number of Animals Affected

TABLE 3. Summary of Ophthalmological Findings

Refer Appendix 2

	At the end of treatment period (Day7)									
PARAMETERS										
Group No.	G1A	G2A	G3A	G4A	G5A	G1B	G2B	G3B	G4B	G5B
Dose (mg/kg/day)	0	30	100	300	1000	0	30	100	300	1000
No. of rats	6 males	6 males	6 males	6 males	6 males	6 males	6 males	6 males	6 males	6 males
EYE EFFECTS	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

Note: Ophthalmological examination carried out during the acclimatization period did not reveal any eye abnormalities.
NAD: No abnormalities detected

TABLE 4. Summary of Body Weights (g)

Refer Appendix 3

Sex: Male		Day(s) Relative to Start Date						
		1	2	3	4	5	6	7
G1A	Mean	310.33	311.79	313.93	318.92	323.46	329.49	332.30
0	SD	21.04	19.86	18.14	19.79	19.45	20.72	20.30
mg/kg/day	N	6	6	6	6	6	6	6
G2A	Mean	312.20	311.75	317.69	319.66	322.58	327.34	332.90
30	SD	22.46	22.55	27.23	25.71	25.56	24.39	23.95
mg/kg/day	N	6	6	6	6	6	6	6
G3A	Mean	313.14	312.75	317.45	318.91	323.69	326.26	331.50
100	SD	21.21	23.04	22.25	21.26	20.82	21.50	21.78
mg/kg/day	N	6	6	6	6	6	6	6
G4A	Mean	313.40	312.81	317.50	321.24	323.73	327.02	330.75
300	SD	23.32	22.77	23.80	24.20	25.93	26.60	24.72
mg/kg/day	N	6	6	6	6	6	6	6
G5A	Mean	309.32	306.71	309.15	309.84	309.46	311.51	311.58
1000	SD	19.99	20.43	18.10	18.37	19.28	20.24	19.52
mg/kg/day	N	6	6	6	6	6	6	6

Statistical analysis: Anova & Dunnett(Log)

TABLE 4 contd. Summary of Body Weights (g)

Refer Appendix 3

Sex: Male		Day(s) Relative to Start Date						
		1	2	3	4	5	6	7
G1B	Mean	307.86	306.65	309.26	313.45	315.30	319.23	323.52
0	SD	19.03	21.68	19.34	22.14	21.51	23.44	22.35
mg/kg/day	N	6	6	6	6	6	6	6
G2B	Mean	313.50	314.92	317.47	319.33	324.75	329.07	331.34
30	SD	18.57	15.20	11.68	11.11	12.46	13.98	14.73
mg/kg/day	N	6	6	6	6	6	6	6
G3B	Mean	311.32	307.23	311.10	316.85	318.77	324.55	328.17
100	SD	23.12	23.22	23.37	24.34	21.93	21.97	23.80
mg/kg/day	N	6	6	6	6	6	6	6
G4B	Mean	310.50	307.19	311.90	314.35	316.36	320.64	322.32
300	SD	21.68	24.47	24.33	23.85	25.58	26.77	26.83
mg/kg/day	N	6	6	6	6	6	6	6
G5B	Mean	312.58	309.00	312.33	311.98	307.44	308.09	308.92
1000	SD	22.40	23.58	24.87	23.54	23.60	21.23	21.94
mg/kg/day	N	6	6	6	6	6	6	6

Statistical analysis: Anova & Dunnett(Log)

TABLE 5. Summary of Body Weight Gains (g)

Refer Appendix 4

Sex: Male		Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]
		1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
G1A	Mean	1.45	2.14	4.99	4.54	6.03	2.82	21.97
0	SD	2.29	2.61	2.54	2.79	3.26	1.92	2.05
mg/kg/day	N	6	6	6	6	6	6	6
G2A	Mean	-0.45	5.94	1.98	2.92	4.76	5.57	20.70
30	SD	2.68	6.48	5.68	4.37	1.92	2.31	6.85
mg/kg/day	N	6	6	6	6	6	6	6
G3A	Mean	-0.39	4.70	1.46	4.78	2.56	5.24	18.36
100	SD	3.30	2.90	2.02	2.87	2.14	1.67	5.17
mg/kg/day	N	6	6	6	6	6	6	6
G4A	Mean	-0.59	4.69	3.74	2.49	3.29	3.73	17.35
300	SD	3.33	2.61	2.48	2.36	2.41	2.74	5.54
mg/kg/day	N	6	6	6	6	6	6	6
G5A	Mean	-2.62	2.45	0.69+	-0.39	2.05	0.07	2.26+
1000	SD	2.50	4.47	1.62	2.88	1.77	4.72	5.41
mg/kg/day	N	6	6	6	6	6	6	6

Statistical analysis: Anova & Dunnett(Log); +: Significantly higher (+) than the vehicle control group

TABLE 5 contd. Summary of Body Weight Gains (g)

Refer Appendix 4

Sex: Male		Total % Weight Gain
		1 → 7
G1A	Mean	7.12
0	SD	1.00
mg/kg/day	N	6
G2A	Mean	6.65
30	SD	2.15
mg/kg/day	N	6
G3A	Mean	5.88
100	SD	1.69
mg/kg/day	N	6
G4A	Mean	5.55
300	SD	1.69
mg/kg/day	N	6
G5A	Mean	0.75
1000	SD	1.73
mg/kg/day	N	6

TABLE 5 contd. Summary of Body Weight Gains (g)

Refer Appendix 4

Sex: Male		Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]	Absolute Weight Gain (g) [a]
		1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
G1B	Mean	-1.21	2.61	4.19	1.86	3.93	4.29	15.66
0	SD	3.36	4.80	3.93	1.25	4.07	2.63	6.54
mg/kg/day	N	6	6	6	6	6	6	6
G2B	Mean	1.42	2.55	1.86	5.42	4.33	2.27	17.84
30	SD	9.35	4.05	2.42	3.01	1.70	1.72	12.43
mg/kg/day	N	6	6	6	6	6	6	6
G3B	Mean	-4.08	3.86	5.76	1.92	5.78	3.62	16.85
100	SD	3.64	1.32	2.06	2.85	1.74	2.48	4.38
mg/kg/day	N	6	6	6	6	6	6	6
G4B	Mean	-3.31	4.71	2.45	2.01	4.28	1.68	11.82
300	SD	2.97	1.64	2.06	2.06	2.16	1.87	5.66
mg/kg/day	N	6	6	6	6	6	6	6
G5B	Mean	-3.58	3.33	-0.35	-4.54	0.66+	0.83	-3.66-
1000	SD	4.61	5.44	1.96	9.23	5.07	4.63	4.74
mg/kg/day	N	6	6	6	6	6	6	6

Statistical analysis: Anova & Dunnett(Log): +/-: Significantly higher (+)/lower (-) than the vehicle control group

TABLE 5 contd. Summary of Body Weight Gains (g)

Refer Appendix 4

Sex: Male		Total % Weight Gain
		1 → 7
G1B	Mean	5.06
0	SD	2.05
mg/kg/day	N	6
G2B	Mean	5.83
30	SD	4.30
mg/kg/day	N	6
G3B	Mean	5.43
100	SD	1.39
mg/kg/day	N	6
G4B	Mean	3.73
300	SD	1.55
mg/kg/day	N	6
G5B	Mean	-1.15
1000	SD	1.57
mg/kg/day	N	6

TABLE 6. Summary of Food Consumption (g/rat/day)

Refer Appendix 5

Sex: Male		Day(s) Relative to Start Date		
		1 → 2	2 → 5	5 → 7
G1A	Mean	25.490	27.066	26.325
0	SD	0.374	0.864	0.425
mg/kg/day	N	6	6	6
G2A	Mean	26.663	27.077	27.405
30	SD	0.495	1.098	0.876
mg/kg/day	N	6	6	6
G3A	Mean	27.313+	27.392	26.427
100	SD	1.213	0.971	1.559
mg/kg/day	N	6	6	6
G4A	Mean	28.268+	28.824+	26.330
300	SD	0.764	1.181	0.570
mg/kg/day	N	6	6	6
G5A	Mean	27.358+	25.593	23.774-
1000	SD	1.326	1.110	0.102
mg/kg/day	N	6	6	6

Statistical analysis: Anova & Dunnett(Log); +/-: Significantly higher (+)/lower (-) than the vehicle control group

TABLE 6 contd. Summary of Food Consumption (g/rat/day)

Refer Appendix 5

Sex: Male		Day(s) Relative to Start Date		
		1 → 2	2 → 5	5 → 7
G1B	Mean	27.445	26.220	25.018
0	SD	1.679	0.782	0.521
mg/kg/day	N	6	6	6
G2B	Mean	28.192	27.183	26.058
30	SD	0.886	0.836	1.595
mg/kg/day	N	6	6	6
G3B	Mean	27.762	26.636	24.659
100	SD	1.710	1.561	1.316
mg/kg/day	N	6	6	6
G4B	Mean	27.585	27.216	24.642
300	SD	1.624	2.501	1.575
mg/kg/day	N	6	6	6
G5B	Mean	26.333	26.238	22.400-
1000	SD	3.242	0.814	1.472
mg/kg/day	N	6	6	6

Statistical analysis: Anova & Dunnett(Log); -: Significantly lower (-) than the vehicle control group

TABLE 7. Summary of Water Consumption (g/rat/day)

Refer Appendix 6

Sex: Male		Day(s) Relative to Start Date	
		1 → 5	5 → 7
G1A	Mean	21.3	22.0
0	SD	1.3	0.6
mg/kg/day	N	6	6
G2A	Mean	21.1	24.3+
30	SD	0.9	1.2
mg/kg/day	N	6	6
G3A	Mean	20.9	21.4
100	SD	0.8	1.0
mg/kg/day	N	6	6
G4A	Mean	21.5	23.8+
300	SD	1.4	1.0
mg/kg/day	N	6	6
G5A	Mean	21.5	21.1
1000	SD	1.1	1.4
mg/kg/day	N	6	6

Statistical analysis: Anova & Dunnett; +: Significantly higher (+) than the vehicle control group

TABLE 7 contd. Summary of Water Consumption (g/rat/day)

Refer Appendix 6

Sex: Male		Day(s) Relative to Start Date	
		1 → 5	5 → 7
G1B	Mean	19.7	19.4
0	SD	1.7	1.1
mg/kg/day	N	6	6
G2B	Mean	18.2	18.5
30	SD	1.0	1.1
mg/kg/day	N	6	6
G3B	Mean	18.2	18.6
100	SD	0.2	0.7
mg/kg/day	N	6	6
G4B	Mean	19.2	20.2
300	SD	0.9	1.9
mg/kg/day	N	6	6
G5B	Mean	17.4	16.6-
1000	SD	2.3	1.8
mg/kg/day	N	6	6

Statistical analysis: Anova & Dunnett; -: Significantly lower (-) than the vehicle control group

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18 FIGURES

FIGURE 1. Body Weights

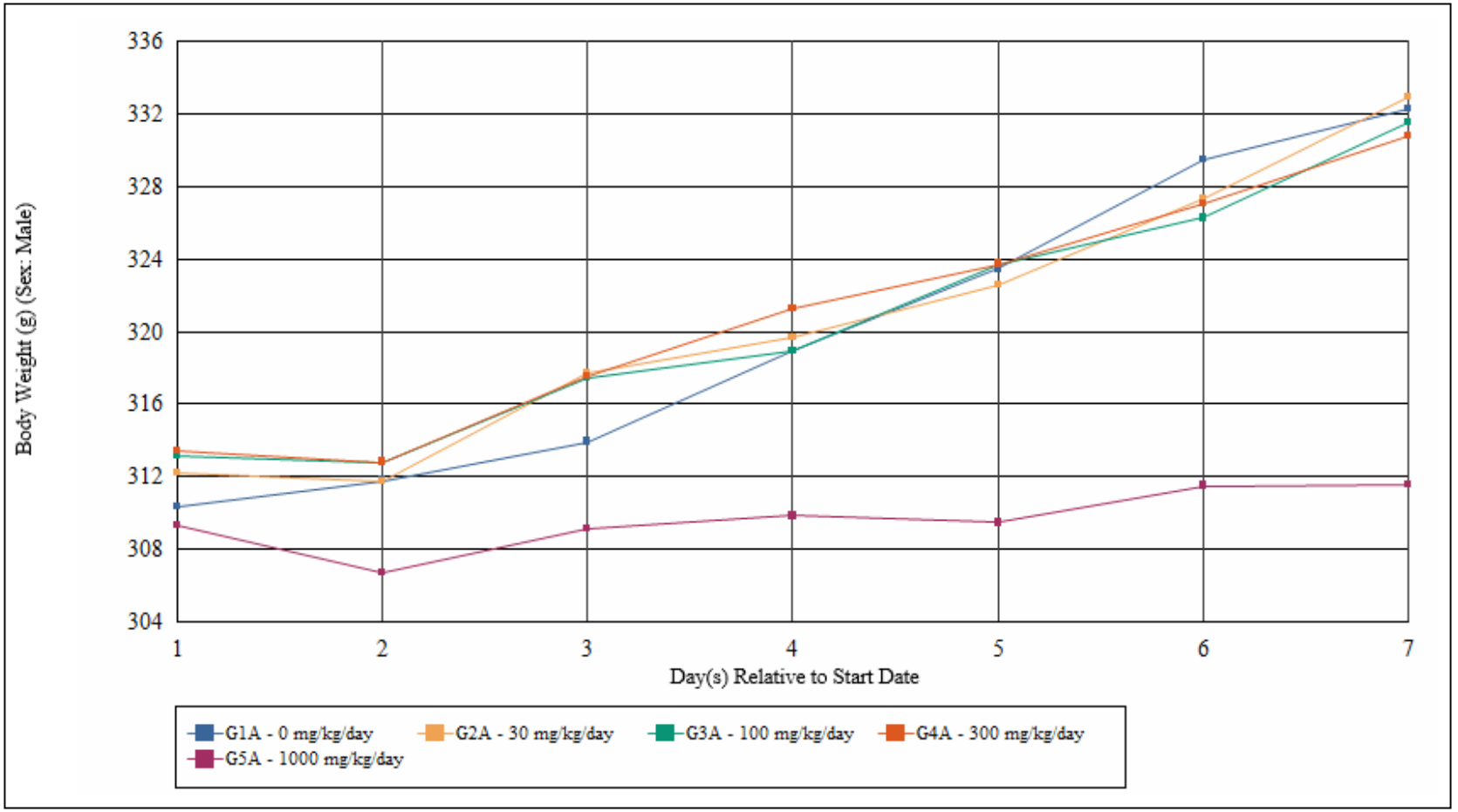


FIGURE 1 contd. Body Weights

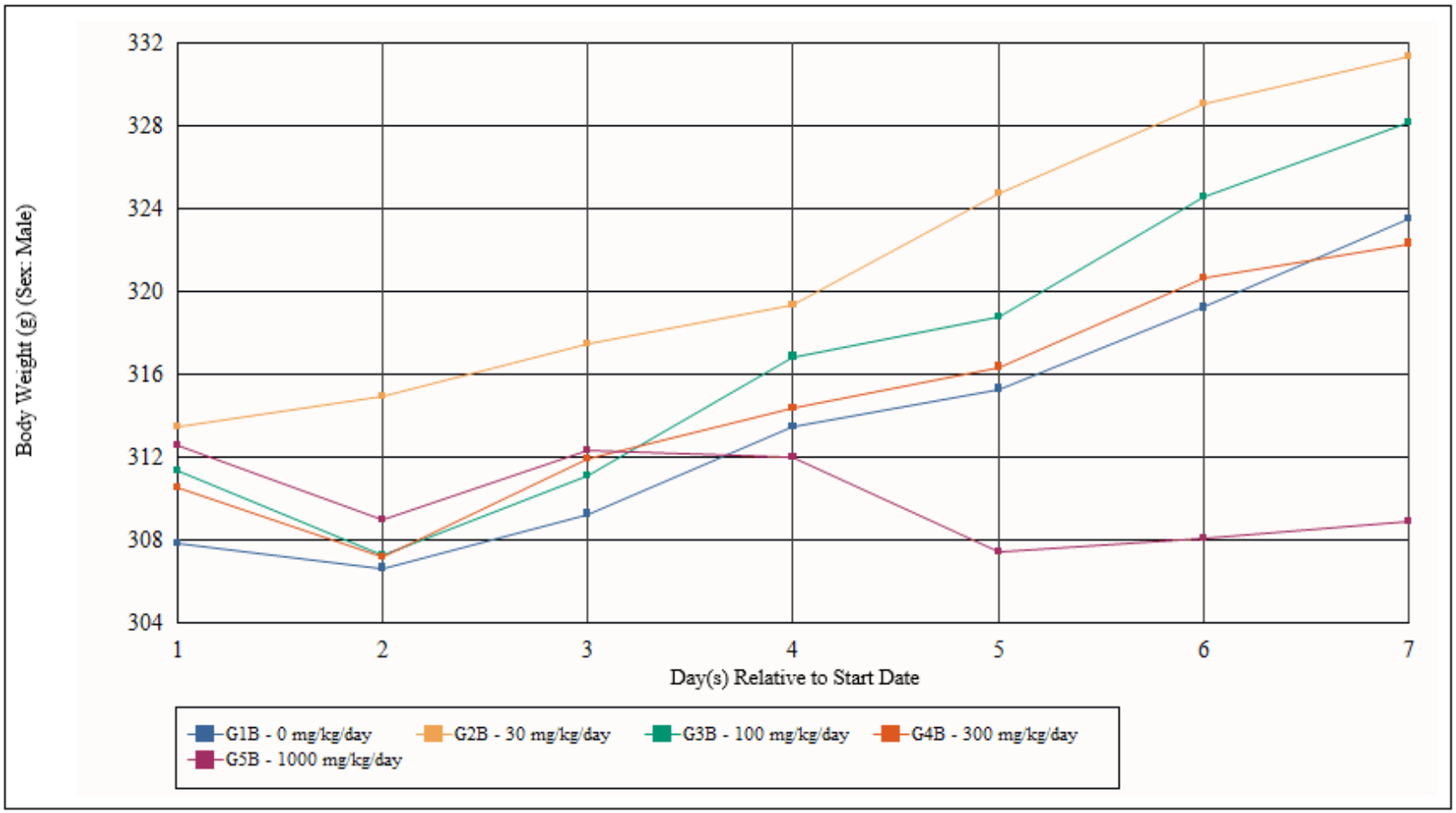


FIGURE 2. Food Consumption

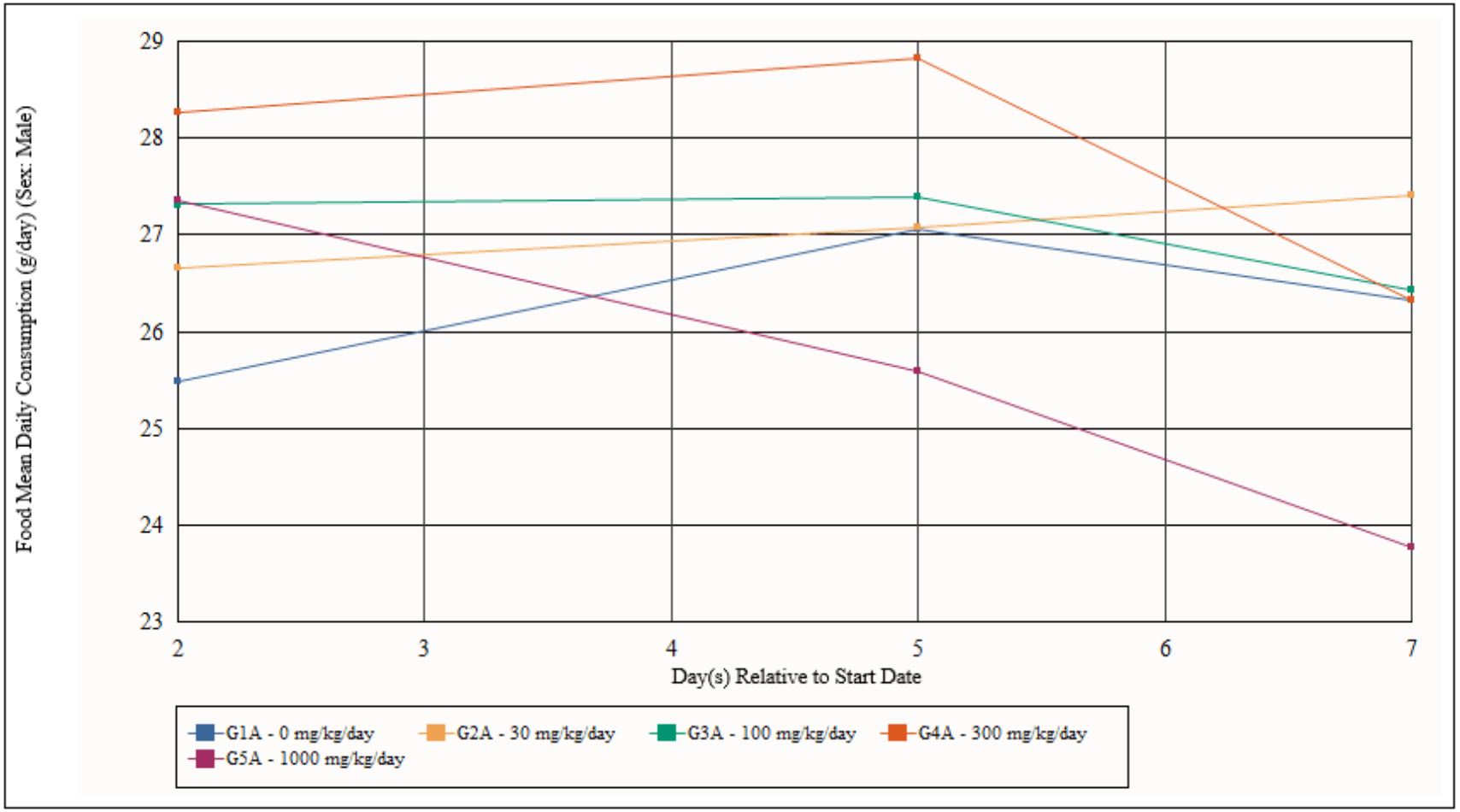


FIGURE 2 contd. Food Consumption

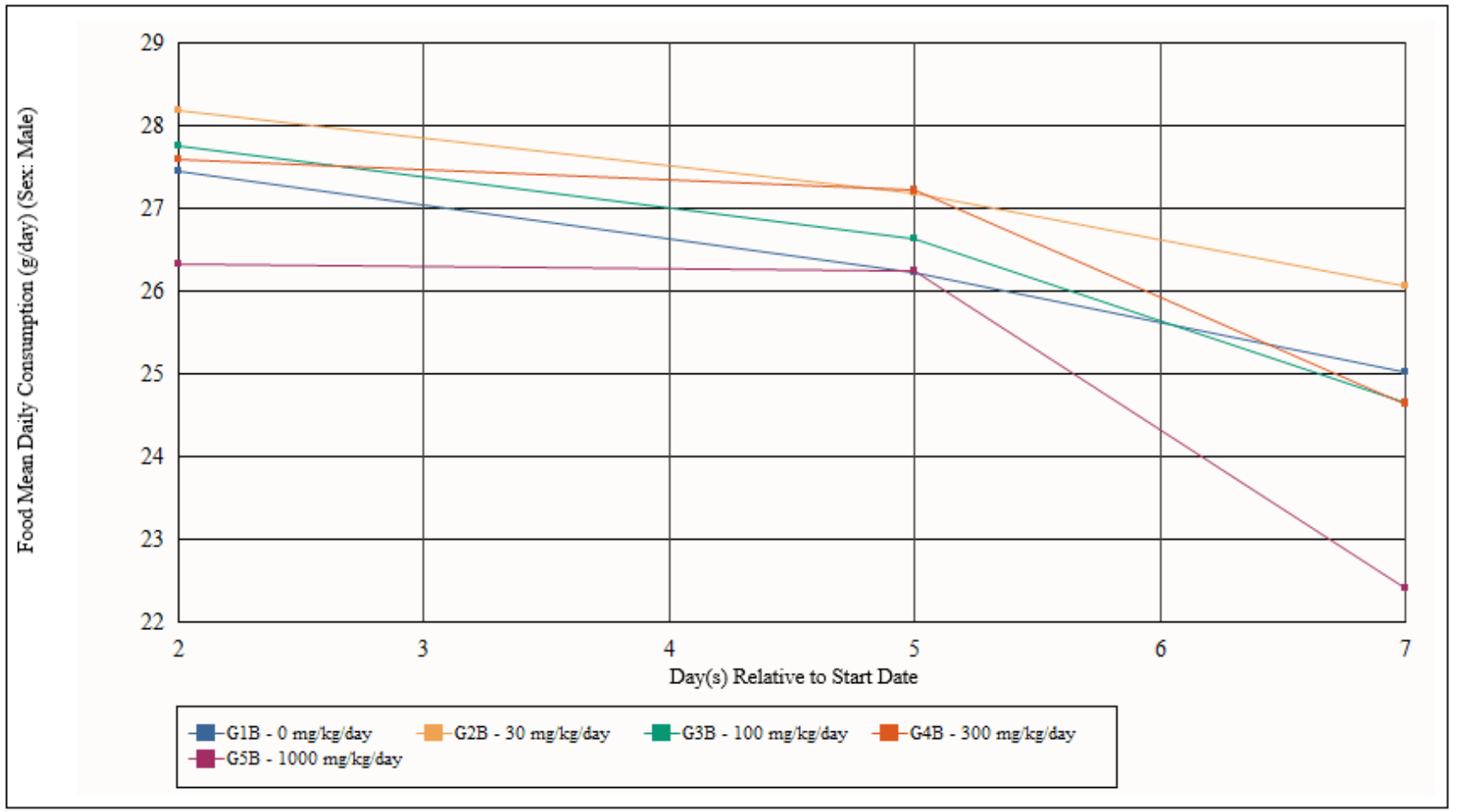


FIGURE 3. Water Consumption

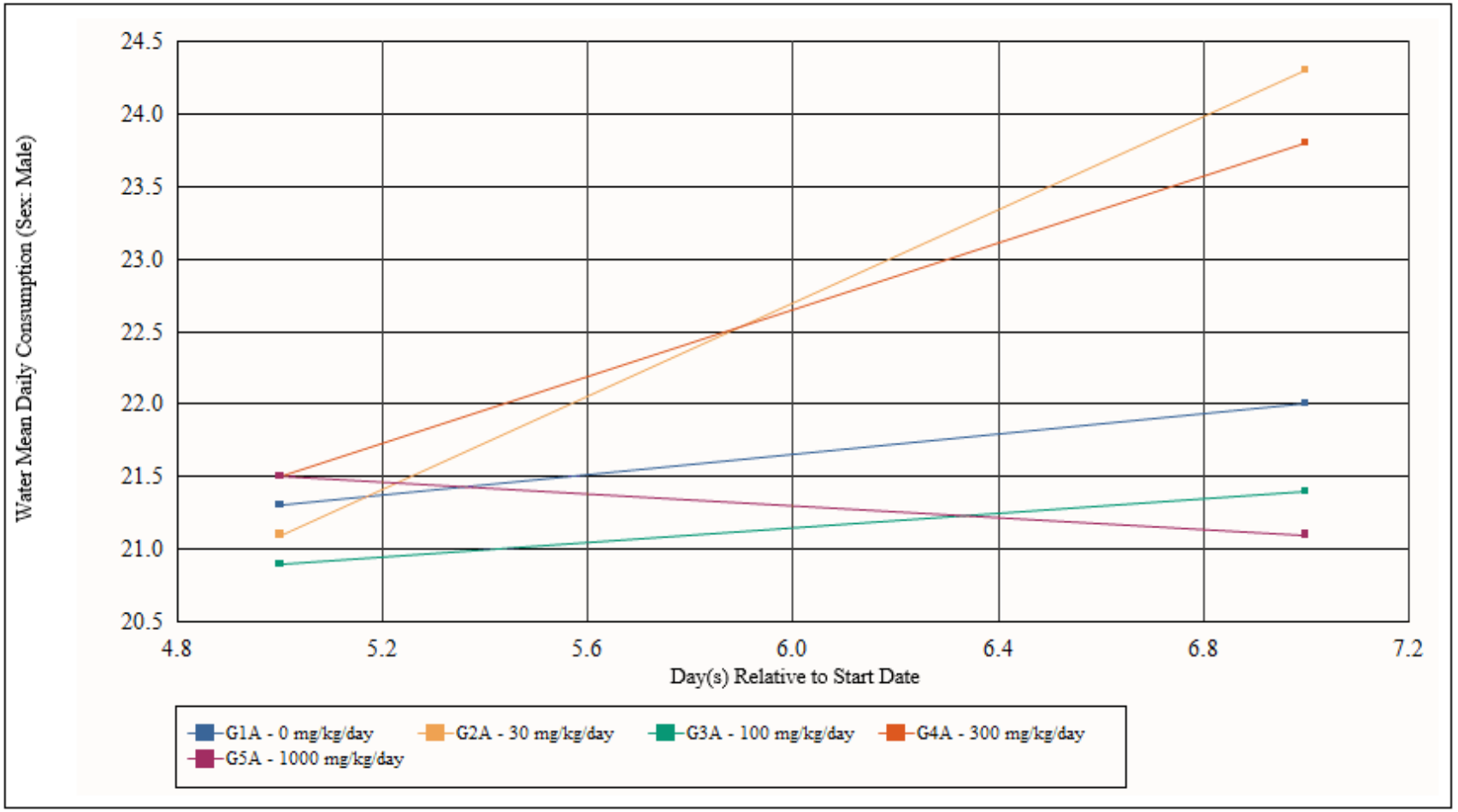
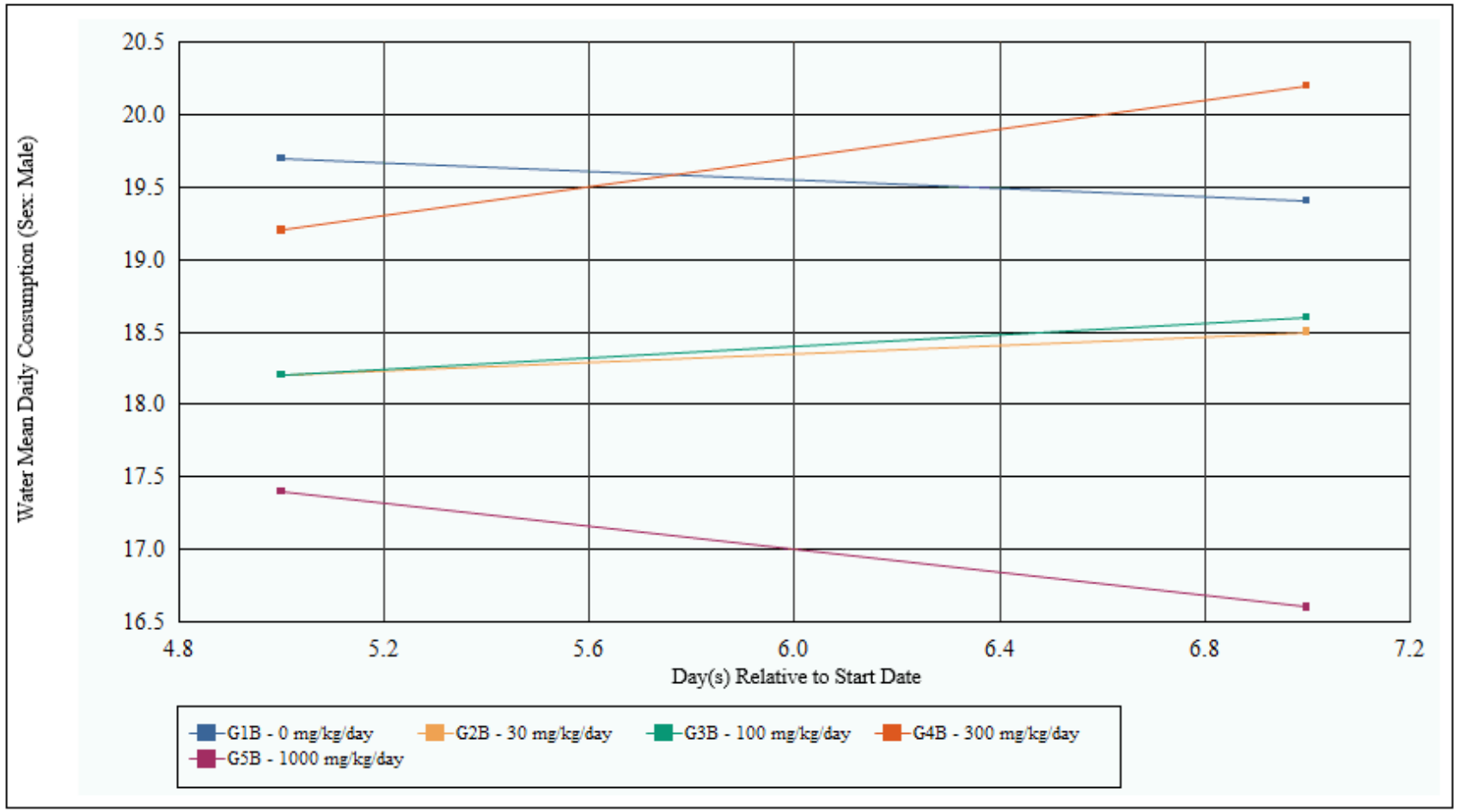


FIGURE 3 contd. Water Consumption



19 APPENDICES

APPENDIX 1. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G1A 0 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5681	Normal	1 to 8
Ru5682	Normal	1 to 8
Ru5683	Normal	1 to 8
Ru5684	Normal	1 to 8
Ru5685	Normal	1 to 8
Ru5686	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G2A 30 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5687	Normal	1 to 8
Ru5688	Normal	1 to 8
Ru5689	Normal	1 to 8
Ru5690	Normal	1 to 8
Ru5691	Normal	1 to 8
Ru5692	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G3A 100 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5693	Normal	1 to 8
Ru5694	Normal	1 to 8
Ru5695	Normal	1 to 8
Ru5696	Normal	1 to 8
Ru5697	Normal	1 to 8
Ru5698	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G4A 300 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5699	Normal	1 to 8
Ru5700	Normal	1 to 8
Ru5701	Normal	1 to 8
Ru5702	Normal	1 to 8
Ru5703	Normal	1 to 8
Ru5704	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G5A 1000 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5705	Normal	1 to 8
Ru5706	Normal	1 to 8
Ru5707	Normal	1 to 8
Ru5708	Normal	1 to 8
Ru5709	Normal	1 to 8
Ru5710	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G1B 0 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5711	Normal	1 to 8
Ru5712	Normal	1 to 8
Ru5713	Normal	1 to 8
Ru5714	Normal	1 to 8
Ru5715	Normal	1 to 8
Ru5716	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G2B 30 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5717	Normal	1 to 8
Ru5718	Normal	1 to 8
Ru5719	Normal	1 to 8
Ru5720	Normal	1 to 8
Ru5721	Normal	1 to 8
Ru5722	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G3B 100 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5723	Normal	1 to 8
Ru5724	Normal	1 to 8
Ru5725	Normal	1 to 8
Ru5726	Normal	1 to 8
Ru5727	Normal	1 to 8
Ru5728	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G4B 300 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5729	Normal	1 to 8
Ru5730	Normal	1 to 8
Ru5731	Normal	1 to 8
Ru5732	Normal	1 to 8
Ru5733	Normal	1 to 8
Ru5734	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 1 contd. Individual Clinical Signs, Detailed Clinical Examination and Mortality

G5B 1000 mg/kg/day Sex: Male	Observation Type: All Types	From Day 1 (Start Date) to 8 (Start Date)
Ru5735	Normal	1 to 8
Ru5736	Normal	1 to 8
Ru5737	Normal	1 to 8
Ru5738	Normal	1 to 8
Ru5739	Normal	1 to 8
Ru5740	Normal	1 to 8

Values=Clin Obs Range

APPENDIX 2. Individual Ophthalmological Examination

Group No. Dose (mg/kg/day)	Rat No.	Day 7	Group No. Dose (mg/kg/day)	Rat No.	Day 7
	Males			Males	
G1A	Ru5681	NAD	G4A	Ru5699	NAD
0	Ru5682	NAD	300	Ru5700	NAD
	Ru5683	NAD		Ru5701	NAD
	Ru5684	NAD		Ru5702	NAD
	Ru5685	NAD		Ru5703	NAD
	Ru5686	NAD		Ru5704	NAD
G2A	Ru5687	NAD	G5A	Ru5705	NAD
30	Ru5688	NAD	1000	Ru5706	NAD
	Ru5689	NAD		Ru5707	NAD
	Ru5690	NAD		Ru5708	NAD
	Ru5691	NAD		Ru5709	NAD
	Ru5692	NAD		Ru5710	NAD
G3A	Ru5693	NAD			
100	Ru5694	NAD			
	Ru5695	NAD			
	Ru5696	NAD			
	Ru5697	NAD			
	Ru5698	NAD			

NAD : No Abnormality Detected

Note: Ophthalmological examination of all acclimatized rats during acclimatization period did not reveal any abnormalities.

APPENDIX 2 contd. Individual Ophthalmological Examination

Group No. Dose (mg/kg/day)	Rat No.	Day 7	Group No. Dose (mg/kg/day)	Rat No.	Day 7
	Males			Males	
G1B	Ru5711	NAD	G4B	Ru5729	NAD
0	Ru5712	NAD	300	Ru5730	NAD
	Ru5713	NAD		Ru5731	NAD
	Ru5714	NAD		Ru5732	NAD
	Ru5715	NAD		Ru5733	NAD
	Ru5716	NAD		Ru5734	NAD
G2B	Ru5717	NAD	G5B	Ru5735	NAD
30	Ru5718	NAD	1000	Ru5736	NAD
	Ru5719	NAD		Ru5737	NAD
	Ru5720	NAD		Ru5738	NAD
	Ru5721	NAD		Ru5739	NAD
	Ru5722	NAD		Ru5740	NAD
G3B	Ru5723	NAD			
100	Ru5724	NAD			
	Ru5725	NAD			
	Ru5726	NAD			
	Ru5727	NAD			
	Ru5728	NAD			

NAD : No Abnormality Detected

Note: Ophthalmological examination of all acclimatized rats during acclimatization period did not reveal any abnormalities.

APPENDIX 3. Individual Body Weights (g)

G1A 0 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5681	334.63	337.19	336.99	344.36	348.49	355.17	355.78
Ru5682	308.02	308.11	307.78	311.06	319.98	323.05	327.62
Ru5683	282.16	286.45	290.34	291.47	297.15	299.52	303.80
Ru5684	327.17	327.78	327.73	334.58	339.92	344.99	349.23
Ru5685	320.56	318.60	322.67	326.84	328.10	339.26	342.14
Ru5686	289.46	292.58	298.04	305.20	307.11	314.92	315.23

APPENDIX 3 contd. Individual Body Weights (g)

G2A 30 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5687	333.62	331.53	347.63	341.16	337.65	342.84	343.83
Ru5688	315.53	318.58	318.58	325.27	332.00	334.84	340.67
Ru5689	304.31	306.11	313.64	311.73	319.58	322.57	329.43
Ru5690	291.90	292.38	297.56	297.45	301.71	309.36	315.10
Ru5691	341.95	340.06	348.75	354.73	357.66	361.35	368.50
Ru5692	285.88	281.81	279.95	287.63	286.88	293.05	299.87

APPENDIX 3 contd. Individual Body Weights (g)

G3A 100 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5693	283.80	278.46	286.36	286.95	290.33	291.89	297.48
Ru5694	327.47	330.64	332.57	333.72	340.12	340.33	346.90
Ru5695	296.73	295.69	301.65	304.89	309.87	310.28	313.93
Ru5696	319.35	322.62	324.67	326.36	330.26	335.15	338.06
Ru5697	309.02	308.88	311.33	315.19	324.43	329.39	336.64
Ru5698	342.45	340.22	348.14	346.35	347.14	350.49	355.97

APPENDIX 3 contd. Individual Body Weights (g)

G4A 300 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5699	308.29	309.07	309.28	313.23	318.10	319.83	320.77
Ru5700	331.01	335.27	340.85	346.02	351.09	357.58	358.53
Ru5701	281.46	281.80	284.75	290.85	290.77	293.60	300.11
Ru5702	313.01	312.37	318.24	322.37	324.39	324.21	330.74
Ru5703	347.45	341.88	348.35	352.45	355.82	359.36	361.29
Ru5704	299.16	296.47	303.55	302.51	302.18	307.51	313.04

APPENDIX 3 contd. Individual Body Weights (g)

G5A 1000 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5705	283.22	279.87	285.58	285.71	285.57	286.45	281.77
Ru5706	311.20	309.70	318.21	317.50	314.24	316.59	316.54
Ru5707	306.05	300.58	299.46	300.73	296.39	298.61	304.32
Ru5708	296.80	293.49	297.56	299.47	300.51	302.66	303.86
Ru5709	316.20	317.97	318.95	317.60	320.84	320.54	324.85
Ru5710	342.46	338.62	335.14	338.05	339.19	344.19	338.12

APPENDIX 3 contd. Individual Body Weights (g)

G1B 0 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5711	291.03	287.67	290.41	296.22	298.92	298.28	300.35
Ru5712	305.75	303.50	312.08	317.92	317.57	325.46	332.03
Ru5713	281.56	275.72	282.03	278.30	281.54	285.42	292.28
Ru5714	332.81	331.89	331.79	337.83	339.47	348.34	348.83
Ru5715	319.25	321.15	324.29	328.99	331.31	335.62	339.33
Ru5716	316.74	319.94	314.93	321.43	323.01	322.26	328.30

APPENDIX 3 contd. Individual Body Weights (g)

G2B 30 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5717	322.33	326.16	325.53	330.30	337.04	342.12	345.50
Ru5718	291.02	308.96	313.77	315.25	321.84	326.69	330.29
Ru5719	299.59	293.15	302.00	303.78	307.01	309.17	309.21
Ru5720	311.48	314.75	314.06	314.81	323.49	326.49	330.79
Ru5721	344.20	337.03	335.85	334.00	340.83	347.80	349.30
Ru5722	312.38	309.44	313.60	317.83	318.27	322.17	322.97

APPENDIX 3 contd. Individual Body Weights (g)

G3B 100 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5723	330.33	330.83	334.00	339.53	341.12	348.53	353.58
Ru5724	296.66	294.91	299.94	303.72	308.30	311.88	313.67
Ru5725	304.84	300.54	304.80	311.58	314.24	319.22	325.08
Ru5726	280.23	276.99	281.02	284.07	288.27	296.45	295.88
Ru5727	344.30	338.74	343.83	350.69	347.34	353.16	358.08
Ru5728	311.54	301.39	302.99	311.53	313.36	318.04	322.70

APPENDIX 3 contd. Individual Body Weights (g)

G4B 300 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5729	321.64	320.14	322.88	326.33	327.94	333.12	333.19
Ru5730	282.14	275.26	279.56	280.72	280.38	284.50	287.24
Ru5731	308.03	304.87	307.97	313.00	315.86	316.73	315.95
Ru5732	344.03	344.06	349.08	349.90	355.52	363.04	365.53
Ru5733	293.70	286.76	293.23	297.47	299.05	303.29	304.53
Ru5734	313.47	312.07	318.68	318.68	319.43	323.17	327.48

APPENDIX 3 contd. Individual Body Weights (g)

G5B 1000 mg/kg/day	Day(s) Relative to Start Date						
	1	2	3	4	5	6	7
Ru5735	315.39	311.42	319.26	317.68	315.15	315.24	311.24
Ru5736	319.17	309.93	305.30	307.61	314.96	312.39	312.09
Ru5737	302.56	294.52	295.41	294.70	280.80	290.71	291.99
Ru5738	340.48	339.00	340.23	337.28	337.17	338.17	337.56
Ru5739	323.50	326.82	337.47	336.76	319.57	314.55	324.16
Ru5740	274.37	272.32	276.30	277.84	276.96	277.50	276.49

APPENDIX 4. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G1A 0 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5681	2.56	-0.20	7.37	4.13	6.68	0.61	21.15
Ru5682	0.09	-0.33	3.28	8.92	3.07	4.57	19.60
Ru5683	4.29	3.89	1.13	5.68	2.37	4.28	21.64
Ru5684	0.61	-0.05	6.85	5.34	5.07	4.24	22.06
Ru5685	-1.96	4.07	4.17	1.26	11.16	2.88	21.58
Ru5686	3.12	5.46	7.16	1.91	7.81	0.31	25.77

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G1A 0 mg/kg/day	Total % Weight Gain
	1 → 7
Ru5681	6.32
Ru5682	6.36
Ru5683	7.67
Ru5684	6.74
Ru5685	6.73
Ru5686	8.90

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G2A 30 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5687	-2.09	16.10	-6.47	-3.51	5.19	0.99	10.21
Ru5688	3.05	0.00	6.69	6.73	2.84	5.83	25.14
Ru5689	1.80	7.53	-1.91	7.85	2.99	6.86	25.12
Ru5690	0.48	5.18	-0.11	4.26	7.65	5.74	23.20
Ru5691	-1.89	8.69	5.98	2.93	3.69	7.15	26.55
Ru5692	-4.07	-1.86	7.68	-0.75	6.17	6.82	13.99

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G2A 30 mg/kg/day	Total % Weight Gain 1 → 7
Ru5687	3.06
Ru5688	7.97
Ru5689	8.25
Ru5690	7.95
Ru5691	7.76
Ru5692	4.89

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G3A 100 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5693	-5.34	7.90	0.59	3.38	1.56	5.59	13.68
Ru5694	3.17	1.93	1.15	6.40	0.21	6.57	19.43
Ru5695	-1.04	5.96	3.24	4.98	0.41	3.65	17.20
Ru5696	3.27	2.05	1.69	3.90	4.89	2.91	18.71
Ru5697	-0.14	2.45	3.86	9.24	4.96	7.25	27.62
Ru5698	-2.23	7.92	-1.79	0.79	3.35	5.48	13.52

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G3A 100 mg/kg/day	Total % Weight Gain 1 → 7
Ru5693	4.82
Ru5694	5.93
Ru5695	5.80
Ru5696	5.86
Ru5697	8.94
Ru5698	3.95

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G4A 300 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5699	0.78	0.21	3.95	4.87	1.73	0.94	12.48
Ru5700	4.26	5.58	5.17	5.07	6.49	0.95	27.52
Ru5701	0.34	2.95	6.10	-0.08	2.83	6.51	18.65
Ru5702	-0.64	5.87	4.13	2.02	-0.18	6.53	17.73
Ru5703	-5.57	6.47	4.10	3.37	3.54	1.93	13.84
Ru5704	-2.69	7.08	-1.04	-0.33	5.33	5.53	13.88

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G4A 300 mg/kg/day	Total % Weight Gain
	1 → 7
Ru5699	4.05
Ru5700	8.31
Ru5701	6.63
Ru5702	5.66
Ru5703	3.98
Ru5704	4.64

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G5A 1000 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5705	-3.35	5.71	0.13	-0.14	0.88	-4.68	-1.45
Ru5706	-1.50	8.51	-0.71	-3.26	2.35	-0.05	5.34
Ru5707	-5.47	-1.12	1.27	-4.34	2.22	5.71	-1.73
Ru5708	-3.31	4.07	1.91	1.04	2.15	1.20	7.06
Ru5709	1.77	0.98	-1.35	3.24	-0.30	4.31	8.65
Ru5710	-3.84	-3.48	2.91	1.14	5.00	-6.07	-4.34

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G5A 1000 mg/kg/day	Total % Weight Gain 1 → 7
Ru5705	-0.51
Ru5706	1.72
Ru5707	-0.57
Ru5708	2.38
Ru5709	2.74
Ru5710	-1.27

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G1B 0 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5711	-3.36	2.74	5.81	2.70	-0.64	2.07	9.32
Ru5712	-2.25	8.58	5.84	-0.35	7.89	6.57	26.28
Ru5713	-5.84	6.31	-3.73	3.24	3.88	6.86	10.72
Ru5714	-0.92	-0.10	6.04	1.64	8.87	0.49	16.02
Ru5715	1.90	3.14	4.70	2.32	4.31	3.71	20.08
Ru5716	3.20	-5.01	6.50	1.58	-0.75	6.04	11.56

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G1B 0 mg/kg/day	Total % Weight Gain
	1 → 7
Ru5711	3.20
Ru5712	8.60
Ru5713	3.81
Ru5714	4.81
Ru5715	6.29
Ru5716	3.65

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G2B 30 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5717	3.83	-0.63	4.77	6.74	5.08	3.38	23.17
Ru5718	17.94	4.81	1.48	6.59	4.85	3.60	39.27
Ru5719	-6.44	8.85	1.78	3.23	2.16	0.04	9.62
Ru5720	3.27	-0.69	0.75	8.68	3.00	4.30	19.31
Ru5721	-7.17	-1.18	-1.85	6.83	6.97	1.50	5.10
Ru5722	-2.94	4.16	4.23	0.44	3.90	0.80	10.59

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G2B 30 mg/kg/day	Total % Weight Gain
	1 → 7
Ru5717	7.19
Ru5718	13.49
Ru5719	3.21
Ru5720	6.20
Ru5721	1.48
Ru5722	3.39

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G3B 100 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5723	0.50	3.17	5.53	1.59	7.41	5.05	23.25
Ru5724	-1.75	5.03	3.78	4.58	3.58	1.79	17.01
Ru5725	-4.30	4.26	6.78	2.66	4.98	5.86	20.24
Ru5726	-3.24	4.03	3.05	4.20	8.18	-0.57	15.65
Ru5727	-5.56	5.09	6.86	-3.35	5.82	4.92	13.78
Ru5728	-10.15	1.60	8.54	1.83	4.68	4.66	11.16

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G3B 100 mg/kg/day	Total % Weight Gain
	1 → 7
Ru5723	7.04
Ru5724	5.73
Ru5725	6.64
Ru5726	5.58
Ru5727	4.00
Ru5728	3.58

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G4B 300 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5729	-1.50	2.74	3.45	1.61	5.18	0.07	11.55
Ru5730	-6.88	4.30	1.16	-0.34	4.12	2.74	5.10
Ru5731	-3.16	3.10	5.03	2.86	0.87	-0.78	7.92
Ru5732	0.03	5.02	0.82	5.62	7.52	2.49	21.50
Ru5733	-6.94	6.47	4.24	1.58	4.24	1.24	10.83
Ru5734	-1.40	6.61	0.00	0.75	3.74	4.31	14.01

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G4B 300 mg/kg/day	Total % Weight Gain
	1 → 7
Ru5729	3.59
Ru5730	1.81
Ru5731	2.57
Ru5732	6.25
Ru5733	3.69
Ru5734	4.47

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G5B 1000 mg/kg/day	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)	Absolute Weight Gain (g)
	1 → 2	2 → 3	3 → 4	4 → 5	5 → 6	6 → 7	1 → 7
Ru5735	-3.97	7.84	-1.58	-2.53	0.09	-4.00	-4.15
Ru5736	-9.24	-4.63	2.31	7.35	-2.57	-0.30	-7.08
Ru5737	-8.04	0.89	-0.71	-13.90	9.91	1.28	-10.57
Ru5738	-1.48	1.23	-2.95	-0.11	1.00	-0.61	-2.92
Ru5739	3.32	10.65	-0.71	-17.19	-5.02	9.61	0.66
Ru5740	-2.05	3.98	1.54	-0.88	0.54	-1.01	2.12

APPENDIX 4 contd. Individual Body Weight Gains (g)

Sex: Male Day(s) Relative to Start Date

G5B 1000 mg/kg/day	Total % Weight Gain 1 → 7
Ru5735	-1.32
Ru5736	-2.22
Ru5737	-3.49
Ru5738	-0.86
Ru5739	0.20
Ru5740	0.77

APPENDIX 5. Individual Food Consumption (g/rat/day)

G1A 0 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5681	25.53	28.07	26.56
Ru5682	25.53	28.07	26.56
Ru5683	25.89	26.99	26.64
Ru5684	25.89	26.99	26.64
Ru5685	25.06	26.14	25.78
Ru5686	25.06	26.14	25.78

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G2A 30 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5687	27.25	26.10	26.28
Ru5688	27.25	26.10	26.28
Ru5689	26.59	26.68	27.95
Ru5690	26.59	26.68	27.95
Ru5691	26.15	28.45	28.00
Ru5692	26.15	28.45	28.00

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G3A 100 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5693	25.81	26.15	25.59
Ru5694	25.81	26.15	25.59
Ru5695	28.45	28.15	25.26
Ru5696	28.45	28.15	25.26
Ru5697	27.69	27.88	28.43
Ru5698	27.69	27.88	28.43

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G4A 300 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5699	27.29	29.78	27.01
Ru5700	27.29	29.78	27.01
Ru5701	28.70	29.37	26.24
Ru5702	28.70	29.37	26.24
Ru5703	28.83	27.32	25.75
Ru5704	28.83	27.32	25.75

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G5A 1000 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5705	27.33	26.01	23.73
Ru5706	27.33	26.01	23.73
Ru5707	25.89	24.20	23.69
Ru5708	25.89	24.20	23.69
Ru5709	28.86	26.57	23.91
Ru5710	28.86	26.57	23.91

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G1B 0 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5711	25.34	26.06	24.35
Ru5712	25.34	26.06	24.35
Ru5713	28.05	25.44	25.28
Ru5714	28.05	25.44	25.28
Ru5715	28.95	27.17	25.42
Ru5716	28.95	27.17	25.42

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G2B 30 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5717	29.17	27.70	27.95
Ru5718	29.17	27.70	27.95
Ru5719	28.22	26.11	24.40
Ru5720	28.22	26.11	24.40
Ru5721	27.19	27.75	25.83
Ru5722	27.19	27.75	25.83

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G3B 100 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5723	29.62	27.76	25.40
Ru5724	29.62	27.76	25.40
Ru5725	25.80	24.63	22.97
Ru5726	25.80	24.63	22.97
Ru5727	27.87	27.52	25.62
Ru5728	27.87	27.52	25.62

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G4B 300 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5729	26.27	24.41	23.30
Ru5730	26.27	24.41	23.30
Ru5731	29.66	30.00	26.64
Ru5732	29.66	30.00	26.64
Ru5733	26.84	27.24	23.99
Ru5734	26.84	27.24	23.99

APPENDIX 5 contd. Individual Food Consumption (g/rat/day)

G5B 1000 mg/kg/day	Day(s) Relative to Start Date		
	1 → 2	2 → 5	5 → 7
Ru5735	22.32	26.98	24.03
Ru5736	22.32	26.98	24.03
Ru5737	27.31	25.22	22.43
Ru5738	27.31	25.22	22.43
Ru5739	29.37	26.51	20.74
Ru5740	29.37	26.51	20.74

APPENDIX 6. Individual Water Consumption (g/rat/day)

G1A 0 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5681	21	22
Ru5682	21	22
Ru5683	20	22
Ru5684	20	22
Ru5685	23	23
Ru5686	23	23

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G2A 30 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5687	20	23
Ru5688	20	23
Ru5689	21	25
Ru5690	21	25
Ru5691	22	25
Ru5692	22	25

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G3A 100 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5693	21	20
Ru5694	21	20
Ru5695	20	21
Ru5696	20	21
Ru5697	22	22
Ru5698	22	22

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G4A 300 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5699	23	25
Ru5700	23	25
Ru5701	21	24
Ru5702	21	24
Ru5703	20	23
Ru5704	20	23

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G5A 1000 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5705	22	22
Ru5706	22	22
Ru5707	20	19
Ru5708	20	19
Ru5709	22	22
Ru5710	22	22

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G1B 0 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5711	22	19
Ru5712	22	19
Ru5713	18	21
Ru5714	18	21
Ru5715	19	18
Ru5716	19	18

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G2B 30 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5717	20	20
Ru5718	20	20
Ru5719	18	17
Ru5720	18	17
Ru5721	17	19
Ru5722	17	19

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G3B 100 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5723	18	19
Ru5724	18	19
Ru5725	18	18
Ru5726	18	18
Ru5727	18	19
Ru5728	18	19

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G4B 300 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5729	18	18
Ru5730	18	18
Ru5731	19	21
Ru5732	19	21
Ru5733	20	21
Ru5734	20	21

APPENDIX 6 contd. Individual Water Consumption (g/rat/day)

G5B 1000 mg/kg/day	Day(s) Relative to Start Date	
	1 → 5	5 → 7
Ru5735	18	17
Ru5736	18	17
Ru5737	20	19
Ru5738	20	19
Ru5739	15	15
Ru5740	15	15

APPENDIX 7. Dose Formulation Analysis Report

DOSE FORMULATION ANALYSIS REPORT

ACETAMIDE: 7-DAY REPEAT DOSE ORAL TOXICITY STUDY IN WISTAR RATS WITH TOXICOGENOMICS

STUDY No.: G16084

SPONSOR

MICHIGAN STATE UNIVERSITY
COLLEGE OF ENGINEERING, MICHIGAN STATE UNIVERSITY
EAST LANSING
MICHIGAN 48824

TEST FACILITY

DEPARTMENT OF DRUG METABOLISM,
PHARMACOKINETICS AND CLINICAL PHARMACOLOGY
EUROFINS ADVINUS LIMITED
POST BOX No. 5813, PLOT Nos. 21 & 22
PEENYA II PHASE, BENGALURU 560 058, INDIA

APPENDIX 7 Contd. Dose Formulation Analysis Report

DECLARATION

This is to declare that the formulation analytical work was performed under the supervision of Formulation Analyst and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the formulation analytical work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Formulation Analyst accepts overall responsibility for the technical conduct of the formulation analysis as well as the interpretation, data analysis, documentation and reporting of the results.

Alagappan L., M. Pharm.,
Formulation Analyst

23 December 2019
Date

APPENDIX 7 Contd. Dose Formulation Analysis Report

LIST OF COMMONLY USED ABBREVIATIONS AND SYMBOLS

%	:	per cent
°C	:	Degree celsius
µg	:	Microgram
µL	:	Microlitre
µm	:	Micrometre
A.I.	:	Active Ingredient
DMPK &CP	:	Drug Metabolism Pharmacokinetics & Clinical Pharmacology
DMSO	:	Dimethyl Sulfoxide
g	:	Gram
HPLC	:	High Performance Liquid Chromatography
i.d.	:	Internal diameter
mg	:	Milligram
mL	:	Millilitre
mm	:	Millimetre
nm	:	Nanometre
No.	:	Number
RSD	:	Relative Standard Deviation
SD	:	Standard Deviation
UV	:	Ultraviolet
v/v	:	Volume/volume
WSTD	:	Working Standard

APPENDIX 7 Contd. Dose Formulation Analysis Report

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APPENDIX 7 Contd. Dose Formulation Analysis Report

1. SUMMARY

The dose formulations were analyzed for active ingredient concentration using a validated analytical method G16081 to support the Eurofins Advinus Study G16084. No test item peak was detected in the vehicle samples at the retention time of Acetamide. The mean concentration of Acetamide prepared at 3, 10, 30 and 100 mg/mL ranged from 92.33 to 105.58% and was within ± 15% of the nominal concentration. The relative standard deviation (% RSD) at each dose level was less than 15 % (5.61 to 9.17%). This indicates that the prepared dose formulations met the acceptance criteria for active ingredient concentration.

APPENDIX 7 Contd. Dose Formulation Analysis Report

2. LIST OF SCIENTISTS / PROFESSIONALS

Study Director	D. P. Santhosh kumar, M.Sc., Ph.D., ERT Department of Safety Assessment
Formulation analysis in-charge and Bioanalyst	L. Alagappan, M.Pharm. DMPK & CP
Sample preparation and operator of instrument	Rajib Satpathy, M.Sc. DMPK & CP
Freezer / Deepfreezer custodian	R. V. Venkatesh, M.Sc. DMPK & CP

3. DOSE FORMULATION ANALYSIS SCHEDULE

Sample receipt date	Sample analysis date	Remarks
30 January 2018	30 January 2018	Data accepted

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Reagents

- Acetamide, Test-item (Used as standard)
- Propionamide (Internal standard)
- Milli-Q water
- Methanol
- Ethyl acetate
- Sodium chloride
- 0.5M HCl: Prepared by adding 20.625 mL of HCl solution (12.1M or ~37%) to 500 mL methanol in a glass bottle.
- 0.7M Potassium hydroxide: Prepared by dissolving 9.88988g of potassium hydroxide in 250 mL of water.
- Xanthydrol 0.25%: Weighed 126.05 mg of Xanthydrol and dissolved in 50 mL of methanol. This solution was protected from light and stored in a Refrigerator (2 to 8 °C). This solution was used within 7 days from the date

of preparation.

- Solvents, chemicals and reagents of high purity

4.1.2 Apparatus

- Analytical balance having readability ± 0.01 mg or better.
- Ultra-sonicator
- Gas Chromatography (GC) equipped with mass spectrometer (MS)
- Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm Film Thickness

4.1.3 Glassware

- Volumetric Flasks: 5, 10, and 25 mL
- Measuring Cylinders: 500 and 1000 mL
- Micro Pipettes: 100 to 1000 µL, 20 to 200µL
- HPLC glass vials (2 mL capacity) with caps
- Beakers: 50 mL, 100 mL

4.1.4 Preparation of Stock solutions and its dilutions

Stock Solution of Acetamide: Accurately weighed 10.021 mg of Acetamide and transferred into a 10 mL volumetric flask. Milli-Q water was added to the flask and the contents were dissolved by vortexing. Then the volume was made up to the mark using Milli-Q water and the content was mixed thoroughly. This resulted in a stock solution of about 1002.10 µg/mL.

Working Standard Solution: Aliquots of 0.025, 0.063, 0.125, 0.250, 0.500 and 0.625 mL of the stock solution of Acetamide were added in respective order in to 25 mL volumetric flask. The solutions were made up to mark with Milli-Q water. These resulted in the working standard solutions at concentrations of about 1.00, 2.53, 5.01, 10.02, 20.04 and 25.05 µg/mL of Acetamide.

4.1.5 Preparation of Sample Solutions

Samples of dose formulations were taken and diluted with diluent in such a way to get the final concentration of about 10 µg/mL.

- **G1 (0 mg/mL):** Diluted 0.1 mL of the sample by adding 0.20 mL of Milli-Q water to give intermediate sample. Diluted 0.250 mL of the intermediate

sample to 25 mL with Milli-Q water.

- **G2 (3 mg/mL):** Diluted 0.1 mL of the sample by adding 0.20 mL of Milli-Q water to give intermediate sample (1 mg/mL). Diluted 0.250 mL of the intermediate sample to 25 mL with Milli-Q water.
- **G3 (10 mg/mL):** Diluted 0.1 mL of the sample by adding 0.90 mL of Milli-Q water to give intermediate sample (1 mg/mL). Diluted 0.250 mL of the intermediate sample to 25 mL with Milli-Q water.
- **G4 (30 mg/mL):** Diluted 0.1 mL of the sample by adding 2.90 mL of Milli-Q water to give intermediate sample (1 mg/mL). Diluted 0.250 mL of the intermediate sample to 25 mL with Milli-Q water.
- **G5 (100 mg/mL):** Diluted 0.1 mL of the sample to 10 mL with Milli-Q water to give intermediate sample (1 mg/mL). Dilute 0.250 mL of the intermediate sample to 25 mL with Milli-Q water.

4.1.6 Preparation of Internal standard Working Solutions

Internal Standard Stock Solution of Propionamide: 10.145 mg of Propionamide reference standard was weighed and transferred into a 10 mL volumetric flask. Methanol was added to the flask and the content was dissolved by vortexing. Then the volume was made up to the mark using methanol and the content was mixed thoroughly. This resulted in a stock solution of about 1010 µg/mL.

Internal Standard Working Solution: Aliquot of 1.490 mL of the stock solution of Propionamide was transferred in to 10 mL volumetric flask. The volume was made up to mark with methanol. This resulted in the working solution at concentrations of about 150 µg/mL of Propionamide.

4.1.7 Derivatization Procedure

1. Transferred accurately 2.5 mL of standard/samples in to a 15 mL polypropylene centrifuge tube.
2. A volume of 50 µL of internal standard (150 µg/mL propionamide in methanol) solution was added to each sample and vortexed to mix.
3. A volume of 2.5 mL of 0.5M HCl in methanol was added to each tube and sample was vortexed to mix.
4. Then 200 µl of xanthydrol (0.25% solution) was added, vortexed to mix and incubate in darkness at 40 ºC for about 90 minutes.
5. After incubation, about 3 g of sodium chloride was added to each tube.
6. All the samples were neutralized by adding 2.0 mL of 0.7 M KOH.
7. Then 3.0 mL of ethyl acetate was added to each tube. All the tubes were vortexed,

sonicated and centrifuged at 4,000 rpm at 4 °C for 5 min.

8. Transferred 1.7 mL of supernatant from each sample to a new RIA vial.
9. All the samples were placed on a nitrogen evaporator until all of the ethyl acetate is been removed.
10. Then 250 µL of ethyl acetate was added to each RIA vial. Vortexed all the samples and centrifuged at 4000 rpm at 4 °C for 5 min.
11. Transferred 150 µL of the supernatant in to an insert placed in the GC vial for analysis by GC-MS.

4.1.8 Chromatographic Conditions

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.

Carrier Gas: Helium

Flow Rate: 1.2 mL/min

Column Oven Temperature:

40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.

Injector Temperature: 250°C

Injection volume: 1 µL

Mass Spectrometry: SIM Mode
Solvent Delay Time: 10.0 min
Ion source temperature: 200°C
Interface temperature: 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5

Mode: Splitless

Run Time: 25 minutes

- All the parameters were maintained constant throughout the analysis.
- The system suitability was determined before each day's sample analysis using working standard solutions.
- Peak area of Acetamide reference standard for each injection was recorded.
- Acetamide peak in the dose formulation sample was identified by comparing the retention time of analyte peak in the standard.
- Calibration curve was prepared for Acetamide by plotting peak area ratio

versus concentration (µg/mL, after correcting for purity) for each standard.

- Best-line fit equation (Y = a + bX) was calculated using the method of least square. In this equation, 'Y' is peak area, 'X' is concentration (µg/mL), 'a' is -intercept and 'b' is the slope of the line.

4.1.9 Calculation

$$\text{Concentration in the sample solution } (\mu g/mL) = \frac{\text{Sample peak area - } a}{b}$$

$$\text{Calculated test item concentration } (mg/mL) = \frac{\text{Concentration in the sample solution } (\mu g/mL)}{1000} \times \frac{\text{Dilution } (mL)}{\text{Sample Volume } (mL)}$$

4.2 System Suitability

System suitability of the instrument was calculated by checking the system precision.

The Chronology of system suitability test was as below:

- One diluent injection.
- Three injections of the highest working standard solution.
- One diluent injection.

System precision was checked by calculating relative standard deviation (% RSD) from the mean peak area values and retention time of three replicate injections of a highest working standard (each peak) solution and the same was not more than 15% in each case. Diluent, injected at the end of system suitability test, did not show injection carryover (was not more than 10.0% of the mean standard response).

APPENDIX 7 Contd. Dose Formulation Analysis Report

5. RESULTS

The list of analytical runs is presented in TABLE 1 and all data for the analysis were found to be acceptable.

5.1 System Suitability

System suitability test results are presented in TABLE 2. All the System suitability test parameters on the day of analysis met the study plan requirements for:

- % RSD of area and retention time of five consecutive injections of working standard was less than 15%.
- Diluent injected at the end of system suitability test did not show injection carryover.

The representative chromatograms of diluent, vehicle, CC standard solution and a representative formulation sample are presented in FIGURE 1 to FIGURE 4, respectively.

5.2 Dose Formulation Sample Analysis

The dose formulation samples prepared on Day 1 at 0, 3, 10, 30 and 100 mg/mL were received from the Department of Safety Assessment for determination of concentration of the test item.

All the dose formulation samples were analyzed using a validated analytical method (Eurofins Advinus Study number G16082).

Peak area observed in the vehicle control sample at the retention time of Acetamide was less than 10% of the peak area in system suitability sample and met the acceptance criteria as per study plan. The mean concentration of prepared at 3, 10, 30 and 100 mg/mL ranged from 92.33 to 105.58% and was within ± 15% of the nominal concentration. The relative standard deviation (% RSD) at each dose level was less than 15% (5.61 to 9.17%). This indicates that the prepared dose formulations met the acceptance criteria for concentration. The results are presented in TABLE 3.

APPENDIX 7 Contd. Dose Formulation Analysis Report

6. CONCLUSION

The dose formulations were analyzed for concentration of Acetamide using a validated analytical method G16082 to support the Advinus Study G16084. Vehicle control sample found to be free of any significant presence of test-item. The mean concentration of Acetamide prepared at 3, 10, 30 and 100 mg/mL was within ± 15% of the nominal concentration and the relative standard deviation (% RSD) at each dose level was less than 15 %. This indicates that the prepared dose formulations met the acceptance criteria for Acetamide concentration.

APPENDIX 7 Contd. Dose Formulation Analysis Report

7. TABLES

APPENDIX 7 Contd. Dose Formulation Analysis Report

TABLE 1. List of Analytical runs

Date of experiment	Runs	Remarks
30 January 2018	System suitability and Day 1 formulation analysis	Batch acquisition interrupted due to instrument malfunction
31 January 2018	System suitability and Day 1 formulation analysis	Data accepted

TABLE 2. System Suitability and Standard Check Tests

Date	% RSD for peak area ratio	% RSD for retention time for analyte	% RSD for retention time for IS
31 January 2018	0.09	0.00	0.00
Acceptance Criteria	*<15*	*<15*	*<15*

TABLE 3. Concentration of Acetamide in Dose Formulation Samples

Nominal Concentration (mg/mL)	Sample details	Obtained Concentration (mg/mL)	Mean Obtained Concentration (mg/mL)	SD	% RSD	% Agreement	Mean % Agreement
0	G1R1	BLQ	BLQ	NA	NA	NA	NA
	G1R2	BLQ					
	G1R3	BLQ					
	G1R4	BLQ					
3.00	G2R1	2.58	2.79	0.233	8.35	86.00	92.83
	G2R2	3.07				102.33	
	G2R3	2.88				96.00	
	G2R4	2.61				87.00	
10.0	G3R1	8.90	9.65	0.881	9.13	89.00	96.48
	G3R2	9.01				90.10	
	G3R3	9.89				98.90	
	G3R4	10.79				107.90	
30.0	G4R1	32.80	30.94	2.139	6.91	109.33	103.13
	G4R2	27.95				93.17	
	G4R3	30.91				103.03	
	G4R4	32.09				106.97	
100.0	G5R1	98.43	105.96	5.924	5.59	98.43	105.96
	G5R2	105.65				105.65	
	G5R3	106.90				106.90	
	G5R4	112.86				112.86	

BLQ: Below the limit of quantitation

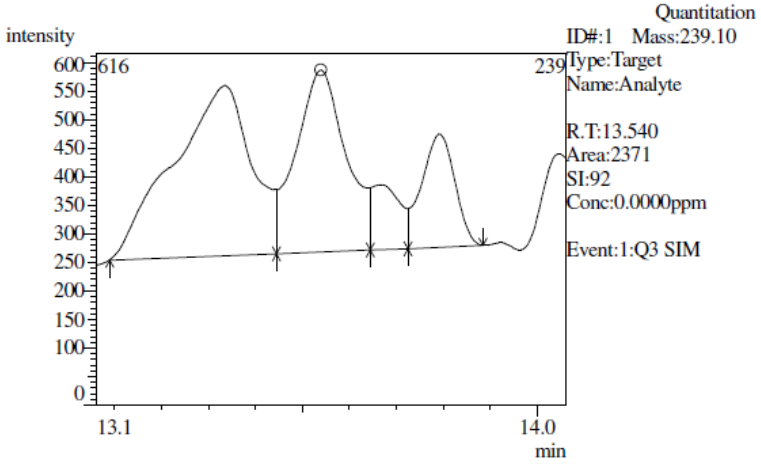
8. FIGURES

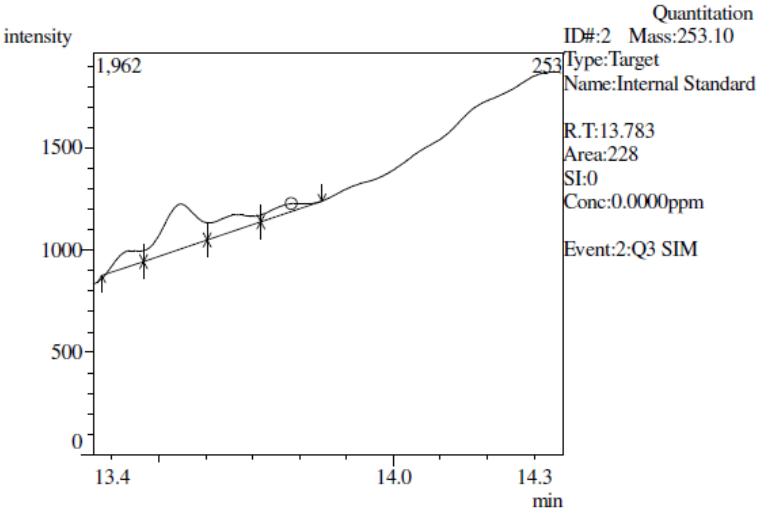
APPENDIX 7 Contd. Dose Formulation Analysis Report

FIGURE 1. Chromatogram for Diluent

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 1/31/2018 7:55:44 PM
Sample Name	: G16084-SSA-Diluent
Vial #	: 5
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-Acetamide-Study\310118-G16084-Formulation SSA-008.qgd
Method File	: E:\GCMSMS\G16084-Acetamide-Study\Acetamide_2_p.qgm
Tuning File	: E:\GCMSMS\G16084-Acetamide-Study\Tune_Column_CID-off_310118.qgt





Quantitative Result Table

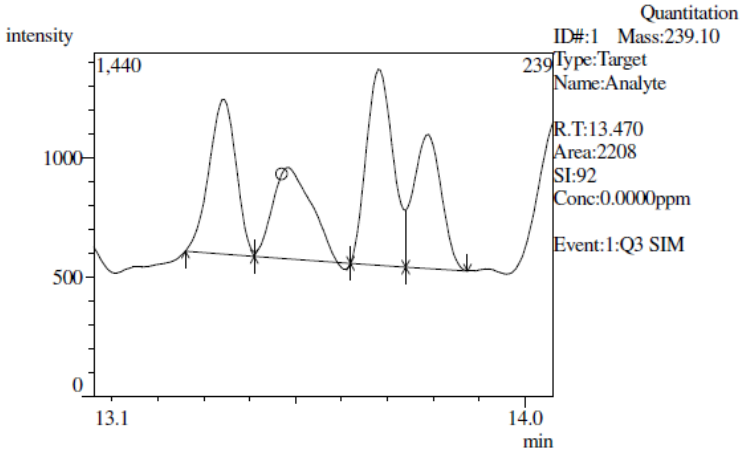
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Analyte	13.540	239.10	2371	317	0
2	Internal Standard	13.783	253.10	228	38	0

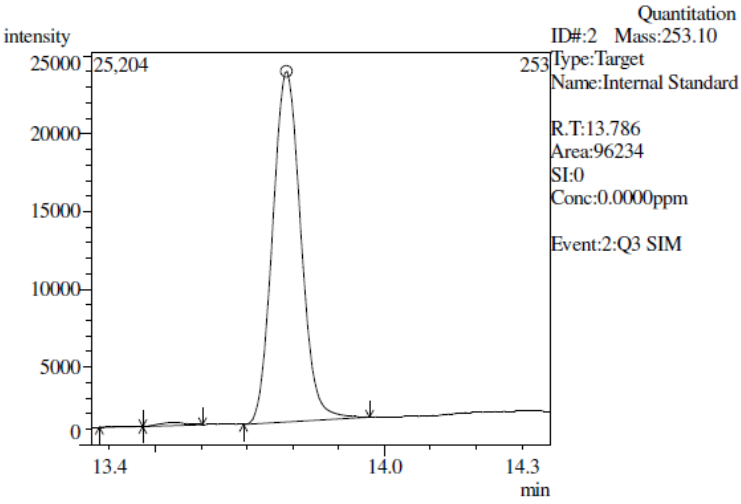
APPENDIX 7 Contd. Dose Formulation Analysis Report

FIGURE 2. Chromatogram for Vehicle

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 1/31/2018 9:38:58 PM
Sample Name	: G16084-SSA-G1-R2
Vial #	: 8
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-Acetamide-Study\310118-G16084-Formulation SSA-011.qgd
Method File	: E:\GCMSMS\G16084-Acetamide-Study\Acetamide_2_p.qgm
Tuning File	: E:\GCMSMS\G16084-Acetamide-Study\Tune_Column_CID-off_310118.qgt





Quantitative Result Table

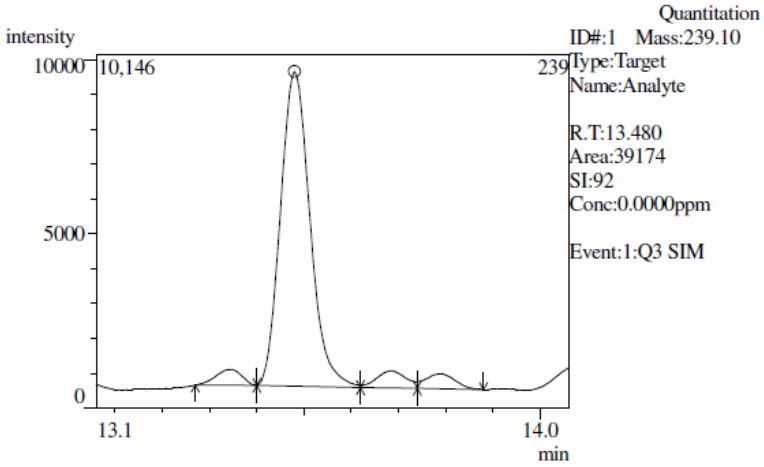
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Analyte	13.470	239.10	2208	353	0
2	Internal Standard	13.786	253.10	96234	22534	0

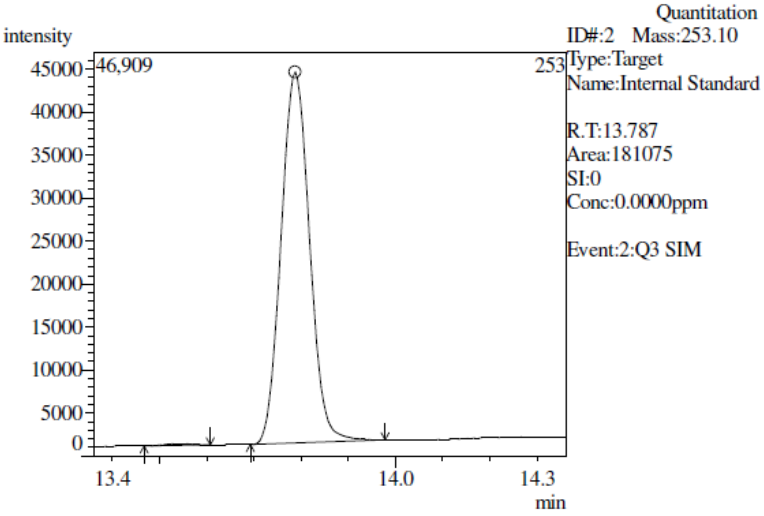
APPENDIX 7 Contd. Dose Formulation Analysis Report

FIGURE 3. Chromatogram for CC Standard Solution (1.00 µg/mL)

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 1/31/2018 8:30:14 PM
Sample Name	: G16084-SSA-CS1
Vial #	: 6
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-Acetamide-Study\310118-G16084-Formulation SSA-009.qgd
Method File	: E:\GCMSMS\G16084-Acetamide-Study\Acetamide_2_p.qgm
Tuning File	: E:\GCMSMS\G16084-Acetamide-Study\Tune_Column_CID-off_310118.qgt





Quantitative Result Table

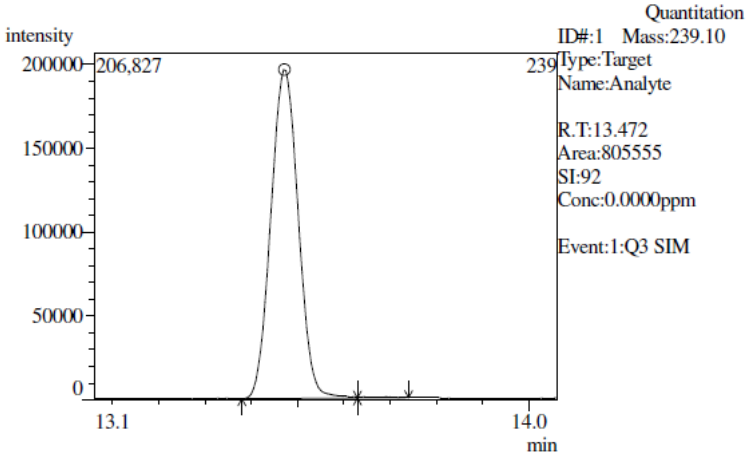
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Analyte	13.480	239.10	39174	9035	0
2	Internal Standard	13.787	253.10	181075	43163	0

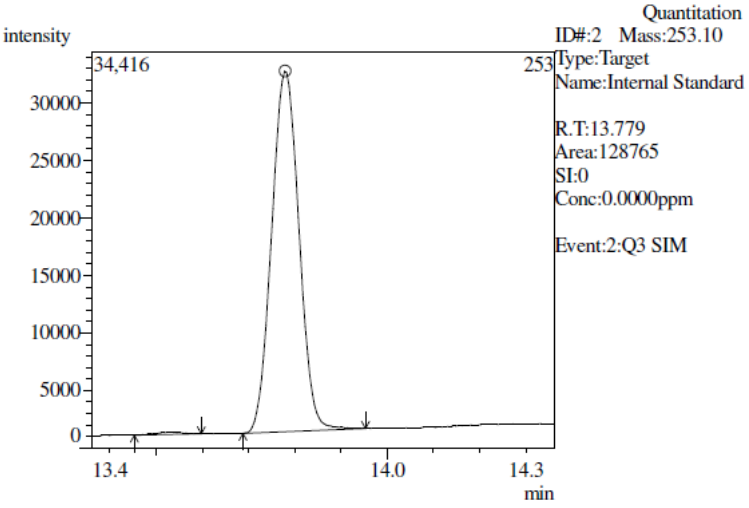
APPENDIX 7 Contd. Dose Formulation Analysis Report

FIGURE 4. Chromatogram for CC Standard Solution (25.00 µg/mL)

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 2/1/2018 10:50:02 AM
Sample Name	: G16084-SSA-CS6
Vial #	: 31
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-Acetamide-Study\310118-G16084-Formulation SSA-034.qgd
Method File	: E:\GCMSMS\G16084-Acetamide-Study\Acetamide_2_p.qgm
Tuning File	: E:\GCMSMS\G16084-Acetamide-Study\Tune_Column_CID-off_310118.qgt





Quantitative Result Table

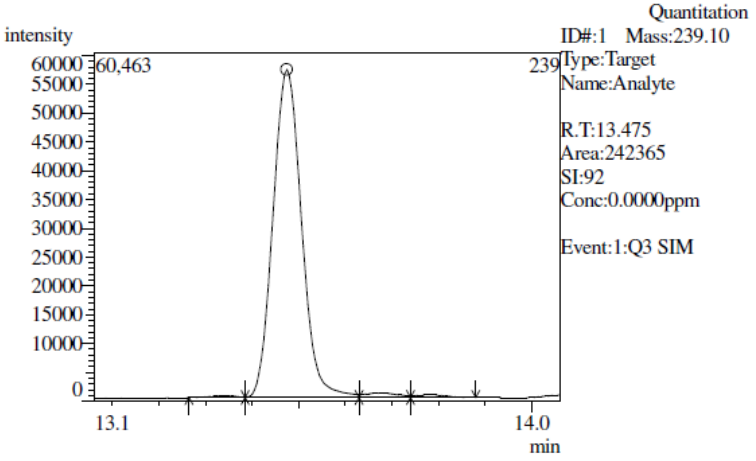
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Analyte	13.472	239.10	805555	196116	0
2	Internal Standard	13.779	253.10	128765	31361	0

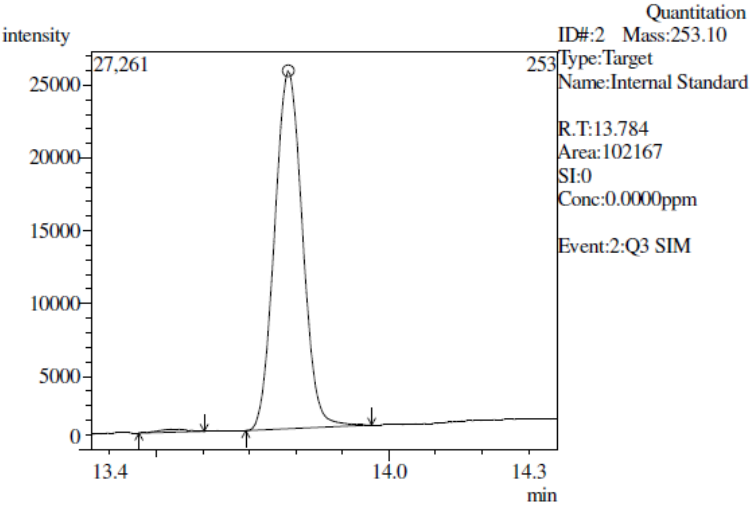
APPENDIX 7 Contd. Dose Formulation Analysis Report

FIGURE 5. Chromatogram for Acetamide in Dose Formulation Sample

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 2/1/2018 3:57:49 AM
Sample Name	: G16084-SSA-G3-R3
Vial #	: 19
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-Acetamide-Study\310118-G16084-Formulation SSA-022.qgd
Method File	: E:\GCMSMS\G16084-Acetamide-Study\Acetamide_2_p.qgm
Tuning File	: E:\GCMSMS\G16084-Acetamide-Study\Tune_Column_CID-off_310118.qgt





Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Analyte	13.475	239.10	242365	56961	0
2	Internal Standard	13.784	253.10	102167	24539	0

9. APPENDIX

APPENDIX 7 Contd. Dose Formulation Analysis Report

APPENDIX 1. Certificate of Analysis for Acetamide

Certificate of Analysis

Dec 19, 2017 (JST)

TOKYO CHEMICAL INDUSTRY CO.,LTD.
4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan

Chemical Name: Acetamide		
Product Number: A0007 CAS: 60-35-5	Lot: XHH4A	

Tests	Results	Specifications
Purity(GC)	100.0 %	min. 98.0 %
Melting point	81.4 deg-C	80.0 to 84.0 deg-C
Solubility in Water	transparency	almost transparency

TCI Lot numbers are 4-5 characters in length. Characters listed after the first 4-5 characters are control numbers for internal purpose only.
The contents of the specifications are subject to change without advance notice. The specification values displayed here are the most up to date values. There may be cases where the product labels display a different specification, however, the product quality still meets the latest specification.

Customer service:
TCI Chemicals (India) Pvt. Ltd.
Tel: 044-2262 0909 / 044-2262 8878
Fax: 044-2262 8902
E-mail: Sales-IN@TCIchemicals.com

APPENDIX 7 Contd. Dose Formulation Analysis Report

APPENDIX 2. Certificate of analysis of Internal standard

Certificate of Analysis

Printed on Jan 8, 2018 (JST)

TCI CHEMICALS (INDIA) PRIVATE LTD.
Plot No.B28, Phase II, 5th Cross Street,MEPZ-SEZ, Tambaram,
Chennai - 600 045

Chemical Name: Propionamide

Product Number: P0499 CAS: 79-05-0	Lot: FIL01	Amount :

Tests	Results	Specifications
Purity(GC)	100.0 %	min. 98.0 %
Purity(Total Nitrogen)	100.6 %	min. 98.0 %
Melting point	81.6 deg-C	77.0 to 82.0 deg-C
Solubility in Water	transparency	almost transparency

Customer service:
TCI CHEMICALS (INDIA) PRIVATE LTD.
Tel :91-44-2262 0909
Fax :91-44-2262 8902
Email : sales-IN@TCIchemicals.com

*Suggested Re-test Date : 1 Year from the date of Invoice
*Quality Complaint Deadline : Within one month upon Invoice Date
* Invoice Dated : Dec/11/2017

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APPENDIX 8. Bioanalytical Report

BIOANALYTICAL REPORT

STUDY TITLE

ACETAMIDE: 7-DAY REPEAT DOSE ORAL TOXICITY STUDY IN WISTAR RATS WITH TOXICOGENOMICS

STUDY No.: G16084

BIOANALYST

L. Alagappan, M.Pharm

SPONSOR

MICHIGAN STATE UNIVERSITY
COLLEGE OF ENGINEERING, MICHIGAN STATE UNIVERSITY
EAST LANSING
MICHIGAN 48824

TEST FACILITY

DEPARTMENT OF DRUG METABOLISM,
PHARMACOKINETICS AND
CLINICAL PHARMACOLOGY
EUROFINS ADVINUS LIMITED
POST BOX No. 5813, PLOT Nos. 21 & 22
PEENYA II PHASE, BENGALURU 560 058, INDIA

APPENDIX 8 contd. Bioanalytical Report

DECLARATION

The Bioanalyst hereby declares that the work was performed under supervision of bioanalyst and in accordance with the described procedures. It is assured that the reported results represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study. Analysis of water, feed, urine samples for Acetamide concentrations and analysis of plasma samples for N-hydroxyacetamide concentrations were performed in a Non-GLP environment.

The Bioanalyst accepts overall responsibility for the technical conduct of the bioanalytical report as well as the interpretation, analysis, documentation and reporting of the results.

L. Alagappan, M.Pharm
Bioanalyst

23 December 2019
Date

APPENDIX 8 contd. Bioanalytical Report

LIST OF COMMONLY USED ABBREVIATIONS AND SYMBOLS

BA	Bioanalysis
BLQ	Below limit of quantification
CC	Calibration curve
CV	Coefficient of Variation
DMPK & CP	Department of Drug Metabolism, Pharmacokinetics and Clinical Pharmacology
DQC	Dilution quality control
GD	Gestation day
GLP	Good Laboratory Practice
HEM	High
HQC	High quality control
IS	Internal standard
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LLOQ	Lower limit of quantification
LLOQQC	Lower limit of quantification quality control
LQC	Low Quality Control
mL	Milliliter
MQC	Middle quality control
N/No.	Number
ng	Nanogram
QC	Quality Control
r^2	Coefficient of Determination
SD	Standard Deviation
SOP	Standard Operating Procedure
STD	Standard
ULOQ	Upper limit of quantification
ULOQQC	Upper limit of quantification quality control
°C	Degree Celsius
%	Per cent

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1. LIST OF SCIENTISTS / PROFESSIONALS

Study Director	D. P. Santhosh kumar, M.Sc., Ph.D., ERT Department of Safety Assessment
Bioanalyst	L. Alagappan, M.Pharm. DMPK & CP
Sample preparation and operator of instrument	Rajib Satpathy, M.Sc. DMPK & CP
Sample preparation and operator of instrument	Sayon Kumar Ghosh, M.Sc. DMPK & CP
Sample preparation and operator of instrument	Pratiksha Kambale, MS.Pharm. DMPK & CP
Freezer / Deepfreezer custodian	R. V. Venkatesh, M.Sc. DMPK & CP
Standby Freezer / Deepfreezer custodian	Murali K Rangain, M.Sc. DMPK & CP

2. BIOANALYSIS SCHEDULE

Date of plasma sample receipt	:	09 February 2018
Date of first plasma sample analysis	:	09 March 2018
Date of last plasma sample analysis	:	20 March 2018
Date of liver sample receipt	:	09 February 2018
Date of first liver sample analysis	:	15 March 2018
Date of last liver sample analysis	:	22 March 2018
Date of urine sample receipt	:	21 December 2018
Date of first urine sample analysis	:	07 January 2019
Date of last urine sample analysis	:	26 February 2019
Date of water and feed sample receipt:		03 January 2019
Date of water sample analysis	:	07 January 2019
Date of feed sample analysis	:	10 January 2019
Date of plasma samples analysis for N-hydroxtacetamide	:	15 February 2019

APPENDIX 8 contd. Bioanalytical Report

3. BIOANALYSIS CONDUCT

This bioanalysis of plasma and liver samples were performed as per Good Laboratory Practice (GLP) Regulations and was audited by the Quality Assurance Unit, Eurofins Advinus Limited. Analysis of water, feed, urine samples for Acetamide concentrations and analysis of plasma samples for N-hydroxyacetamide concentrations were performed in a Non-GLP environment. All appropriate study documentation is maintained in the Bioanalytical study file.

4. INTRODUCTION

Plasma samples collected 24 hours after dosing on day 7 (i.e., on day 8), liver tissue samples collected on day 8, urine samples collected on day 7 from the male and female rats, water and feed samples were received from the Department of Safety Assessment, Eurofins Advinus Limited, from Study No. G16084, Study title: Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats with Toxicogenomics.

A total of 60 each of plasma and liver tissue samples and 30 urine samples were received in frozen and good condition. All samples were stored at -70°C until analysis. One each of water and feed sample were received in good condition and the samples were stored at -20°C until analysis.

5. MATERIALS AND METHODS

5.1 Bioanalytical Method

The plasma samples were analyzed for Acetamide concentrations as per the bioanalytical method validated in Eurofins Advinus Study No. G16082 and as described in the Eurofins Advinus's SOP. The liver samples were analyzed as per the bioanalytical method validated in Eurofins Advinus Study No. G16083 and as described in the Eurofins Advinus's SOP. Urine, water and feed samples were analyzed for Acetamide concentrations using fit-for-purpose GC-MS method. Bioanalytical method for estimation of Acetamide in plasma, liver, urine, water and feed samples are presented in Appendix 4 to Appendix 8. Plasma samples were analyzed for N-hydroxyacetamide using fit-for-purpose colorimetric method. Bioanalytical method for estimation of N-hydroxyacetamide in plasma is presented in Appendix 9.

APPENDIX 8 contd. Bioanalytical Report

5.2 Reference Materials

Reference Standard Information:

Name of the compound	:	Acetamide-2,2,2-D3
Name to be used in study	:	Acetamide D3 (Surrogate standard for Acetamide)
Batch No.	:	CS/SI/AAA-0680/01
CAS No.	:	23724-60-9
Source	:	Clearsynth Labs Ltd. 17, Lotus Business park, Off New Link road, Andheri (W) Mumbai – 400053, India
Date of analysis	:	22/8/2017
Retest Date	:	22/8/2019
Purity by GC	:	99.77 %
Physical Appearance	:	White Crystals
Recommended Storage Condition	:	Refrigerator (+2 to +8ºC)

Internal standard

Compound name	:	Propionamide
Batch no.	:	FIL01
Source	:	TCI chemicals (India) private Ltd., Plot B28, Phase II, 5th cross street, MEPZ-SEZ, Tambaram, Chennai - 600045
Retest date	:	11 Dec 2018
Purity (GC)	:	100%
Recommended storage condition	:	Ambient, Protect from moisture

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N-hydroxyacetamide:

Name	:	N-hydroxyacetamide (Alternate name: Acetohydroxamic acid)
CAS No.	:	546-88-3
Batch no.	:	MKCF9793
Source	:	Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103, USA
QC Release date	:	28 Feb 2018
Purity	:	98%
Storage condition	:	2 to 8 °C

5.3 Blank matrices

The blank Wistar rat plasma was used for preparing calibration curve (CC) standards, quality control (QC) samples and to dilute the samples during the plasma samples analysis. The blank Wistar rat liver homogenate was used for preparing CC standards, QC samples and to dilute the samples during the liver samples analysis. Milli-Q water was used for preparing CC standards, QC samples during the water samples analysis. The blank Wistar rat urine was used for preparing CC standards, QC samples during the urine samples analysis. Milli-Q water was used to dilute the urine samples. Grinded feed was used to prepare CC standards and QC sample during feed analysis.

5.4 Analysis of Plasma Samples

Plasma samples were analyzed from 09 March 2018 to 20 March 2018. The analytical batches included extracted blank (duplicate), standard zero (duplicate), calibration curve standards, quality control samples and study samples. System suitability was performed on prior to sample analysis.

5.5 Dilution Integrity in plasma (20 folds)

A 5 folds dilution was established as part of the method validation, however as some plasma samples required a higher dilution, a 20 folds dilution was evaluated as part of the plasma sample analysis in Run 6. DQC samples (n=6) with a dilution factor of 20 were analyzed using the following acceptance crietria: 4 out of 6 samples (67% samples) should have calculated concentration values within ±15% of the nominal concentration, and the % CV should not exceed 15%. Results of %variability of the calculated concentrations of all QC samples were within ±15% for all the 6 QC samples and %CV of the calculated concentrations

was 2.89, which met the criteria, qualifying the 20 folds dilution for all up-coming sample analysis.

5.6 Analysis of Liver Samples

One part of liver tissue was homogenized with four parts of phosphate buffered saline pH 7.4 to give liver homogenate. Liver samples were analyzed 15 March 2018 to 22 March 2018. The analytical batches included extracted blank (duplicate), standard zero (duplicate), calibration curve standards, quality control samples and study samples. System suitability was performed on prior to sample analysis.

5.7 Analysis of Urine Samples

Urine samples were analyzed for Acetamide concentrations using Acetamide D3 as surrogate calibration standard and propionamide as internal standard. Samples were derivatized using xanthydrol and analyzed using fit-for-purpose GC-MS method. Samples were analyzed from 07 January 2019 to 26 February 2019. The analytical batches included extracted blank (duplicate), standard zero (duplicate), calibration curve standards, quality control samples and study samples. System suitability was performed on prior to sample analysis. Urine samples were initially analyzed with a CC range of 0.1 to 50 µg/mL and all the urine samples from dosed animals were found to be much above the limit of quantification. Hence these samples were reanalyzed with a revised CC range of 1 to 500 µg/mL with appropriate dilution of samples.

5.8 Analysis of Water Samples

Water sample was analyzed for Acetamide concentrations covering a CC range of 0.1 to 50 µg/mL using Acetamide D3 as surrogate calibration standard and propionamide as internal standard. Samples were derivatized using xanthydrol and analyzed using fit-for-purpose GC-MS method. Water sample was analyzed in triplicate on 07 January 2019. The analytical batch included blank sample (duplicate), standard zero (duplicate), calibration curve standards, quality control samples and study samples. System suitability was performed on prior to sample analysis.

5.9 Analysis of Feed Samples

Feed sample was analyzed for Acetamide concentrations covering a CC range of 0.1 to 50 µg/mL using Acetamide D3 as surrogate calibration standard and propionamide as internal standard. Samples were derivatized using xanthydrol and analyzed using fit-for-purpose GC-MS method. Feed sample was analyzed in triplicate on 10 January 2019 against a set of calibration standard. Recovery of Acetamide from feed was evaluated by fortifying Acetmide at MQC concentration. System suitability was performed on prior to sample analysis.

APPENDIX 8 contd. Bioanalytical Report

5.10 Analysis of plasma samples for N-hydroxyacetamide

Plasma samples were analyzed for N-hydroxyacetamide using fit-for-purpose colorimetric method covering a CC range of 5 to 100 µg/mL, applying blank substraction at 515 nm. Plasma samples were analyzed on 15 February 2019.

5.11 Data Evaluation

Calibration curves were obtained by plotting the peak area ratio of test item to internal standard against the amount of test item added. A weighted ($w = 1/x^2$, where x is concentration) least squares regression analysis was used to obtain a linear equation over the range of the calibration. The origin was not used in the standard curve calculations. Watson LIMS version 7.2 was used for data regression for Acetamide analysis.

6. RESULTS

Sample analysis were carried out from 09-Mar-2018 to 26-Feb-2019 and the details are given in Table 1.

Analysis of plasma and liver samples for Acetamide concentrations: The sample analysis batches were accepted when at least 6 of the total 8 calibration standards had back calculated values within ±15% variability from nominal value except for the LLOQ standards which were accepted with ±20% variability. Batches were accepted when at least two thirds of the overall QC samples and 50% at each QC level had back calculated values within ±15% variability from nominal value.

The results of the back-calculated concentrations of the CC standards and QC samples in rat plasma are given in Table 2 and Table 3, respectively. The final accepted concentrations of Acetamide in the rat plasma on day 8 are presented in Table 4. A representative calibration curve in rat plasma, representative chromatogram of samples corresponding to standard blank, standard zero, LLOQ standard, ULOQ standard, QC samples, study sample (P_B-023) from plasma analysis are presented in FIGURE 1 to FIGURE 9.

The results of the back-calculated concentrations of the CC standards and QC samples in rat liver homogenate are given in Table 5 and Table 6, respectively. The final accepted concentrations of Acetamide in the rat liver samples on day 8 are presented in Table 7, respectively. A representative calibration curve in rat liver homogenate, representative chromatogram of samples corresponding to standard blank, standard zero, LLOQ standard, ULOQ standard, QC samples, study sample (L_B-021) from liver analysis are presented in FIGURE 10 to FIGURE 18.

Analysis of urine, water and feed samples for Acetamide concentrations: The sample analysis batches were accepted when at least 6 of the total 8 calibration standards had back calculated values within ±20% variability from nominal value. Batches were accepted when at least two thirds of the overall QC samples and 50% at each QC level had back calculated values within ±20% variability from nominal value.

The results of the back-calculated concentrations of the CC standards and QC samples in rat urine are given in Table 8 and Table 11, respectively. The final accepted concentrations of Acetamide in the rat urine on day 7 are presented in Table 12. A representative calibration curve in rat urine, representative chromatogram of samples corresponding to standard blank, LLOQ standard, ULOQ standard, study sample (Ru5687) from urine analysis are presented in FIGURE 19 to FIGURE 23.

The results of the back-calculated concentrations of the CC standards and QC samples in water are given in Table 13 and Table 14, respectively. The final accepted concentrations of Acetamide in the water sample is presented in Table 15. A representative calibration curve in water, representative chromatogram of samples corresponding to standard blank, LLOQ standard, ULOQ standard, water sample are presented in FIGURE 24 to FIGURE 28.

The results of the back-calculated concentrations of the CC standards and recovery QC of Acetamide in feed are given in Table 16 and Table 17, respectively. The final accepted concentrations of Acetamide in the feed sample is presented in Table 18. A representative calibration curve in feed, representative chromatogram of samples corresponding to standard blank, LLOQ standard, ULOQ standard, feed sample are presented in FIGURE 29 to FIGURE 33.

Analysis of plasma samples for N-hydroxyacetamide concentrations: Calibration curve obtained was linear with a coefficient of determination of 0.9986. Calibration curve data is included in Table 19. The final accepted concentrations of N-hydroxyacetamide in the plasma on day 8 is presented in Table 20. Calibration curve for N-hydroxyacetamide in the plasma is presented in FIGURE 34.

7. SAMPLE DISPOSITION

All samples of the study were disposed in consultation with the sponsor before finalization of the report.

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8. CONCLUSION

Following repeated daily oral gavage administration of Acetamide alone and Acetamide in presence of BrdU in male Sprague-Dawley rats (n=6) for 7 Days, liver and plasma samples were collected at 24h from last dosing (on Day 8) and the plasma and liver concentrations of Acetamide were determined by validated GC-MS method. The plasma and liver concentrations of Acetamide are presented in table below:

Dose (mg/kg/day)	Day Nominal	Acetamide groups		Acetamide + BrdU groups	
		Mean Plasma Concentration (µg/mL)	Mean Liver Concentration (µg/g)	Mean Plasma Concentration (µg/mL)	Mean Liver Concentration (µg/g)
0	8	0.350±0.116	2.85±0.233	0.310±0.026	3.51±2.030
30	8	25.8±3.53	19.5±1.62	29.7±3.96	26.7±2.94
100	8	80.5±14.3	57.1±14.7	112±11.8	78.6±10.3
300	8	222±22.9	126±25.3	314±51.1	179±35.9
1000	8	513±48.4	297±64.5	849±85.7	506±42.3

Acetamide levels were estimated from the pooled urine collected on day 7 using fit-for-purpose GC-MS method and the observed concentrations are as below

Dose (mg/kg/day)	Day Nominal	Mean Urine Concentration in Acetamide group (µg/mL)
0	7	4.71 ± 1.14
30	7	833 ± 358
100	7	2482 ± 817
300	7	5647 ± 1313
1000	7	15150 ± 2616

The results demonstrate that the plasma, liver and urine Acetamide concentrations increase with dose.

A sample of water was collected from the Aquaguard during the study which was monitored for Acetamide levels using fit-for-purpose GC-MS method and concentration was below the limit of quantification (LLOQ 0.1 µg/mL) suggesting absence of significant level of Acetamide.

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A sample of feed was collected during the study which was monitored for Acetamide levels using fit-for-purpose GC-MS method and concentration was found to be 1.15 µg/g of feed.

N-hydroxyacetamide (putative metabolite of Acetamide) levels were estimated from the plasma samples collected on day 8 using fit-for-purpose colorimetric method and the observed concentrations are as below:

Dose (mg/kg/day)	Individual N-hydroxyacetamide concentration (µg/mL) in plasma on day 8						Mean concentration ± SD (µg/mL)	n
0	14.3	5.32	6.11	BLQ	BLQ	BLQ	8.59 ± 5.0	3
30	BLQ	BLQ	BLQ	BLQ	6.61	BLQ	6.61[a]	1
100	BLQ	6.71	9.68	5.02	BLQ	BLQ	7.14 ± 2.4	3
300	BLQ	9.19	BLQ	BLQ	9.48	BLQ	9.34[b]	2
1000	21.5	BLQ	39	7.3	BLQ	35.5	25.8 ± 14	4

[a] n=1 animal data; [b] n=2 animal data, values were below quantification limit for other animals in particular group

No consistent dose-dependent increase in N-hydroxyacetamide was observed. The plasma N-hydroxyacetamide concentrations were comparable in 0, 30, 100, 300 mg/kg group except 1000 mg/kg group. At the highest dose (1000 mkd), 4/6 animals showed higher N-hydroxyacetamide levels whereas the other 2 were not distinguishable from controls or below limit of quantification. The results suggest that no significant presence of N-hydroxyacetamide in plasma following Acetamide administration except 1000 mg/kg dose group. Given the potential for interference in colorimetric assays and the number of samples below LOQ, the interpretation of these data is left to the sponsor.

9. TABLES

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Table 1. Status of Analytical Run

Run ID	Extraction date	Assay date	Description	Run status
1	09-Mar-2018	10-Mar-2018	Plasma samples analysis batch-1	Accepted
2	13-Mar-2018	13-Mar-2018	Plasma samples analysis batch-2	Accepted
3	14-Mar-2018	16-Mar-2018	Plasma samples analysis batch-3	Rejected^
4	15-Mar-2018	15-Mar-2018	Liver samples analysis batch-1	Accepted
5	16-Mar-2018	17-Mar-2018	Liver samples analysis batch-2	Rejected*
6	20-Mar-2018	21-Mar-2018	Plasma samples analysis batch-4	Accepted
7	22-Mar-2018	22-Mar-2018	Liver samples analysis batch-3	Accepted
8	22-Mar-2018	23-Mar-2018	Liver samples analysis batch-4	Accepted
9	07-Jan-2019	08-Jan-2019	Water sample analysis	Accepted
10	07-Jan-2019	09-Jan-2019	Urine sample analysis	Accepted
11	10-Jan-2019	10-Jan-2019	Feed sample analysis	Accepted
NA	15-Feb-2019	15-Feb-2019	Plasma samples analysis for N-hydroxyacetamide	Accepted
12	21-Feb-2019	22-Feb-2019	Urine repeat analysis	Accepted
13	26-Feb-2019	26-Feb-2019	Urine repeat analysis 2	Accepted

^: Acquisition error occured
*: CC standards failed to meet acceptance criteria

Table 2. Back-calculated values of CC standards for Acetamide D3 in rat plasma during Acetamide analysis

Concentration [µg/mL]	PLM STD1 0.100 µg/mL	PLM STD2 0.200 µg/mL	PLM STD3 0.500 µg/mL	PLM STD4 2.00 µg/mL	PLM STD5 5.00 µg/mL	PLM STD6 20.0 µg/mL	PLM STD7 40.0 µg/mL	PLM STD8 50.0 µg/mL	Slope	Intercept	r^2
Run 1	0.0998	0.204	0.492	1.78	4.87	21.7	41.9	50.1	0.0497873	-0.0000132375	0.9958
Run 2	0.103	0.190	0.478	*1.61	4.89	20.4	40.5	52.4	0.0497966	-0.000629151	0.9981
Run 6	0.101	0.198	0.476	2.01	4.87	20.7	39.9	51.5	0.0478657	-0.000137560	0.9990
n	3	3	3	2	3	3	3	3	NA		
Overall Mean	0.101	0.197	0.482	1.90	4.88	20.9	40.8	51.3			
S.D.	0.00162	0.00702	0.00872	0.163	0.0115	0.681	1.03	1.16			
%CV	1.60	3.56	1.81	8.58	0.24	3.26	2.52	2.26			
%Bias	1.00	-1.50	-3.60	-5.00	-2.40	4.50	2.00	2.60			

*: CC standard had failed to meet the acceptance criteria (i.e., beyond 15% from nominal value) and excluded from the curve and calculation

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Table 3. Back-Calculated Concentration of Quality Control Samples for Acetamide D3 in rat plasma during Acetamide analysis

Assay date	Run number	PLM LQC 0.300 µg/mL	PLM MQC 20.0 µg/mL	PLM HQC 40.0 µg/mL	PLM DQC5F 112 µg/mL	PLM DQC20F 410 µg/mL
10-Mar-2018	1	0.306	18.7	41.6	-	-
		0.305	20.7	42.6	-	-
13-Mar-2018	2	0.278	21.3	40.8	107	-
		0.259	20.8	40.6	104	-
21-Mar-2018	6	0.267	20.3	40.5	-	386
		0.279	20.3	39.7	-	381
		-	-	-	-	388
		-	-	-	-	360
		-	-	-	-	368
		-	-	-	-	379
Mean Concentration Found (µg/mL)		0.282	20.4	41.0	106	377
Inter-run SD		0.0194	0.889	1.01	2.12	10.9
Inter-run %CV		6.88	4.36	2.46	2.00	2.89
Inter-run %Bias		-6.00	2.00	2.50	-5.36	-8.05
n		6	6	6	2	6

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Table 4. Concentration of Acetamide in rat plasma on day 8

Group	Treatment Description	Dose (mg/kg/day)	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	Acetamide Concentration in Plasma (µg/mL)
G1A	Control	0	Ru5681	Male	8	0	P_A-001	0.296
G1A	Control	0	Ru5682	Male	8	0	P_A-002	0.286
G1A	Control	0	Ru5683	Male	8	0	P_A-003	0.358
G1A	Control	0	Ru5684	Male	8	0	P_A-004	0.283
G1A	Control	0	Ru5685	Male	8	0	P_A-005	0.581
G1A	Control	0	Ru5686	Male	8	0	P_A-006	0.298
Mean								0.350
SD								0.116
%CV								33.14
G1B	Control	0	Ru5711	Male	8	0	P_C-001	0.26
G1B	Control	0	Ru5712	Male	8	0	P_C-002	0.313
G1B	Control	0	Ru5713	Male	8	0	P_C-003	0.312
G1B	Control	0	Ru5714	Male	8	0	P_C-004	0.313
G1B	Control	0	Ru5715	Male	8	0	P_C-005	0.326
G1B	Control	0	Ru5716	Male	8	0	P_C-006	0.336
Mean								0.310
SD								0.026
%CV								8.39
G2A	Low	30	Ru5687	Male	8	0	P_B-001	23.3
G2A	Low	30	Ru5688	Male	8	0	P_B-002	31.6
G2A	Low	30	Ru5689	Male	8	0	P_B-003	26.4
G2A	Low	30	Ru5690	Male	8	0	P_B-004	23.4
G2A	Low	30	Ru5691	Male	8	0	P_B-005	27.6
G2A	Low	30	Ru5692	Male	8	0	P_B-006	22.2
Mean								25.8
SD								3.53
%CV								13.68
G2B	Low	30	Ru5717	Male	8	0	P_D-001	33
G2B	Low	30	Ru5718	Male	8	0	P_D-002	28.8
G2B	Low	30	Ru5719	Male	8	0	P_D-003	32.8
G2B	Low	30	Ru5720	Male	8	0	P_D-004	24.7
G2B	Low	30	Ru5721	Male	8	0	P_D-005	25.4
G2B	Low	30	Ru5722	Male	8	0	P_D-006	33.4
Mean								29.7
SD								3.96
%CV								13.33

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Table 4 Continued… Concentration of Acetamide in rat plasma on day 8

Group	Treatment Description	Dose (mg/kg/day)	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	Acetamide Concentration in Plasma (µg/mL)
G3A	Mid	100	Ru5693	Male	8	0	P_B-007	92.0
G3A	Mid	100	Ru5694	Male	8	0	P_B-008	102
G3A	Mid	100	Ru5695	Male	8	0	P_B-009	63.5
G3A	Mid	100	Ru5696	Male	8	0	P_B-010	75.5
G3A	Mid	100	Ru5697	Male	8	0	P_B-011	80.0
G3A	Mid	100	Ru5698	Male	8	0	P_B-012	70.0
Mean								80.5
SD								14.3
%CV								17.76
G3B	Mid	100	Ru5723	Male	8	0	P_D-007	98
G3B	Mid	100	Ru5724	Male	8	0	P_D-008	121
G3B	Mid	100	Ru5725	Male	8	0	P_D-009	123
G3B	Mid	100	Ru5726	Male	8	0	P_D-010	118
G3B	Mid	100	Ru5727	Male	8	0	P_D-011	96.0
G3B	Mid	100	Ru5728	Male	8	0	P_D-012	115
Mean								112
SD								11.8
%CV								10.54
G4A	Int.	300	Ru5699	Male	8	0	P_B-013	209
G4A	Int.	300	Ru5700	Male	8	0	P_B-014	205
G4A	Int.	300	Ru5701	Male	8	0	P_B-015	200
G4A	Int.	300	Ru5702	Male	8	0	P_B-016	223
G4A	Int.	300	Ru5703	Male	8	0	P_B-017	260
G4A	Int.	300	Ru5704	Male	8	0	P_B-018	237
Mean								222
SD								22.9
%CV								10.32
G4B	Int.	300	Ru5729	Male	8	0	P_D-013	251
G4B	Int.	300	Ru5730	Male	8	0	P_D-014	401
G4B	Int.	300	Ru5731	Male	8	0	P_D-015	311
G4B	Int.	300	Ru5732	Male	8	0	P_D-016	312
G4B	Int.	300	Ru5733	Male	8	0	P_D-017	329
G4B	Int.	300	Ru5734	Male	8	0	P_D-018	278
Mean								314
SD								51.1
%CV								16.27

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Table 4 Continued… Concentration of Acetamide in rat plasma on day 8

Group	Treatment Description	Dose (mg/kg/day)	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	Acetamide Concentration in Plasma (µg/mL)
G5A	High	1000	Ru5705	Male	8	0	P_B-019	550
G5A	High	1000	Ru5706	Male	8	0	P_B-020	534
G5A	High	1000	Ru5707	Male	8	0	P_B-021	432
G5A	High	1000	Ru5708	Male	8	0	P_B-022	552
G5A	High	1000	Ru5709	Male	8	0	P_B-023	534
G5A	High	1000	Ru5710	Male	8	0	P_B-024	476
Mean								513
SD								48.4
%CV								9.43
G5B	High	1000	Ru5735	Male	8	0	P_D-019	806
G5B	High	1000	Ru5736	Male	8	0	P_D-020	988
G5B	High	1000	Ru5737	Male	8	0	P_D-021	858
G5B	High	1000	Ru5738	Male	8	0	P_D-022	726
G5B	High	1000	Ru5739	Male	8	0	P_D-023	850
G5B	High	1000	Ru5740	Male	8	0	P_D-024	868
Mean								849
SD								85.7
%CV								10.09

Table 5. Back-calculated values of CC standards for Acetamide D3 in rat liver homogenate during Acetamide analysis

Concentration [µg/mL]	Liver STD1 0.100 µg/mL	Liver STD2 0.200 µg/mL	Liver STD3 0.500 µg/mL	Liver STD4 2.00 µg/mL	Liver STD5 5.00 µg/mL	Liver STD6 20.0 µg/mL	Liver STD7 40.0 µg/mL	Liver STD8 50.0 µg/mL	Slope	Intercept	r^2
Run 4	0.101	0.201	0.475	2.00	5.11	19.9	40.0	50.9	0.0446757	0.000344169	0.9994
Run 7	0.0981	0.212	0.484	1.86	4.95	19.8	41.5	52.3	0.0453776	-0.000202701	0.9976
Run 8	0.0999	0.202	0.492	1.95	4.82	19.7	43.0	50.4	0.0466174	0.000112042	0.9985
n	3	3	3	3	3	3	3	3	NA		
Overall Mean	0.0997	0.205	0.484	1.94	4.96	19.8	41.5	51.2			
S.D.	0.00146	0.00608	0.00850	0.0709	0.145	0.100	1.50	0.985			
%CV	1.46	2.97	1.76	3.65	2.92	0.51	3.61	1.92			
%Bias	-0.30	2.50	-3.20	-3.00	-0.80	-1.00	3.75	2.40			

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Table 6. Back-Calculated Concentration of Quality Control Samples for Acetamide D3 in rat liver homogenate during Acetamide analysis

Assay date	Run number	Liver LQC 0.300 µg/mL	Liver MQC 20.0 µg/mL	Liver HQC 40.0 µg/mL	Liver DQC5F 112 µg/mL
15-Mar-2018	4	0.312	20.3	41.1	-
		0.294	20.9	45.6	-
22-Mar-2018	7	0.297	20.4	39.6	-
		0.302	20.9	40.3	-
23-Mar-2018	8	0.295	19.9	38.5	108
		0.283	20.6	40.4	117
Mean Concentration Found (µg/mL)		0.297	20.5	40.9	113
Inter-run SD		0.00958	0.385	2.46	6.36
Inter-run %CV		3.23	1.88	6.01	5.63
Inter-run %Bias		-1.00	2.50	2.25	0.89
n		6	6	6	2

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Table 7. Concentration of Acetamide in rat liver on day 8

Group	Treatment Description	Dose (mg/kg/day)	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	Acetamide Concentration in Liver tissue (µg/g)
G1A	Control	0	Ru5681	Male	8	0	L_A-001	2.88
G1A	Control	0	Ru5682	Male	8	0	L_A-002	3.04
G1A	Control	0	Ru5683	Male	8	0	L_A-003	2.85
G1A	Control	0	Ru5684	Male	8	0	L_A-004	2.83
G1A	Control	0	Ru5685	Male	8	0	L_A-005	2.43
G1A	Control	0	Ru5686	Male	8	0	L_A-006	3.09
Mean								2.85
SD								0.233
%CV								8.18
G1B	Control	0	Ru5711	Male	8	0	L_C-001	2.01
G1B	Control	0	Ru5712	Male	8	0	L_C-002	2.38
G1B	Control	0	Ru5713	Male	8	0	L_C-003	2.27
G1B	Control	0	Ru5714	Male	8	0	L_C-004	7.45
G1B	Control	0	Ru5715	Male	8	0	L_C-005	3.39
G1B	Control	0	Ru5716	Male	8	0	L_C-006	3.57
Mean								3.51
SD								2.030
%CV								57.83
G2A	Low	30	Ru5687	Male	8	0	L_B-001	16.8
G2A	Low	30	Ru5688	Male	8	0	L_B-002	18.9
G2A	Low	30	Ru5689	Male	8	0	L_B-003	20.6
G2A	Low	30	Ru5690	Male	8	0	L_B-004	21.3
G2A	Low	30	Ru5691	Male	8	0	L_B-005	20.3
G2A	Low	30	Ru5692	Male	8	0	L_B-006	18.9
Mean								19.5
SD								1.62
%CV								8.31
G2B	Low	30	Ru5717	Male	8	0	L_D-001	30.1
G2B	Low	30	Ru5718	Male	8	0	L_D-002	26.5
G2B	Low	30	Ru5719	Male	8	0	L_D-003	29.8
G2B	Low	30	Ru5720	Male	8	0	L_D-004	23.6
G2B	Low	30	Ru5721	Male	8	0	L_D-005	23.2
G2B	Low	30	Ru5722	Male	8	0	L_D-006	27.0
Mean								26.7
SD								2.94
%CV								11.01

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Table 7 Continued… Concentration of Acetamide in rat liver on day 8

Group	Treatment Description	Dose (mg/kg/day)	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	Acetamide Concentration in Liver tissue (µg/g)
G3A	Mid	100	Ru5693	Male	8	0	L_B-007	58.0
G3A	Mid	100	Ru5694	Male	8	0	L_B-008	75.0
G3A	Mid	100	Ru5695	Male	8	0	L_B-009	36.6
G3A	Mid	100	Ru5696	Male	8	0	L_B-010	64.0
G3A	Mid	100	Ru5697	Male	8	0	L_B-011	42.7
G3A	Mid	100	Ru5698	Male	8	0	L_B-012	66.5
Mean								57.1
SD								14.7
%CV								25.74
G3B	Mid	100	Ru5723	Male	8	0	L_D-007	79.5
G3B	Mid	100	Ru5724	Male	8	0	L_D-008	68.0
G3B	Mid	100	Ru5725	Male	8	0	L_D-009	87.5
G3B	Mid	100	Ru5726	Male	8	0	L_D-010	77.5
G3B	Mid	100	Ru5727	Male	8	0	L_D-011	66.5
G3B	Mid	100	Ru5728	Male	8	0	L_D-012	92.5
Mean								78.6
SD								10.3
%CV								13.1
G4A	Int.	300	Ru5699	Male	8	0	L_B-013	115
G4A	Int.	300	Ru5700	Male	8	0	L_B-014	148
G4A	Int.	300	Ru5701	Male	8	0	L_B-015	98.5
G4A	Int.	300	Ru5702	Male	8	0	L_B-016	129
G4A	Int.	300	Ru5703	Male	8	0	L_B-017	162
G4A	Int.	300	Ru5704	Male	8	0	L_B-018	103
Mean								126
SD								25.3
%CV								20.08
G4B	Int.	300	Ru5729	Male	8	0	L_D-013	150
G4B	Int.	300	Ru5730	Male	8	0	L_D-014	241
G4B	Int.	300	Ru5731	Male	8	0	L_D-015	169
G4B	Int.	300	Ru5732	Male	8	0	L_D-016	195
G4B	Int.	300	Ru5733	Male	8	0	L_D-017	174
G4B	Int.	300	Ru5734	Male	8	0	L_D-018	142
Mean								179
SD								35.9
%CV								20.06

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Table 7 Continued… Concentration of Acetamide in rat liver on day 8

Group	Treatment Description	Dose (mg/kg/day)	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	Acetamide Concentration in Liver tissue (µg/g)
G5A	High	1000	Ru5705	Male	8	0	L_B-019	301
G5A	High	1000	Ru5706	Male	8	0	L_B-020	303
G5A	High	1000	Ru5707	Male	8	0	L_B-021	193
G5A	High	1000	Ru5708	Male	8	0	L_B-022	352
G5A	High	1000	Ru5709	Male	8	0	L_B-023	372
G5A	High	1000	Ru5710	Male	8	0	L_B-024	261
Mean								297
SD								64.5
%CV								21.72
G5B	High	1000	Ru5735	Male	8	0	L_D-019	488
G5B	High	1000	Ru5736	Male	8	0	L_D-020	565
G5B	High	1000	Ru5737	Male	8	0	L_D-021	455
G5B	High	1000	Ru5738	Male	8	0	L_D-022	485
G5B	High	1000	Ru5739	Male	8	0	L_D-023	550
G5B	High	1000	Ru5740	Male	8	0	L_D-024	493
Mean								506
SD								42.3
%CV								8.36

Table 8. Back-calculated values of CC standards for Acetamide D3 in Urine during Acetamide analysis

Concentration [µg/mL]	Urine STD1 0.100 µg/mL	Urine STD2 0.200 µg/mL	Urine STD3 0.500 µg/mL	Urine STD4 2.00 µg/mL	Urine STD5 5.00 µg/mL	Urine STD6 20.0 µg/mL	Urine STD7 40.0 µg/mL	Urine STD8 50.0 µg/mL	Slope	Intercept	r^2
Run 10	0.109	0.171	0.460	2.17	4.71	18.7	41.9	56.6	0.0232142	-0.000233045	0.9877
%Bias	9.00	-14.50	-8.00	8.50	-5.80	-6.50	4.75	13.20	-		

Table 9. Back-calculated values of CC standards for Acetamide D3 in Urine during Acetamide analysis

Concentration [µg/mL]	Urine STD1 1.00 µg/mL	Urine STD2 2.00 µg/mL	Urine STD3 5.00 µg/mL	Urine STD4 20.0 µg/mL	Urine STD5 50.0 µg/mL	Urine STD6 200 µg/mL	Urine STD7 400 µg/mL	Urine STD8 500 µg/mL	Slope	Intercept	r^2
Run 12	0.992	2.13	4.38	19.9	51.5	219	408	466	0.0798262	0.00648313	0.9938
Run 13	1.02	1.94	4.81	19.7	53.6	201	398	495	0.0370772	0.00125594	0.9985
n	2	2	2	2	2	2	2	2	-		
Overall Mean	1.01	2.04	4.60	19.8	52.6	210	403	481			
S.D.	0.0198	0.134	0.304	0.141	1.48	12.7	7.07	20.5			
%CV	1.96	6.57	6.61	0.71	2.81	6.05	1.75	4.26			
%Bias	1.00	2.00	-8.00	-1.00	5.20	5.00	0.75	-3.80			

APPENDIX 8 contd. Bioanalytical Report

Table 10. Back-Calculated Concentration of Quality Control Samples for Acetamide D3 in urine during Acetamide analysis

Assay date	Run number	Urine_LQC 0.300 µg/mL	Urine_MQC 20.0 µg/mL	Urine_HQC 40.0 µg/mL
09-Jan-2019	10	0.351	24.0	45.7
		0.310	22.8	46.4
Intrarun Mean		0.331	23.4	46.1
Intrarun SD		0.0290	0.849	0.495
Intrarun %CV		8.76	3.63	1.07
Intrarun %Bias		10.33	17.00	15.25
n		2	2	2

Table 11. Back-Calculated Concentration of Quality Control Samples for Acetamide D3 in urine during Acetamide analysis

Assay date	Run number	Urine LQC 5.00 µg/mL	Urine MQC 200 µg/mL	Urine HQC 400 µg/mL	Urine DQC100F 500 µg/mL	Urine DQC40F 500 µg/mL
22-Feb-2019	12	4.83	195	398	-	574
		4.32	208	368	-	548
26-Feb-2019	13	5.50	211	405	#627	-
		4.90	208	403	528	-
Mean Concentration Found (µg/mL)		4.89	206	394	578	561
Inter-run SD		0.483	7.14	17.3	70.0	18.4
Inter-run %CV		9.88	3.47	4.39	12.11	3.28
Inter-run %Bias		-2.20	3.00	-1.50	15.60	12.20
n		4	4	4	2	2

#: QC sample beyond acceptance criteria; %bias beyond 20% from nominal

APPENDIX 8 contd. Bioanalytical Report

Table 12. Concentration of Acetamide in rat urine on day 7

Dose (mg/kg/day)	Treatment Description	Group	Rat No.	Sex	Day Nominal	Acetamide Concentration in Urine (µg/mL)
0	CONTROL	G1A	Ru5681	Male	7	3.47
0	CONTROL	G1A	Ru5682	Male	7	4.97
0	CONTROL	G1A	Ru5683	Male	7	6.59
0	CONTROL	G1A	Ru5684	Male	7	5.16
0	CONTROL	G1A	Ru5685	Male	7	4.36
0	CONTROL	G1A	Ru5686	Male	7	3.70
					Mean	4.71
					SD	1.14
					CV	24.19
30	Low	G2A	Ru5687	Male	7	765
30	Low	G2A	Ru5688	Male	7	1550
30	Low	G2A	Ru5689	Male	7	762
30	Low	G2A	Ru5690	Male	7	614
30	Low	G2A	Ru5691	Male	7	711
30	Low	G2A	Ru5692	Male	7	598
					Mean	833
					SD	358
					CV	42.99
100	Mid	G3A	Ru5693	Male	7	4030
100	Mid	G3A	Ru5694	Male	7	1580
100	Mid	G3A	Ru5695	Male	7	2300
100	Mid	G3A	Ru5696	Male	7	2210
100	Mid	G3A	Ru5697	Male	7	2400
100	Mid	G3A	Ru5698	Male	7	2370
					Mean	2482
					SD	817
					CV	32.92
300	Int.	G4A	Ru5699	Male	7	4730
300	Int.	G4A	Ru5700	Male	7	4600
300	Int.	G4A	Ru5701	Male	7	7650
300	Int.	G4A	Ru5702	Male	7	6490
300	Int.	G4A	Ru5703	Male	7	4320
300	Int.	G4A	Ru5704	Male	7	6090
					Mean	5647
					SD	1313
					CV	23.25

APPENDIX 8 contd. Bioanalytical Report

Table 12 Continued… Concentration of Acetamide in rat urine on day 7

Dose (mg/kg/day)	Treatment Description	Group	Rat No.	Sex	Day Nominal	Acetamide Concentration in Urine (µg/mL)
1000	High	G5A	Ru5705	Male	7	26400
1000	High	G5A	Ru5706	Male	7	20800
1000	High	G5A	Ru5707	Male	7	11700
1000	High	G5A	Ru5708	Male	7	17000
1000	High	G5A	Ru5709	Male	7	16800
1000	High	G5A	Ru5710	Male	7	13300
					Mean	15150
					SD	2616
					CV	17.27

Table 13. Back-calculated values of CC standards for Acetamide D3 in Water during Acetamide analysis

Concentration [µg/mL]	Water STD1 0.100 µg/mL	Water STD2 0.200 µg/mL	Water STD3 0.500 µg/mL	Water STD4 2.00 µg/mL	Water STD5 5.00 µg/mL	Water STD6 20.0 µg/mL	Water STD7 40.0 µg/mL	Water STD8 50.0 µg/mL	Slope	Intercept	r^2
Run 9	0.0998	0.197	0.520	2.00	5.44	16.9	42.0	49.9	0.0537139	-0.00101028	0.9935
%Bias	-0.20	-1.50	4.00	0.00	8.80	-15.50	5.00	-0.20	-		

APPENDIX 8 contd. Bioanalytical Report

Table 14. Back-Calculated Concentration of Quality Control Samples for Acetamide D3 in urine during Acetamide analysis

Assay date	Run number	Water_LQC 0.300 µg/mL	Water_MQC 20.0 µg/mL	Water_HQC 40.0 µg/mL
08-Jan-2019	9	0.287	#14.5	39.9
		0.251	19.8	38.0
Intrarun Mean		0.269	17.2	39.0
Intrarun SD		0.0255	3.75	1.34
Intrarun %CV		9.48	21.80	3.44
Intrarun %Bias		-10.33	-14.00	-2.50
n		2	2	2

#: QC sample beyond acceptance criteria; %bias beyond 20% from nominal

APPENDIX 8 contd. Bioanalytical Report

Table 15. Concentration of Acetamide in Water sample

Sample ID	Concentration of Acetamide in Water (µg/mL)
Water sample Aliquot 1	BLQ <(0.100)
Water sample Aliquot 2	BLQ <(0.100)
Water sample Aliquot 3	BLQ <(0.100)
Mean	BLQ <(0.100)

Table 16. Back-calculated values of CC standards for Acetamide D3 in Feed during Acetamide analysis

Concentration [µg/mL]	Feed_STD1 0.100 µg/mL	Feed_STD2 0.200 µg/mL	Feed_STD3 0.500 µg/mL	Feed_STD4 2.00 µg/mL	Feed_STD5 5.00 µg/mL	Feed_STD6 20.0 µg/mL	Feed_STD7 40.0 µg/mL	Feed_STD8 50.0 µg/mL	Slope	Intercept	r^2
Run 11	0.0976	0.210	0.499	1.98	4.95	20.8	39.4	48.6	0.0540308	0.000908810	0.9990
%Bias	-2.40	5.00	-0.20	-1.00	-1.00	4.00	-1.50	-2.80	-		

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Table 17. Recovery for Acetamide from feed

Assay date	Run number	Feed_REC MQC 20.0 µg/mL
10-Jan-2019	11	21.4
		21.3
		21.6
Intrarun Mean		21.4
Intrarun SD		0.153
Intrarun %CV		0.71
Intrarun %Bias		7.00
n		3

APPENDIX 8 contd. Bioanalytical Report

Table 18. Concentration of Acetamide in Feed sample

Sample ID	Concentration of Acetamide in Feed (µg/g)
Feed sample Aliquot 1	1.15
Feed sample Aliquot 2	1.19
Feed sample Aliquot 3	1.12
Mean	1.15

APPENDIX 8 contd. Bioanalytical Report

Table 19. Calibration curve data for N-Hydroxyacetamide in Plasma

Nominal concentration (µg/mL)	5.00	10.0	20.0	40.0	80.0	100
Instrument response	0.0017	0.0053	0.0195	0.0363	0.0765	0.0982
Slope: 0.0010		Intercept: -0.0033			r^2: 0.9986	

APPENDIX 8 contd. Bioanalytical Report

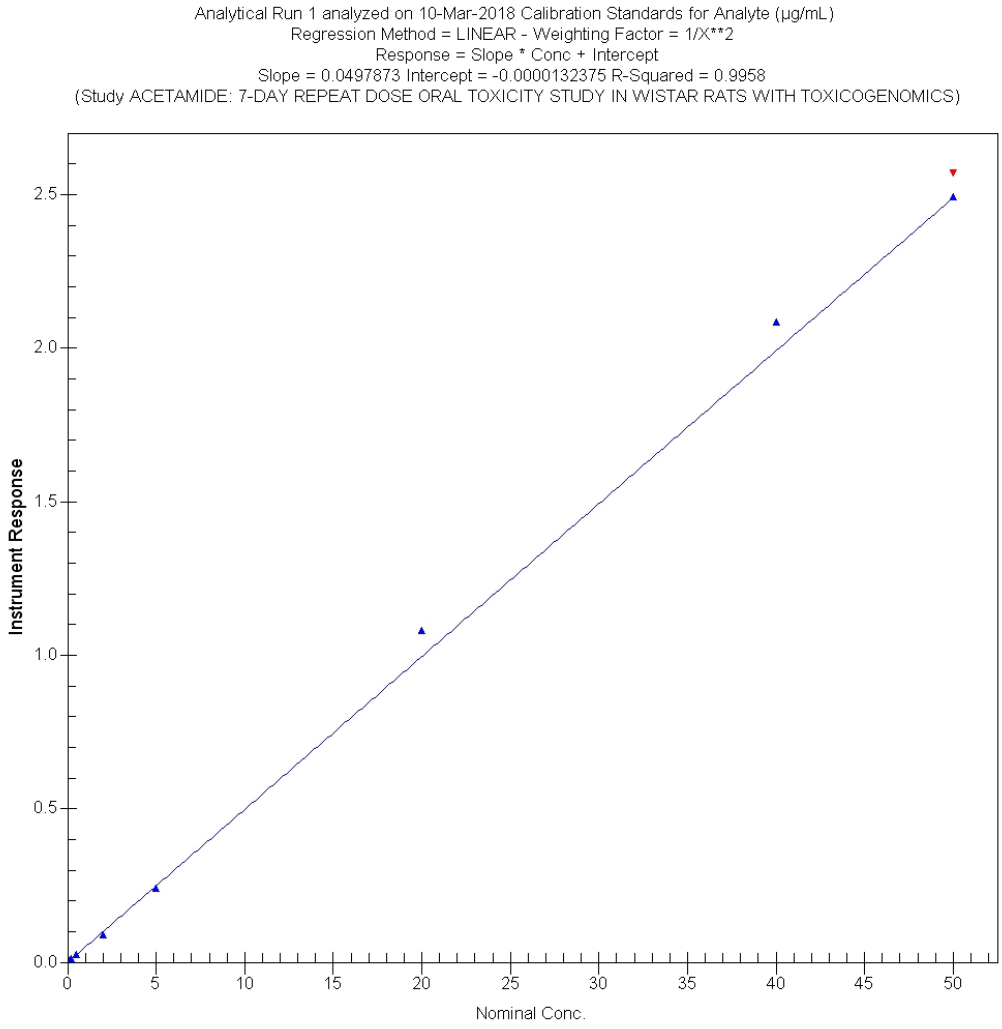
Table 20. Concentration of N-Hydroxyacetamide in plasma sample

Dose (mg/kg/day)	Treatment Description	Group	Rat No.	Sex	Day Nominal	Hour Nominal	Custom ID	N-Hydroxyacetamide Concentration in Plasma (µg/mL)
0	Control	G1A	Ru5681	Male	8	0	P_A-001	14.3
0	Control	G1A	Ru5682	Male	8	0	P_A-002	5.32
0	Control	G1A	Ru5683	Male	8	0	P_A-003	6.11
0	Control	G1A	Ru5684	Male	8	0	P_A-004	BLQ
0	Control	G1A	Ru5685	Male	8	0	P_A-005	BLQ
0	Control	G1A	Ru5686	Male	8	0	P_A-006	BLQ
30	Low	G2A	Ru5687	Male	8	0	P_B-001	BLQ
30	Low	G2A	Ru5688	Male	8	0	P_B-002	BLQ
30	Low	G2A	Ru5689	Male	8	0	P_B-003	BLQ
30	Low	G2A	Ru5690	Male	8	0	P_B-004	BLQ
30	Low	G2A	Ru5691	Male	8	0	P_B-005	6.61
30	Low	G2A	Ru5692	Male	8	0	P_B-006	BLQ
100	Mid	G3A	Ru5693	Male	8	0	P_B-007	BLQ
100	Mid	G3A	Ru5694	Male	8	0	P_B-008	6.71
100	Mid	G3A	Ru5695	Male	8	0	P_B-009	9.68
100	Mid	G3A	Ru5696	Male	8	0	P_B-010	5.02
100	Mid	G3A	Ru5697	Male	8	0	P_B-011	BLQ
100	Mid	G3A	Ru5698	Male	8	0	P_B-012	BLQ
300	Int.	G4A	Ru5699	Male	8	0	P_B-013	BLQ
300	Int.	G4A	Ru5700	Male	8	0	P_B-014	9.19
300	Int.	G4A	Ru5701	Male	8	0	P_B-015	BLQ
300	Int.	G4A	Ru5702	Male	8	0	P_B-016	BLQ
300	Int.	G4A	Ru5703	Male	8	0	P_B-017	9.48
300	Int.	G4A	Ru5704	Male	8	0	P_B-018	BLQ
1000	High	G5A	Ru5705	Male	8	0	P_B-019	21.5
1000	High	G5A	Ru5706	Male	8	0	P_B-020	BLQ
1000	High	G5A	Ru5707	Male	8	0	P_B-021	39.0
1000	High	G5A	Ru5708	Male	8	0	P_B-022	7.30
1000	High	G5A	Ru5709	Male	8	0	P_B-023	BLQ
1000	High	G5A	Ru5710	Male	8	0	P_B-024	35.5

10. FIGURES

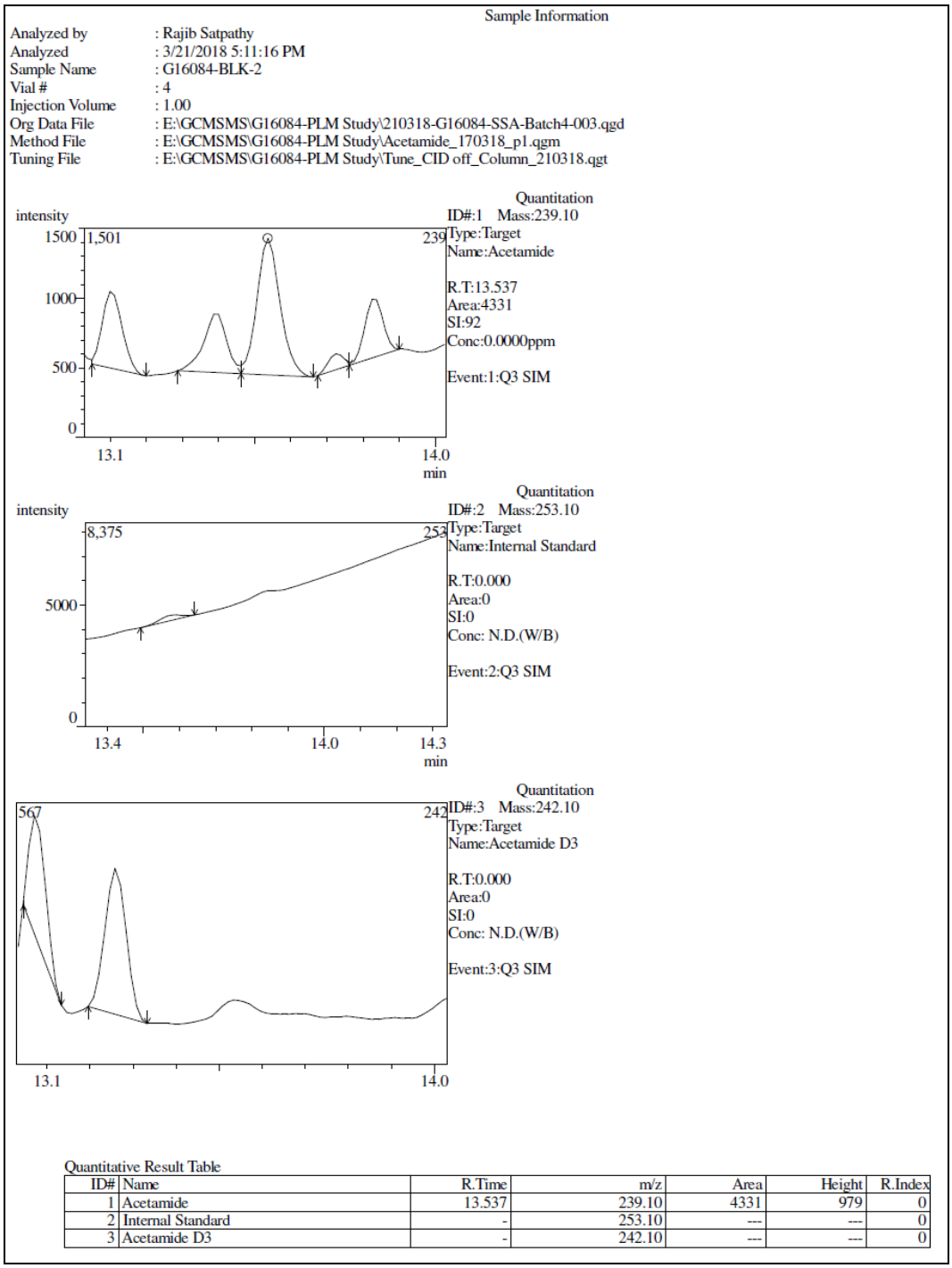
APPENDIX 8 contd. Bioanalytical Report

FIGURE 1. Representative calibration curve of Acetamide D3 in rat plasma



APPENDIX 8 contd. Bioanalytical Report

FIGURE 2. Chromatogram of Acetamide, Acetamide D3 and IS in standard blank sample in rat plasma



Quantitative Result Table

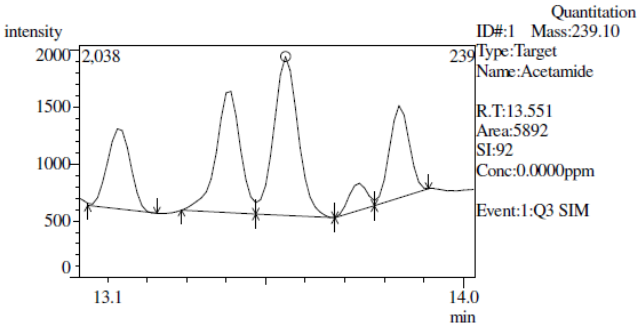
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.537	239.10	4331	979	0
2	Internal Standard	-	253.10	---	---	0
3	Acetamide D3	-	242.10	---	---	0

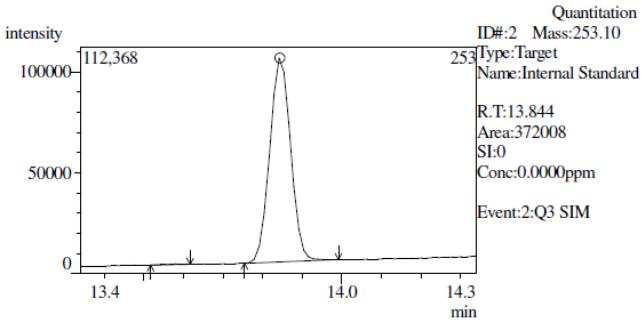
APPENDIX 8 contd. Bioanalytical Report

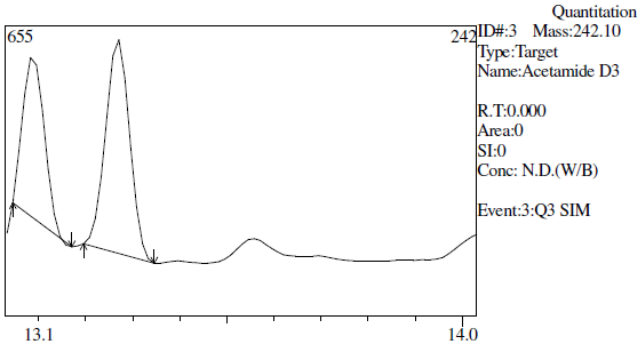
FIGURE 3. Chromatogram of Acetamide, Acetamide D3 and IS in standard zero sample in rat plasma

Sample Information

Analyzed by : Rajib Satpathy
Analyzed : 3/21/2018 6:21:07 PM
Sample Name : G16084-Zero-2
Vial # : 6
Injection Volume : 1.00
Org Data File : E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-005.qgd
Method File : E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File : E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

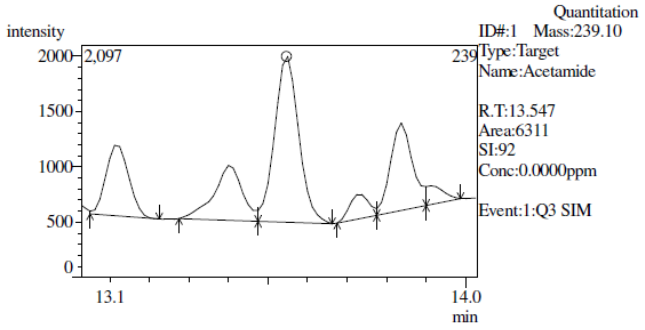
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.551	239.10	5892	1391	0
2	Internal Standard	13.844	253.10	372008	101150	0
3	Acetamide D3	-	242.10	---	---	0

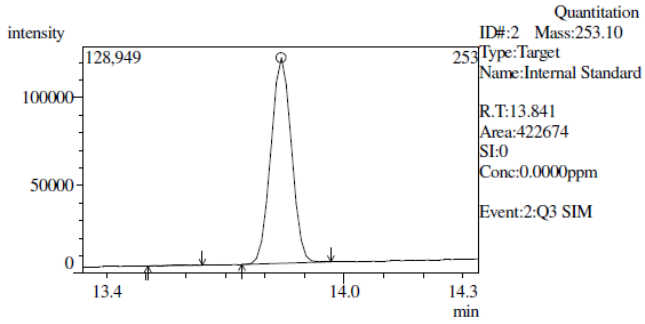
APPENDIX 8 contd. Bioanalytical Report

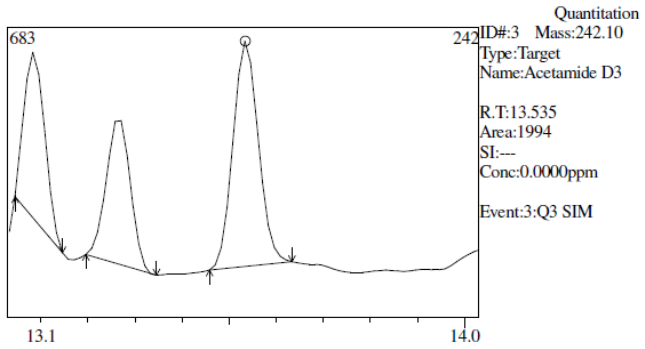
FIGURE 4. Chromatogram of Acetamide, Acetamide D3 and IS in LLOQ standard in rat plasma

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/21/2018 6:55:56 PM
Sample Name	: G16084-STD1-1
Vial #	: 7
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-006.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

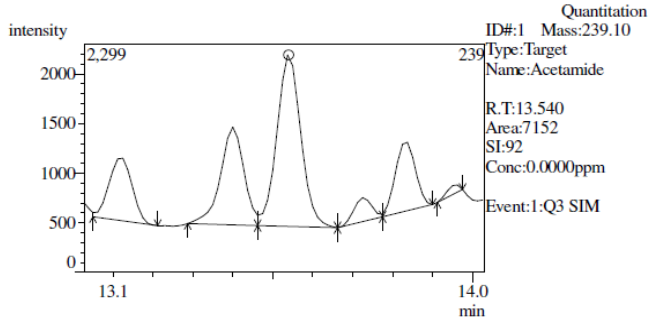
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.547	239.10	6311	1496	0
2	Internal Standard	13.841	253.10	422674	117123	0
3	Acetamide D3	13.535	242.10	1994	530	0

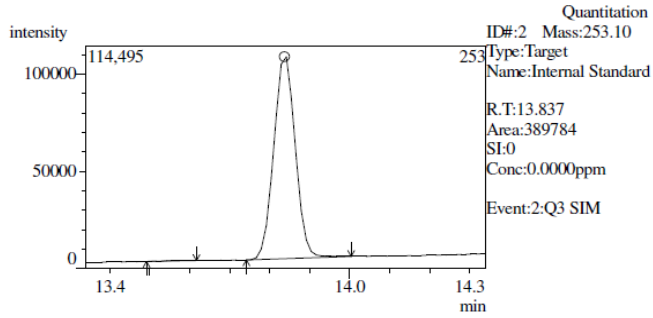
APPENDIX 8 contd. Bioanalytical Report

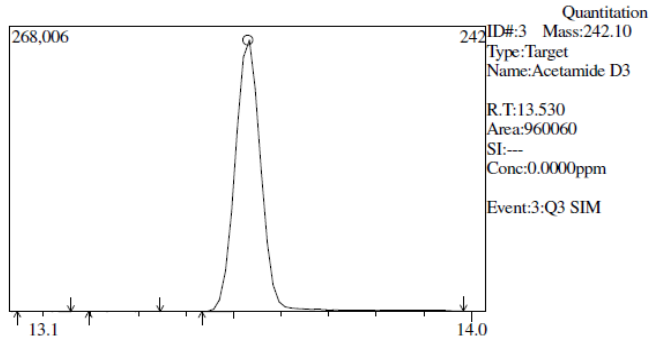
FIGURE 5. Chromatogram of Acetamide, Acetamide D3 and IS in ULOQ standard in rat plasma

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/21/2018 11:35:13 PM
Sample Name	: G16084-STD8-1
Vial #	: 15
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-014.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

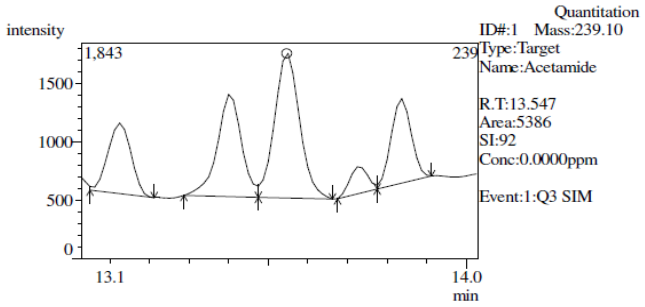
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.540	239.10	7152	1724	0
2	Internal Standard	13.837	253.10	389784	103885	0
3	Acetamide D3	13.530	242.10	960060	255011	0

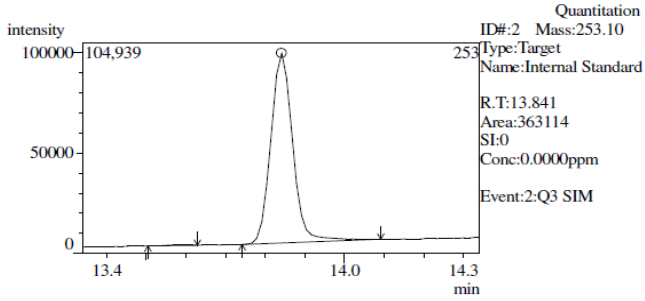
APPENDIX 8 contd. Bioanalytical Report

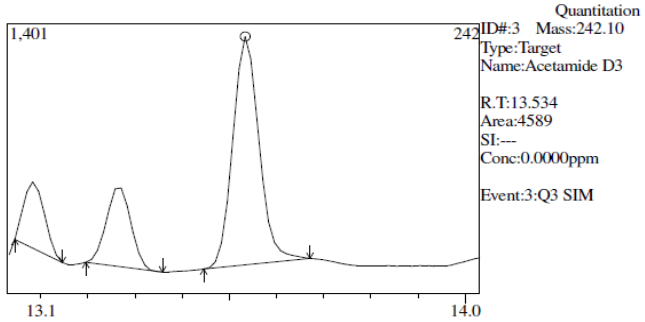
FIGURE 6. Chromatogram of Acetamide, Acetamide D3 and IS in LQC sample in rat plasma

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/22/2018 3:05:55 AM
Sample Name	: G16084-LQC-1
Vial #	: 20
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-020.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

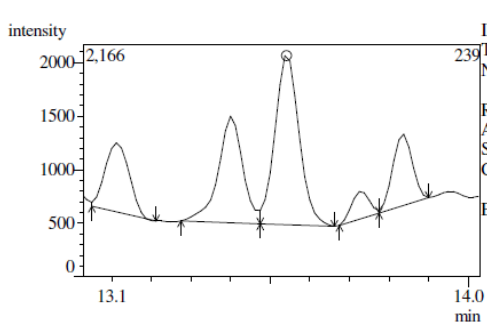
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.547	239.10	5386	1233	0
2	Internal Standard	13.841	253.10	363114	94661	0
3	Acetamide D3	13.534	242.10	4589	1184	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 7. Chromatogram of Acetamide, Acetamide D3 and IS in MQC sample in rat plasma

Sample Information

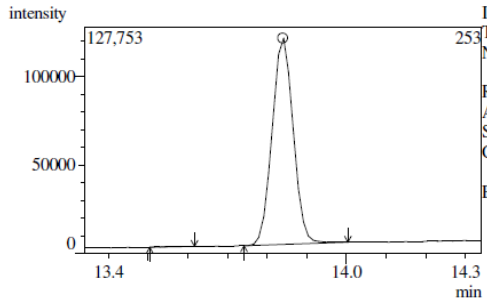
Analyzed by	: Rajib Satpathy
Analyzed	: 3/22/2018 3:41:13 AM
Sample Name	: G16084-MQC-1
Vial #	: 21
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-021.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt



Quantitation
ID#:1 Mass:239.10
Type:Target
Name:Acetamide

R.T:13.542
Area:6663
SI:92
Conc:0.0000ppm

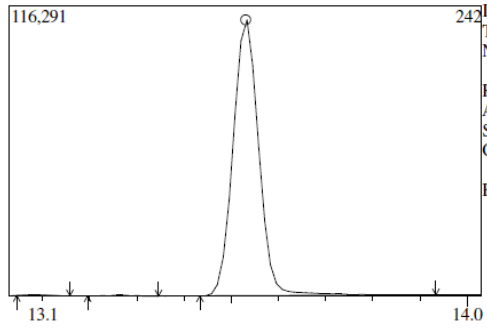
Event:1:Q3 SIM



Quantitation
ID#:2 Mass:253.10
Type:Target
Name:Internal Standard

R.T:13.839
Area:431416
SI:0
Conc:0.0000ppm

Event:2:Q3 SIM



Quantitation
ID#:3 Mass:242.10
Type:Target
Name:Acetamide D3

R.T:13.531
Area:418994
SI:---
Conc:0.0000ppm

Event:3:Q3 SIM

Quantitative Result Table

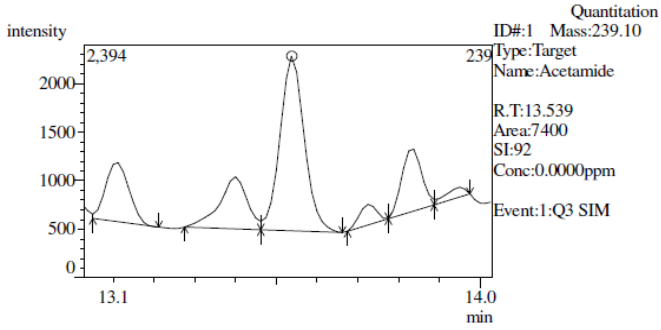
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.542	239.10	6663	1575	0
2	Internal Standard	13.839	253.10	431416	116476	0
3	Acetamide D3	13.531	242.10	418994	110547	0

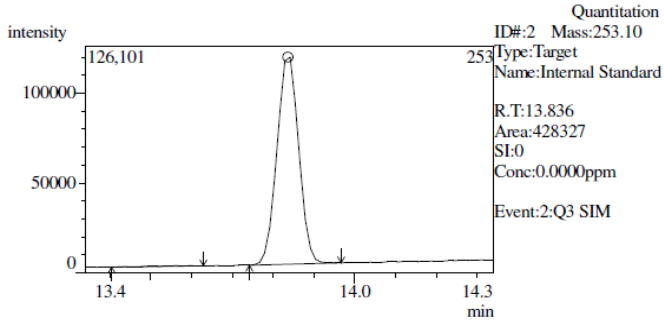
APPENDIX 8 contd. Bioanalytical Report

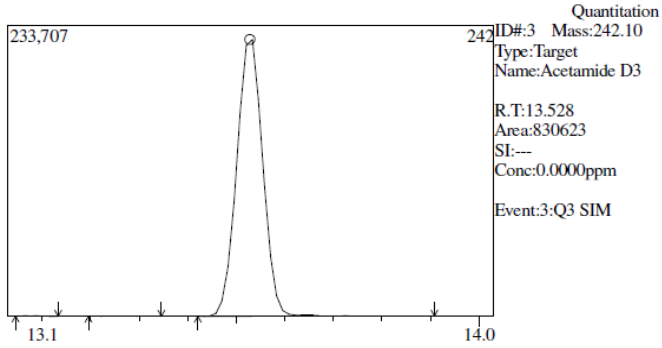
FIGURE 8. Chromatogram of Acetamide, Acetamide D3 and IS in HQC sample in rat Plasma

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/22/2018 4:16:22 AM
Sample Name	: G16084-HQC-1
Vial #	: 22
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-022.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

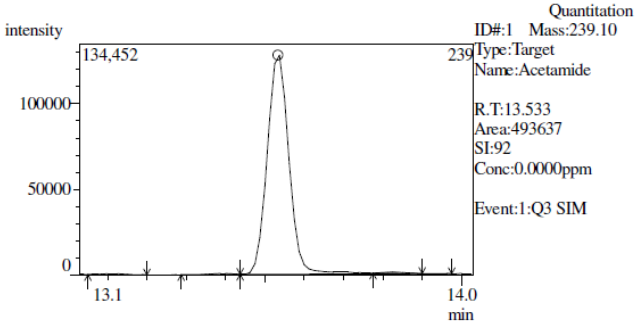
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.539	239.10	7400	1795	0
2	Internal Standard	13.836	253.10	428327	115044	0
3	Acetamide D3	13.528	242.10	830623	222422	0

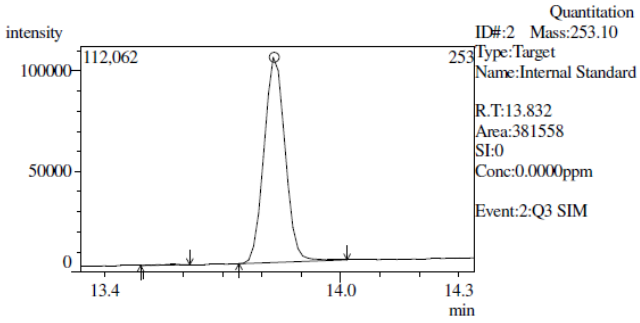
APPENDIX 8 contd. Bioanalytical Report

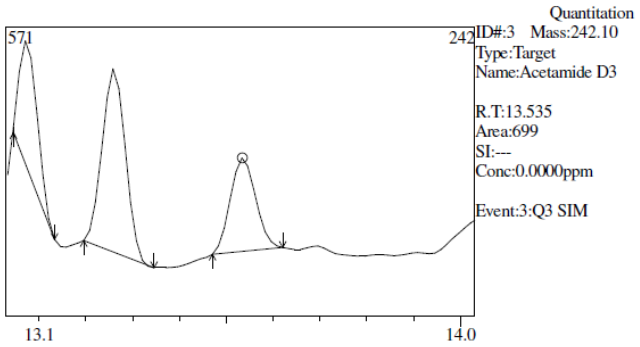
FIGURE 9. Representative chromatogram of Acetamide, Acetamide D3 and IS in rat plasma study sample (P_B-023)

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/22/2018 12:26:14 PM
Sample Name	: G16084-B023
Vial #	: 36
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\210318-G16084-SSA-Batch4-036.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt





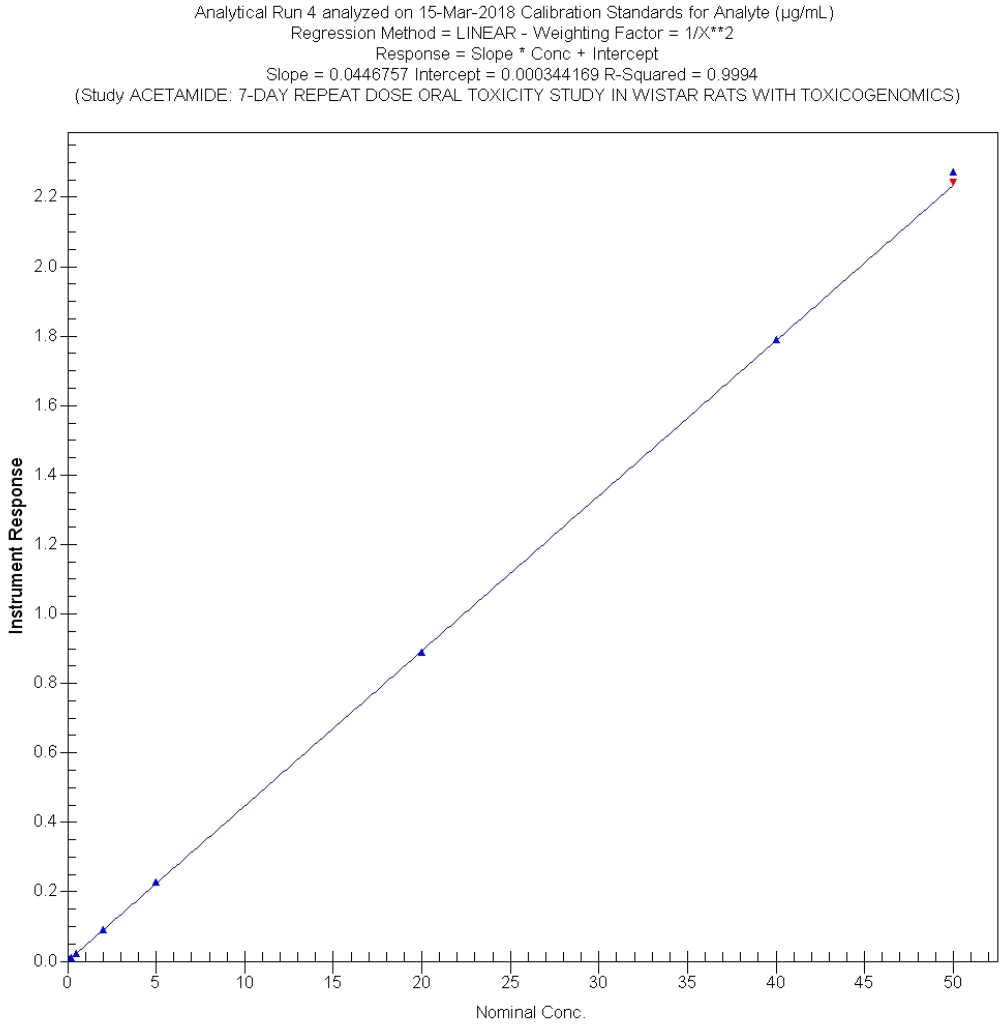


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.533	239.10	493637	127264	0
2	Internal Standard	13.832	253.10	381558	101938	0
3	Acetamide D3	13.535	242.10	699	184	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 10. Representative calibration curve of Acetamide D3 in rat liver homogenate

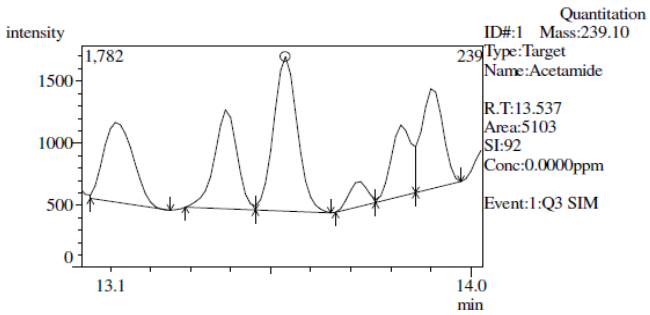


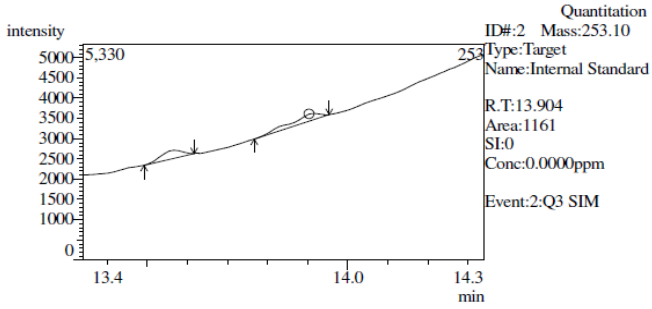
APPENDIX 8 contd. Bioanalytical Report

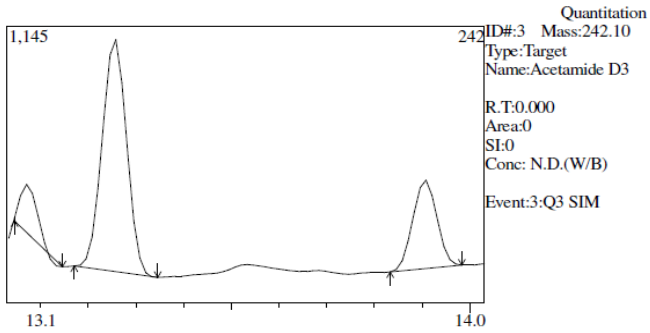
FIGURE 11. Chromatogram of Acetamide, Acetamide D3 and IS in standard blank sample in rat liver homogenate

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/24/2018 9:25:33 AM
Sample Name	: G16084-Ti-BLK-2
Vial #	: 107
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-003.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

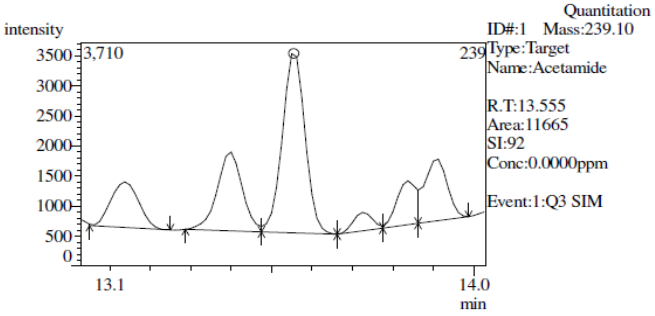
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.537	239.10	5103	1244	0
2	Internal Standard	13.904	253.10	1161	174	0
3	Acetamide D3	-	242.10	---	---	0

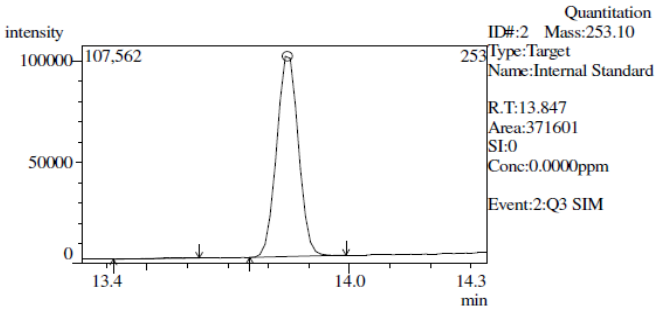
APPENDIX 8 contd. Bioanalytical Report

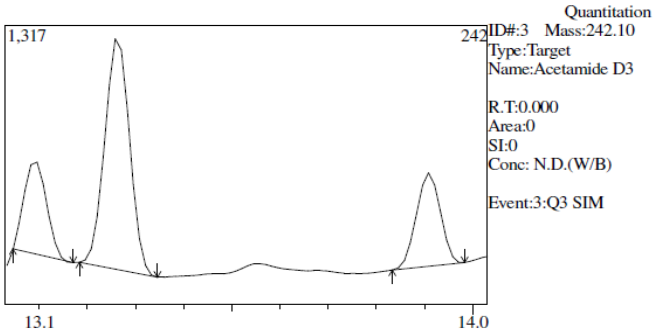
FIGURE 12. Chromatogram of Acetamide, Acetamide D3 and IS in standard zero sample in rat liver homogenate

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/24/2018 10:00:32 AM
Sample Name	: G16084-Ti-Zero-1
Vial #	: 108
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-004.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

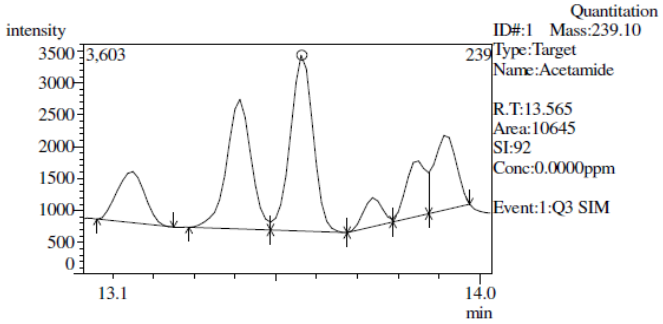
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.555	239.10	11665	2978	0
2	Internal Standard	13.847	253.10	371601	98959	0
3	Acetamide D3	-	242.10	---	---	0

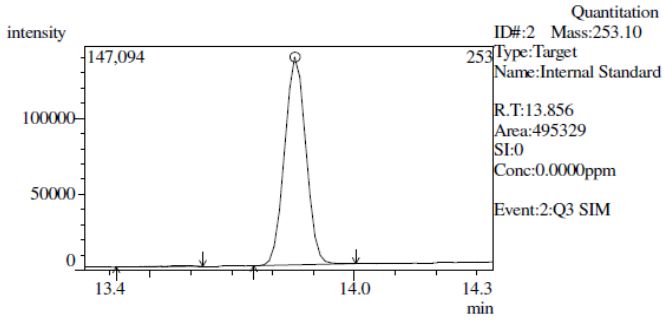
APPENDIX 8 contd. Bioanalytical Report

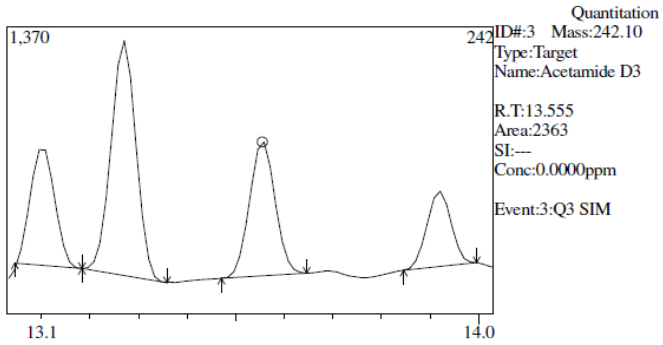
FIGURE 13. Chromatogram of Acetamide, Acetamide D3 and IS in LLOQ standard in rat liver homogenate

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/24/2018 11:10:23 AM
Sample Name	: G16084-Ti-STD1-1
Vial #	: 110
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-006.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

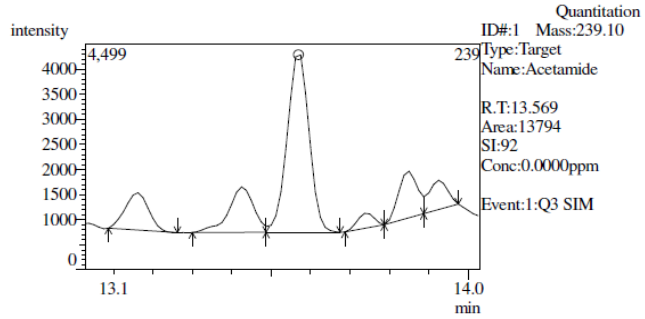
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.565	239.10	10645	2755	0
2	Internal Standard	13.856	253.10	495329	136348	0
3	Acetamide D3	13.555	242.10	2363	638	0

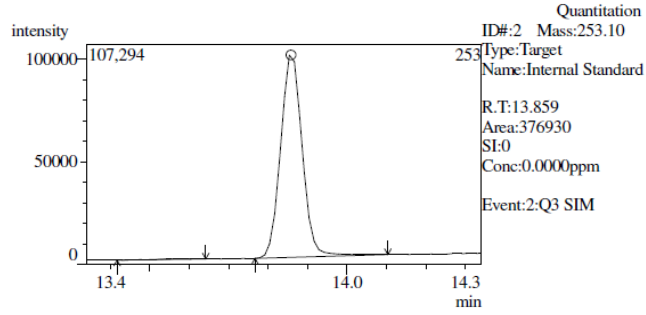
APPENDIX 8 contd. Bioanalytical Report

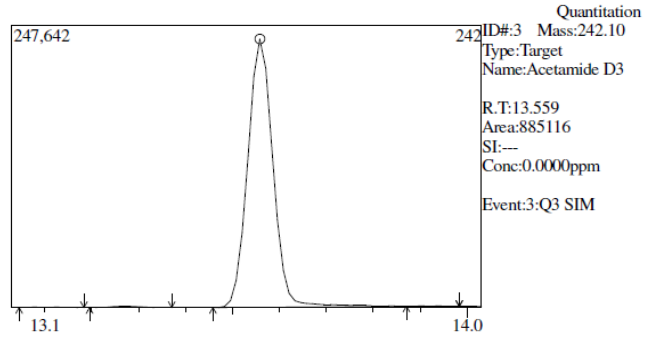
FIGURE 14. Chromatogram of Acetamide, Acetamide D3 and IS in ULOQ standard in rat liver homogenate

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/24/2018 3:49:45 PM
Sample Name	: G16084-Ti-STD8-1
Vial #	: 118
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-014.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

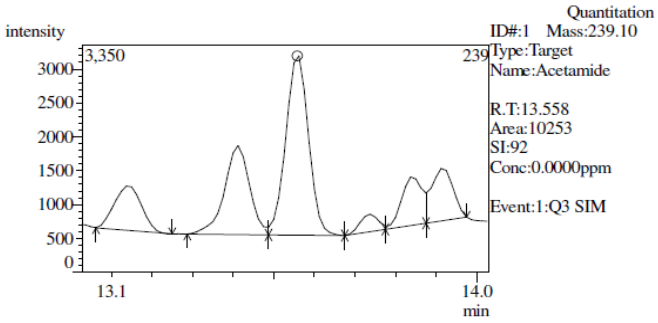
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.569	239.10	13794	3535	0
2	Internal Standard	13.859	253.10	376930	98616	0
3	Acetamide D3	13.559	242.10	885116	235483	0

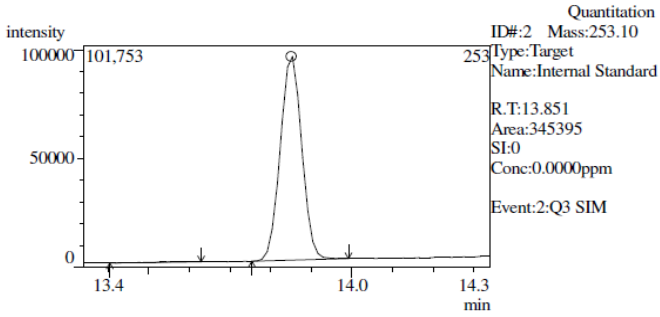
APPENDIX 8 contd. Bioanalytical Report

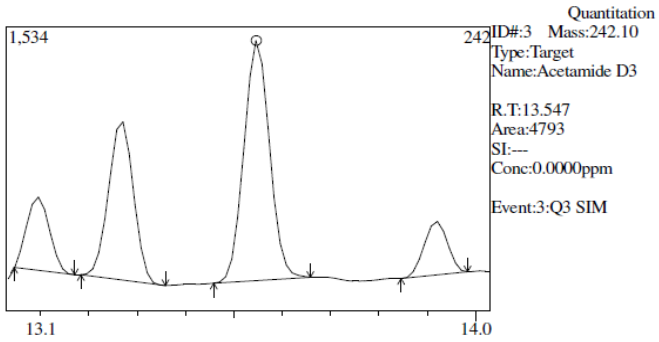
FIGURE 15. Chromatogram of Acetamide, Acetamide D3 and IS in LQC sample in rat liver homogenate

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/24/2018 7:23:13 PM
Sample Name	: G16084-Ti-LQC-1
Vial #	: 123
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-020.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt





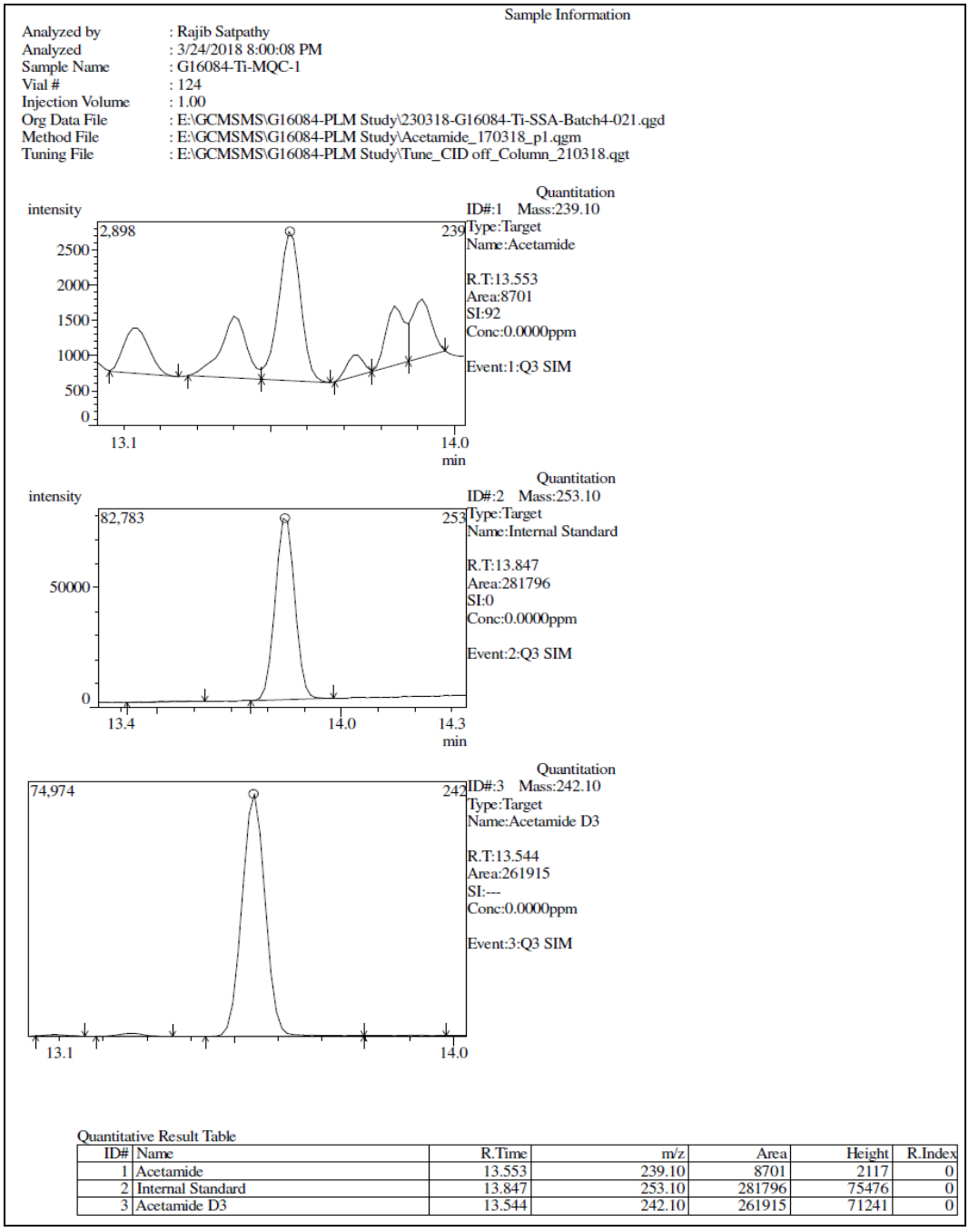


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.558	239.10	10253	2639	0
2	Internal Standard	13.851	253.10	345395	93542	0
3	Acetamide D3	13.547	242.10	4793	1296	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 16. Chromatogram of Acetamide, Acetamide D3 and IS in MQC sample in rat liver homogenate



Quantitative Result Table

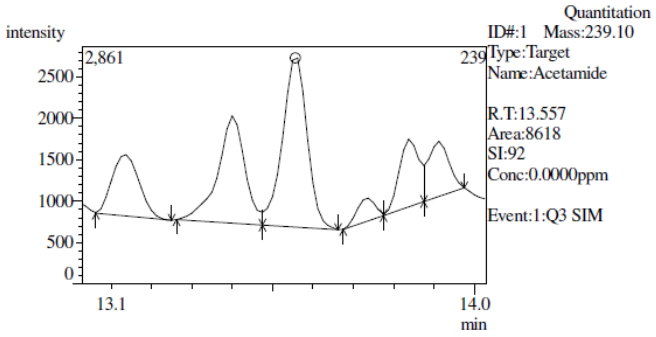
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.553	239.10	8701	2117	0
2	Internal Standard	13.847	253.10	281796	75476	0
3	Acetamide D3	13.544	242.10	261915	71241	0

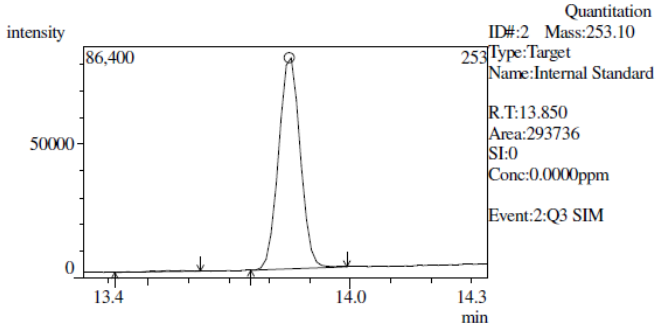
APPENDIX 8 contd. Bioanalytical Report

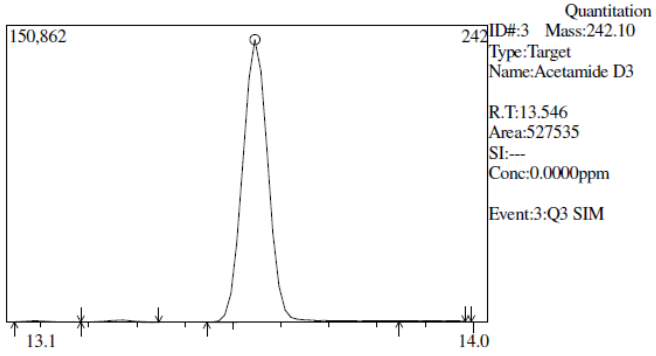
FIGURE 17. Chromatogram of Acetamide, Acetamide D3 and IS in HQC sample in rat liver homogenate

Sample Information

Analyzed by	: Rajib Satpathy
Analyzed	: 3/24/2018 8:35:48 PM
Sample Name	: G16084-Ti-HQC-1
Vial #	: 125
Injection Volume	: 1.00
Org Data File	: E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-022.qgd
Method File	: E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File	: E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt







Quantitative Result Table

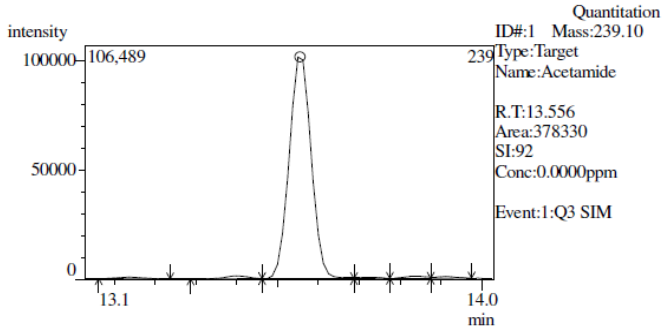
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.557	239.10	8618	2035	0
2	Internal Standard	13.850	253.10	293736	78759	0
3	Acetamide D3	13.546	242.10	527535	143478	0

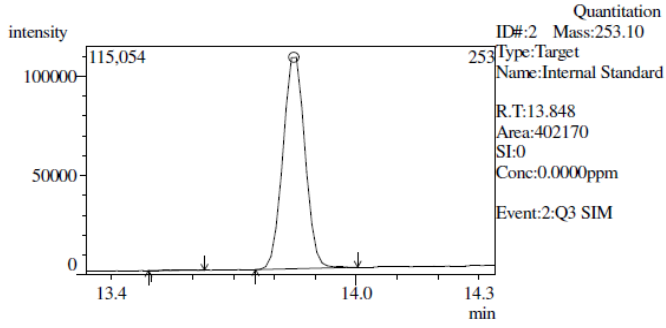
APPENDIX 8 contd. Bioanalytical Report

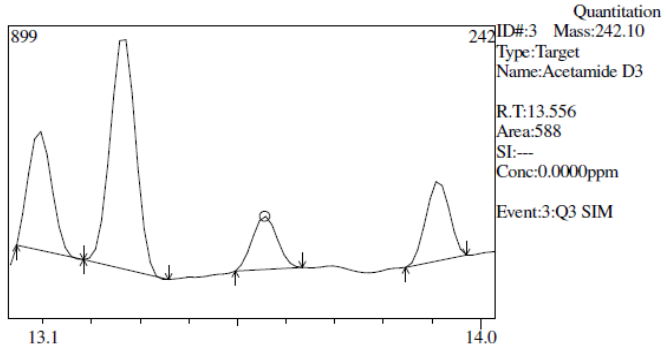
FIGURE 18. Representative chromatogram of Acetamide, Acetamide D3 and IS in rat liver study sample (L_B-021)

Sample Information

Analyzed by : Rajib Satpathy
Analyzed : 3/25/2018 2:23:36 AM
Sample Name : G16084-Ti-B-021
Vial # : 135
Injection Volume : 1.00
Org Data File : E:\GCMSMS\G16084-PLM Study\230318-G16084-Ti-SSA-Batch4-032.qgd
Method File : E:\GCMSMS\G16084-PLM Study\Acetamide_170318_p1.qgm
Tuning File : E:\GCMSMS\G16084-PLM Study\Tune_CID off_Column_210318.qgt





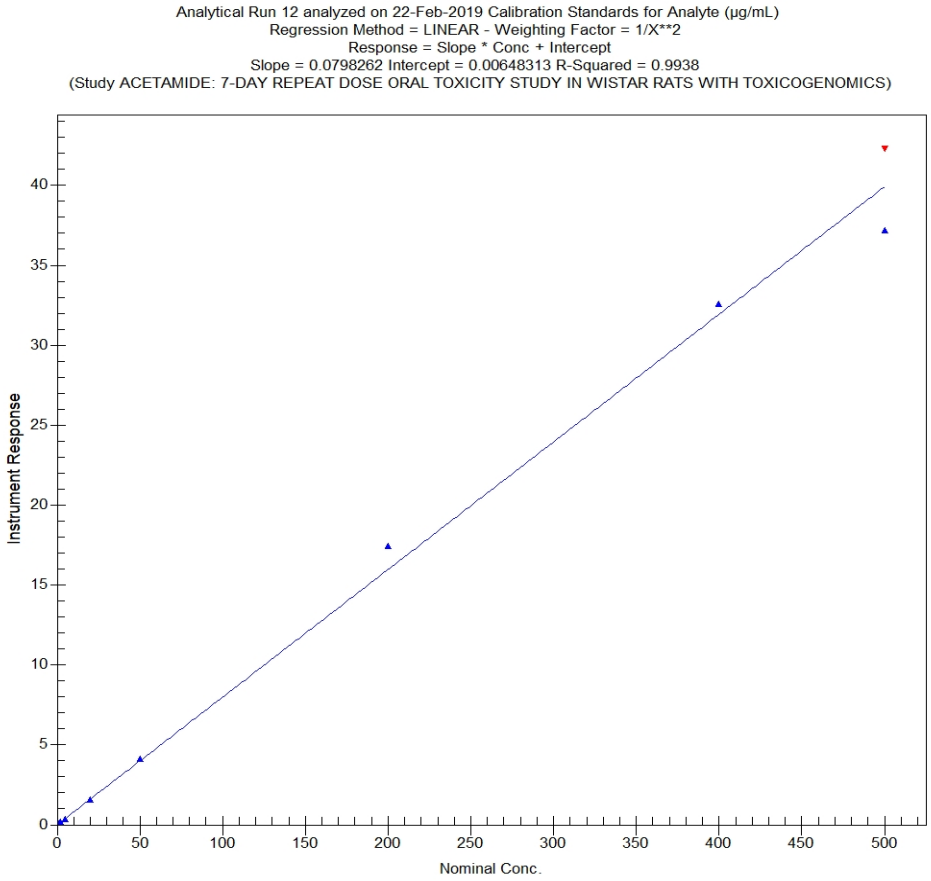


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamide	13.556	239.10	378330	100675	0
2	Internal Standard	13.848	253.10	402170	106420	0
3	Acetamide D3	13.556	242.10	588	163	0

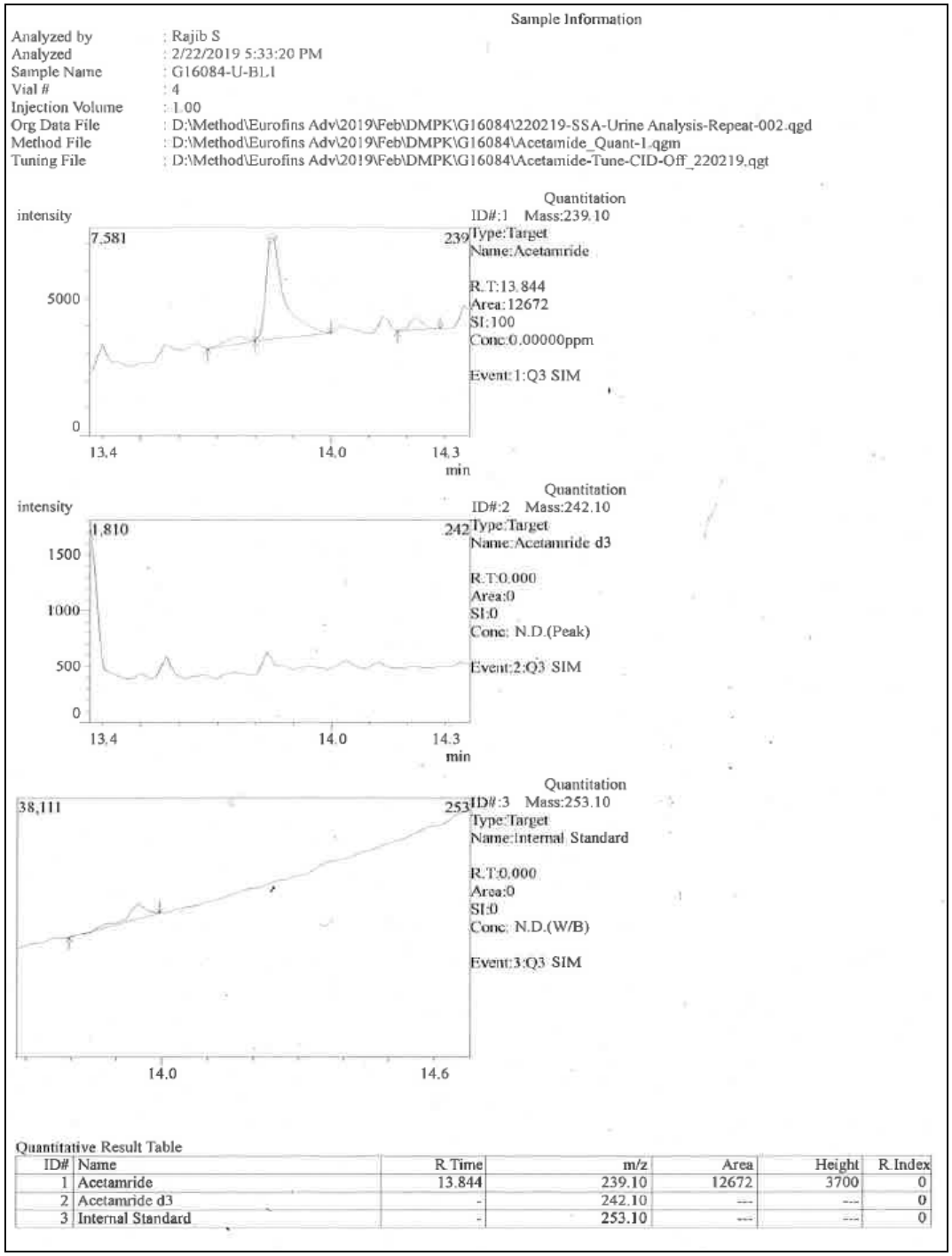
APPENDIX 8 contd. Bioanalytical Report

FIGURE 19. Representative calibration curve of Acetamide D3 in rat urine



APPENDIX 8 contd. Bioanalytical Report

FIGURE 20. Chromatogram of Acetamide, Acetamide D3 and IS in standard blank sample in rat urine

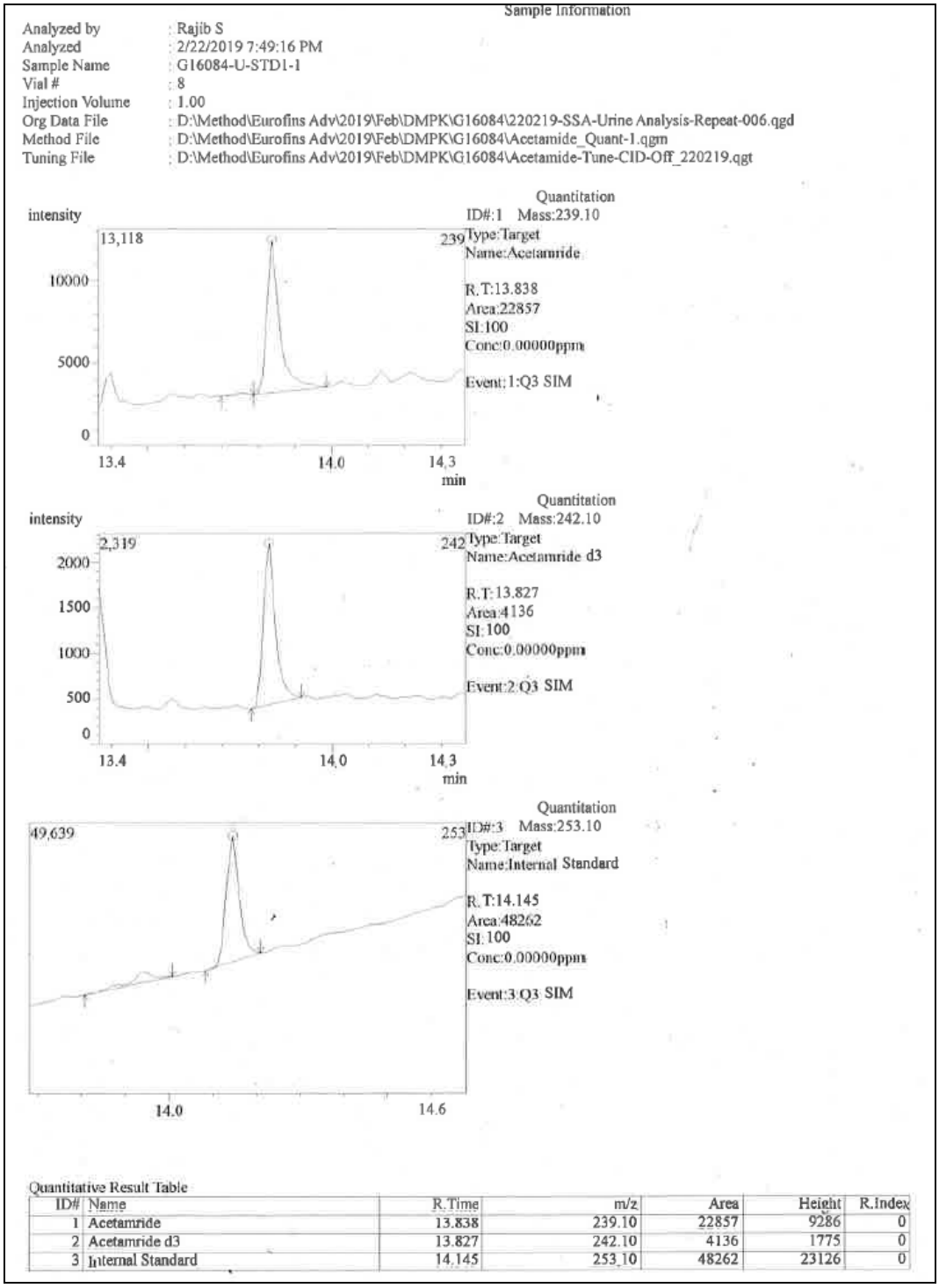


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.844	239.10	12672	3700	0
2	Acetamride d3	-	242.10	---	---	0
3	Internal Standard	-	253.10	---	---	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 21. Chromatogram of Acetamide, Acetamide D3 and IS in LLOQ standard in rat urine

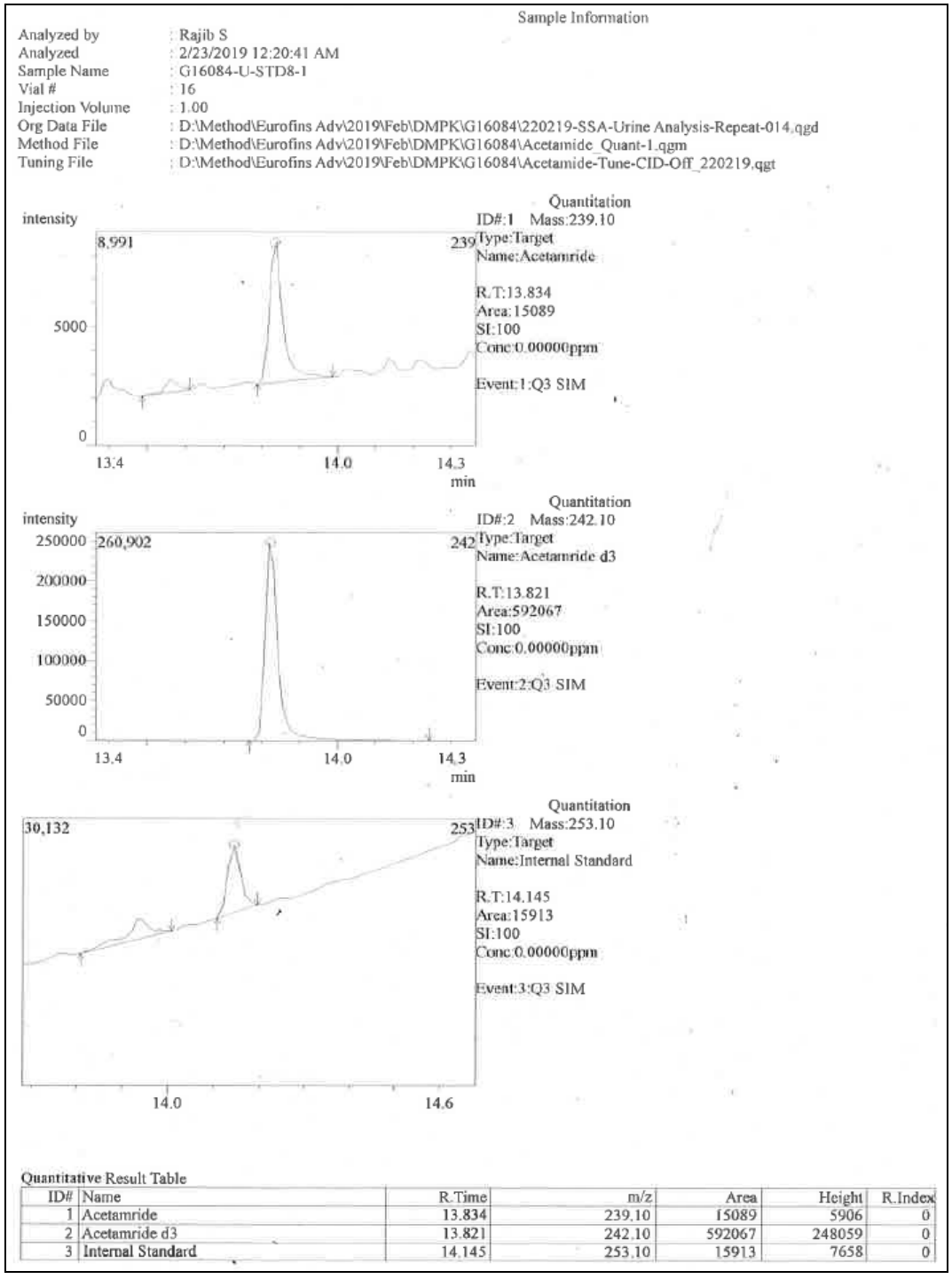


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.838	239.10	22857	9286	0
2	Acetamride d3	13.827	242.10	4136	1775	0
3	Internal Standard	14.145	253.10	48262	23126	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 22. Chromatogram of Acetamide, Acetamide D3 and IS in ULOQ standard in rat urine

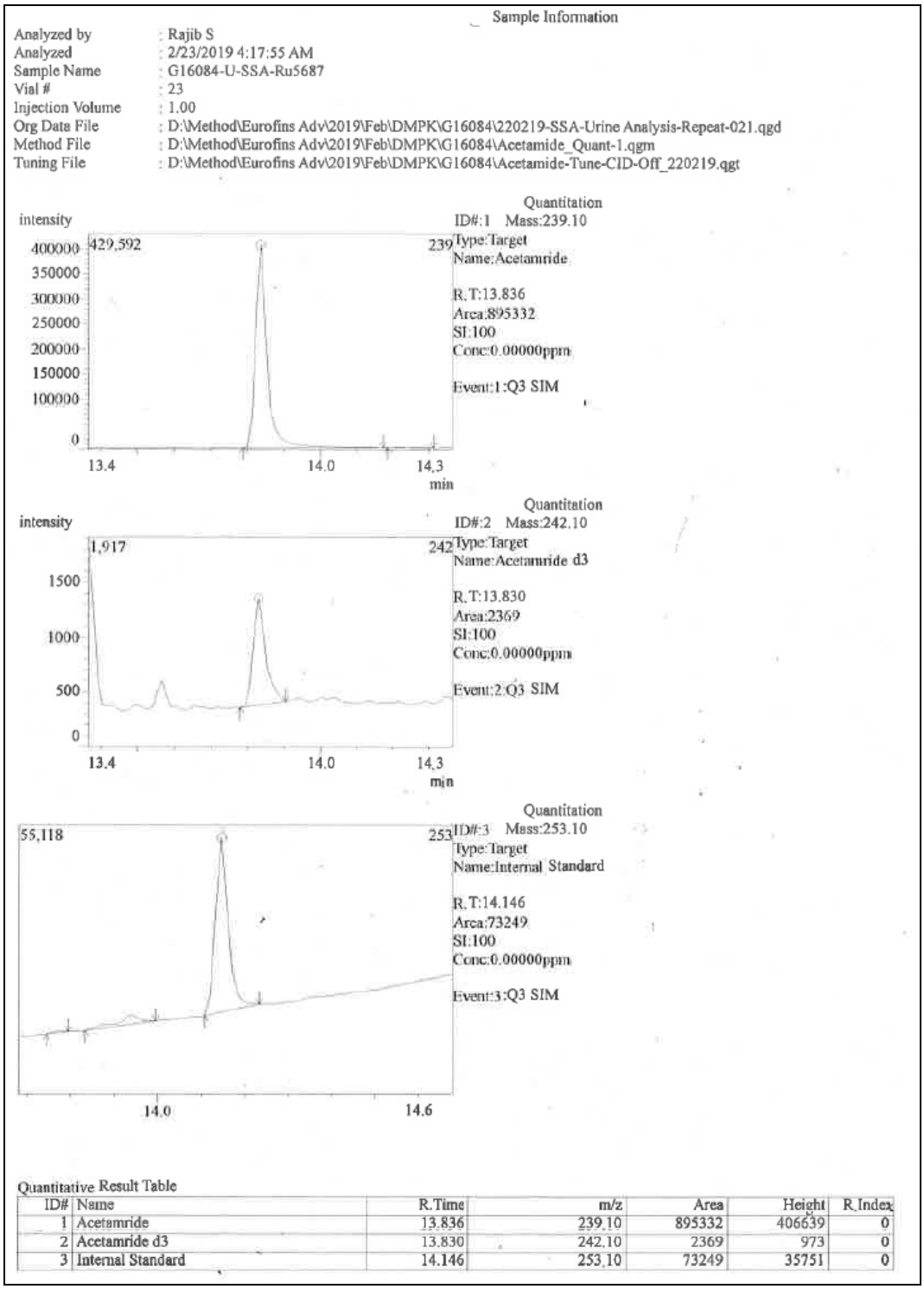


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.834	239.10	15089	5906	0
2	Acetamride d3	13.821	242.10	592067	248059	0
3	Internal Standard	14.145	253.10	15913	7658	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 23. Chromatogram of Acetamide, Acetamide D3 and IS in rat urine study sample

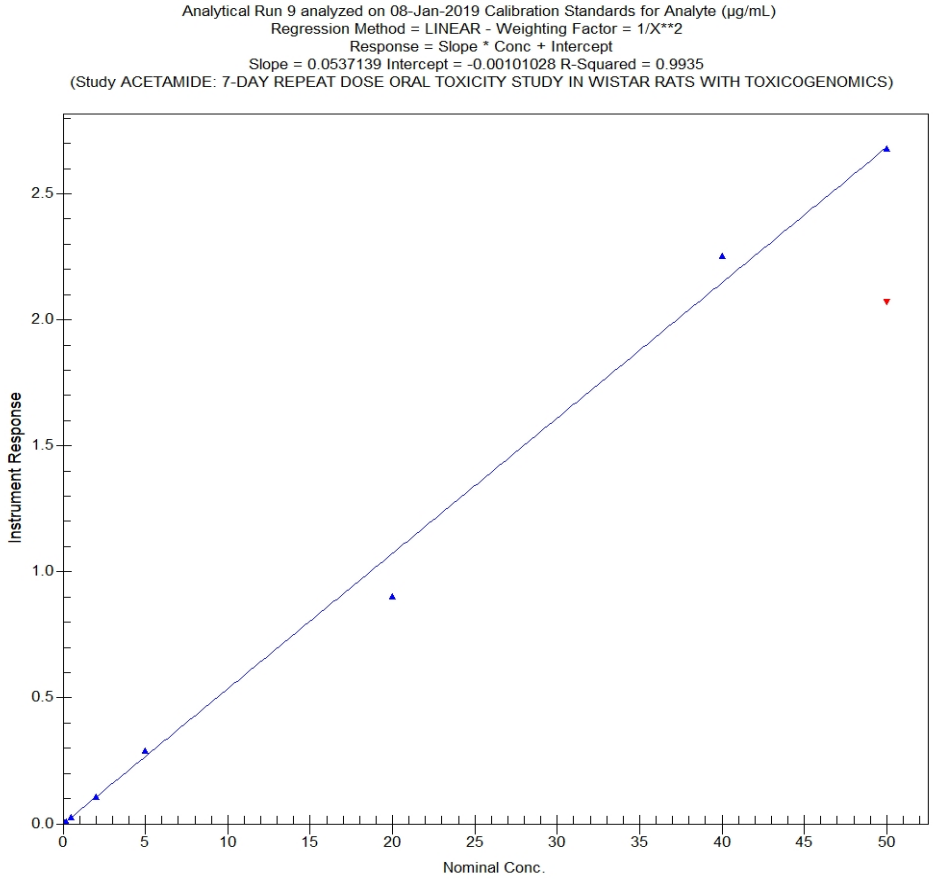


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.836	239.10	895332	406639	0
2	Acetamride d3	13.830	242.10	2369	973	0
3	Internal Standard	14.146	253.10	73249	35751	0

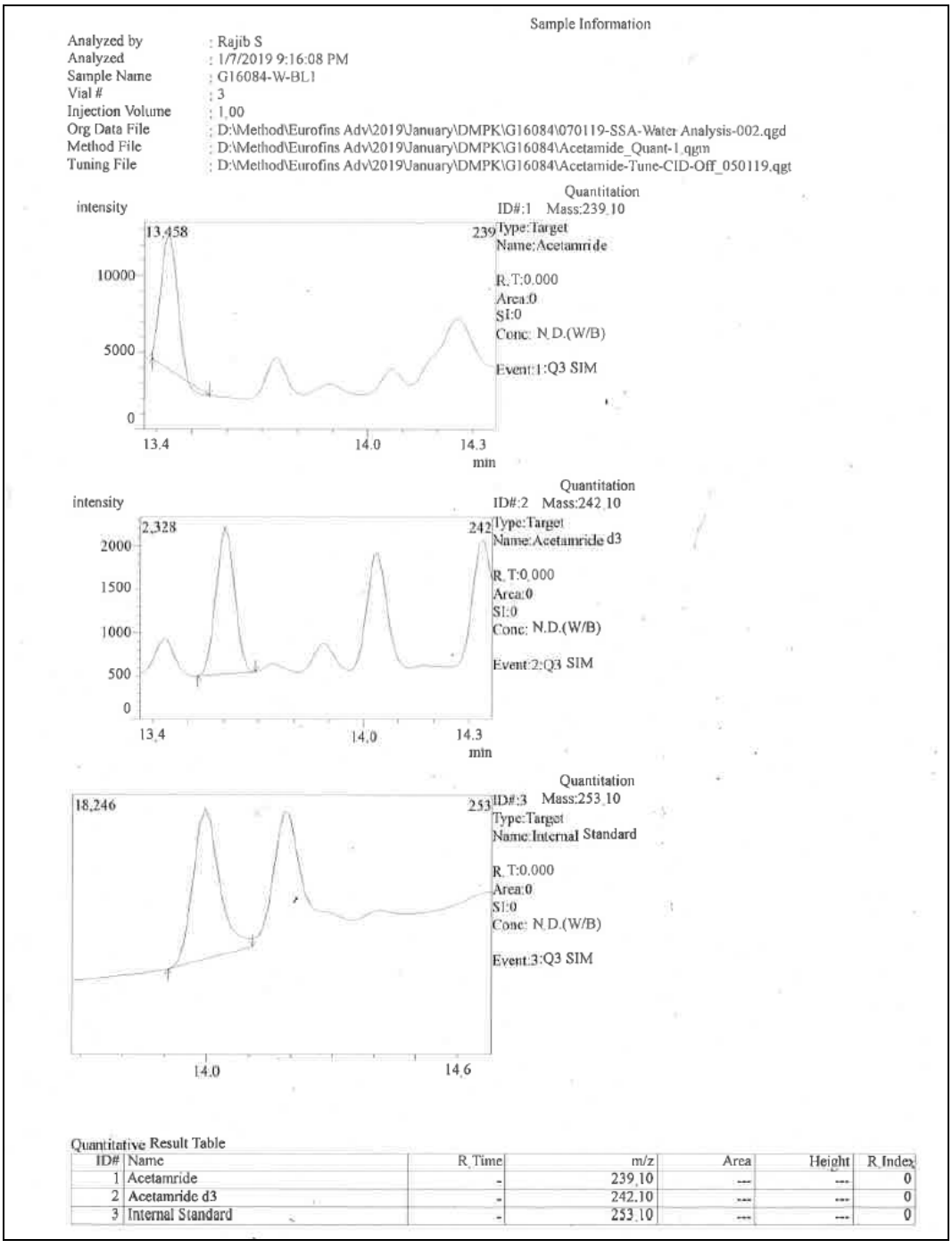
APPENDIX 8 contd. Bioanalytical Report

FIGURE 24. Calibration curve of Acetamide D3 in water



APPENDIX 8 contd. Bioanalytical Report

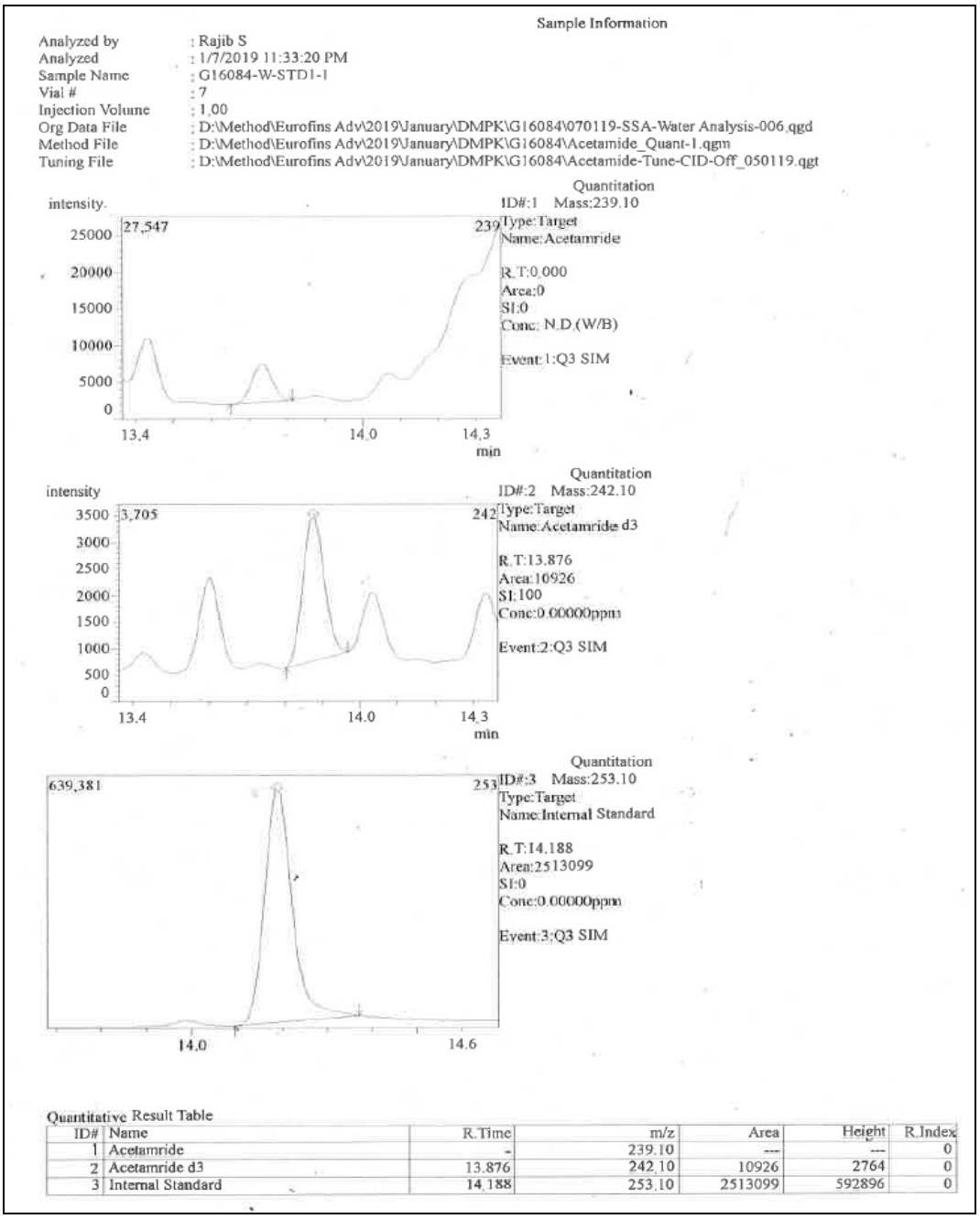
FIGURE 25. Chromatogram of Acetamide, Acetamide D3 and IS in standard blank sample in water



ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	-	239.10	---	---	0
2	Acetamride d3	-	242.10	---	---	0
3	Internal Standard	-	253.10	---	---	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 26. Chromatogram of Acetamide, Acetamide D3 and IS in LLOQ standard in water

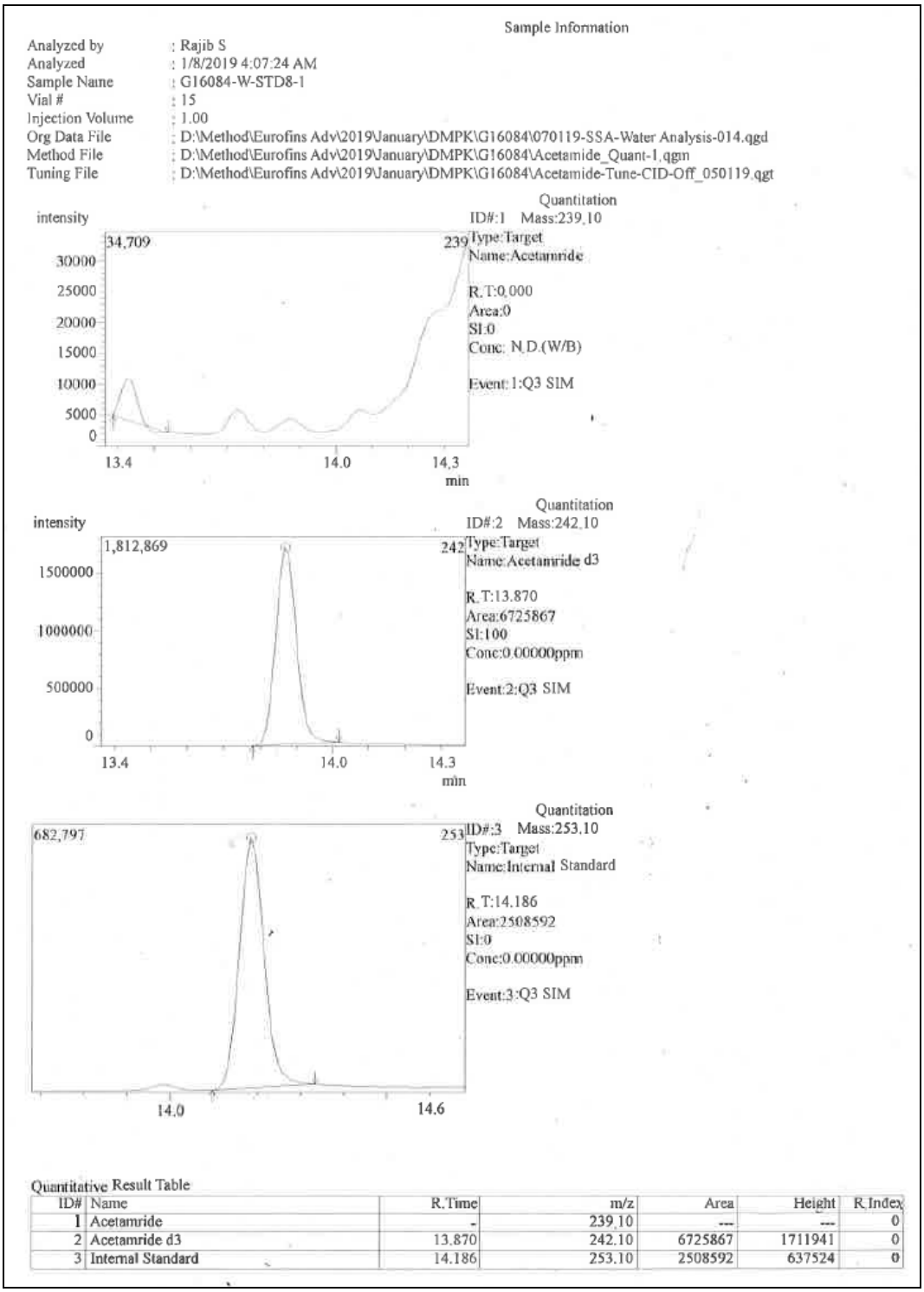


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	-	239.10	---	---	0
2	Acetamride d3	13.876	242.10	10926	2764	0
3	Internal Standard	14.188	253.10	2513099	592896	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 27. Chromatogram of Acetamide, Acetamide D3 and IS in ULOQ standard in water

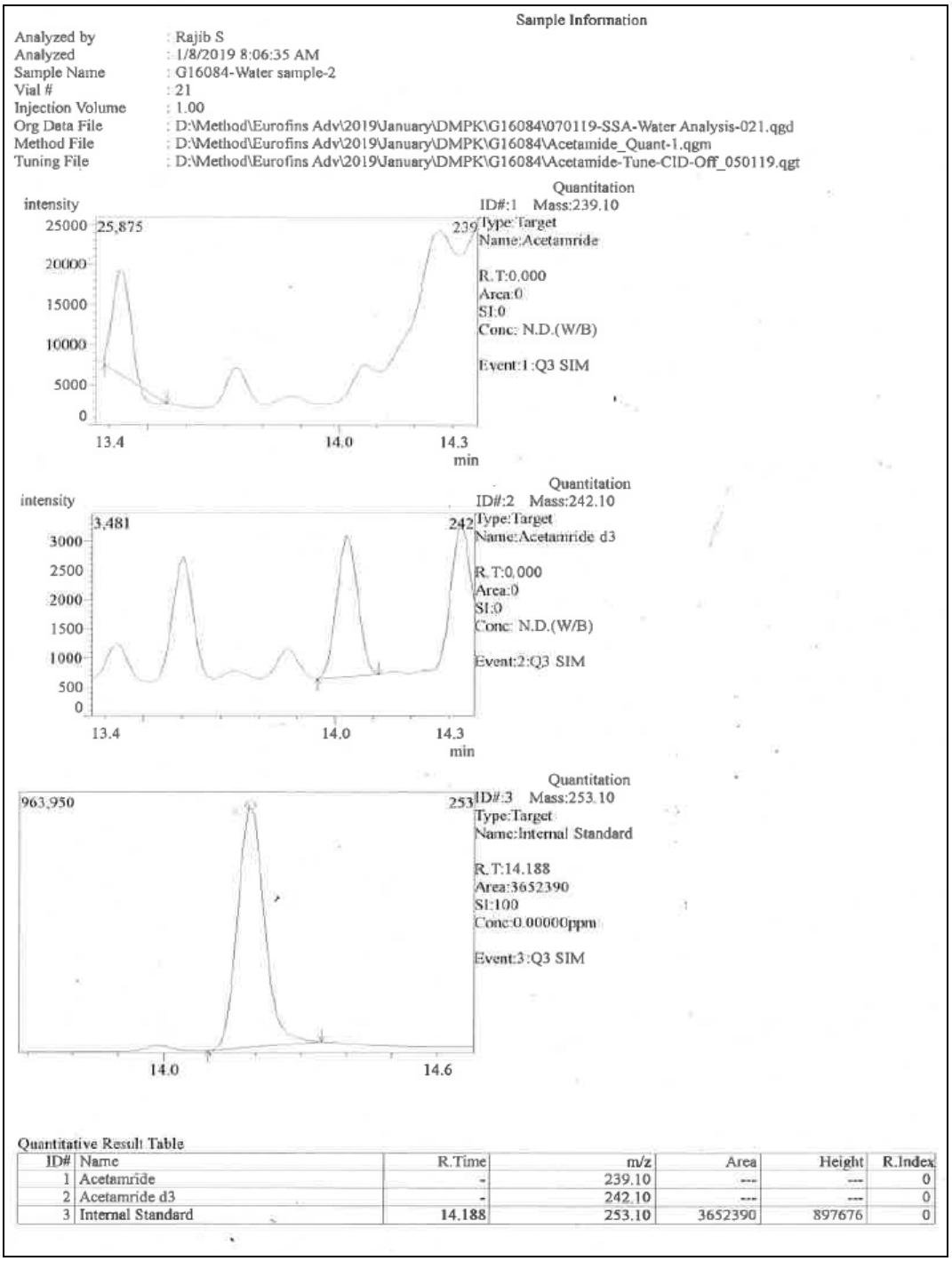


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	-	239.10	---	---	0
2	Acetamride d3	13.870	242.10	6725867	1711941	0
3	Internal Standard	14.186	253.10	2508592	637524	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 28. Chromatogram of Acetamide, Acetamide D3 and IS in water sample

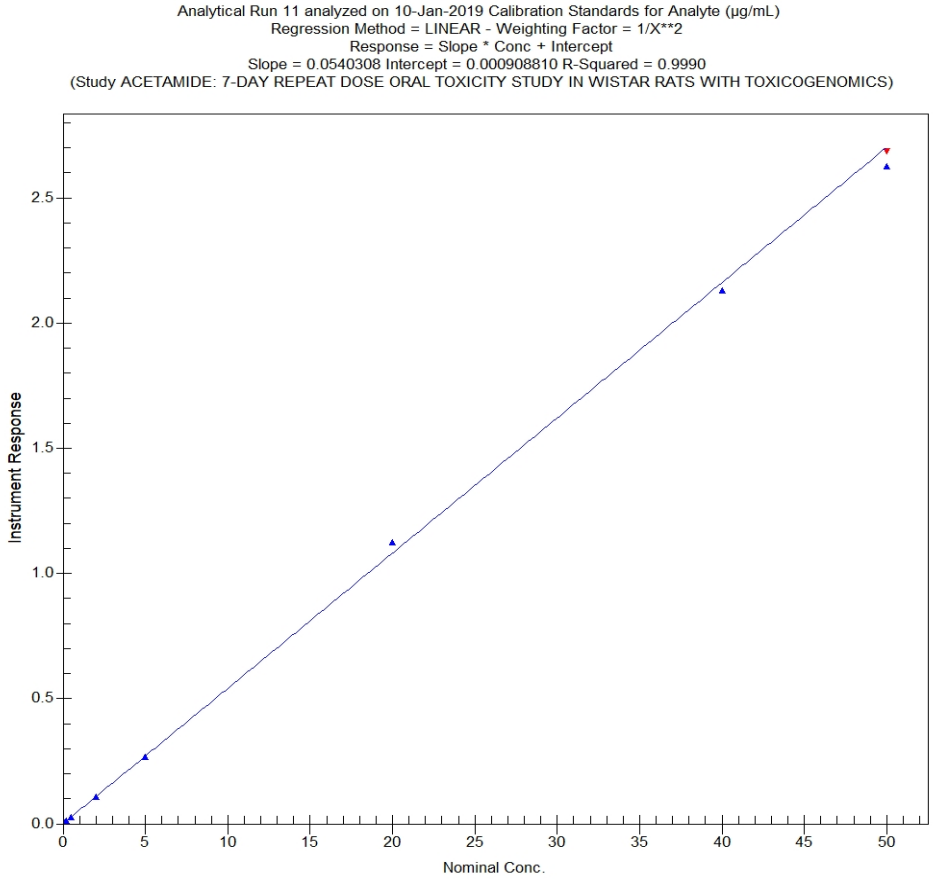


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	-	239.10	---	---	0
2	Acetamride d3	-	242.10	---	---	0
3	Internal Standard	14.188	253.10	3652390	897676	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 29. Calibration curve of Acetamide D3 in feed

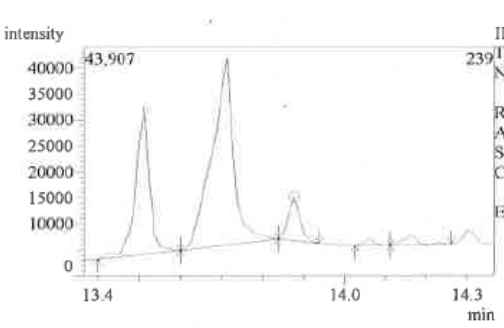


APPENDIX 8 contd. Bioanalytical Report

FIGURE 30. Chromatogram of Acetamide, Acetamide D3 and IS in standard blank sample in feed

Sample Information

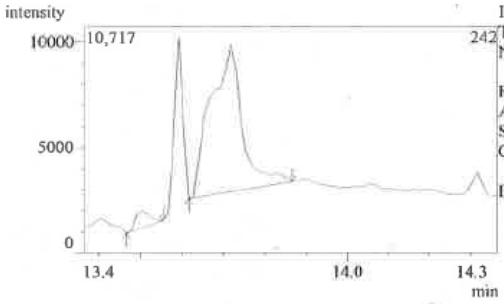
Analyzed by	: Rajib S
Analyzed	: 1/11/2019 1:45:25 AM
Sample Name	: G16084-Feed-BL1
Vial #	: 4
Injection Volume	: 1.00
Org Data File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\100119-SSA-Feed Analysis-002.qgd
Method File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\Acetamide_Quant-3.qgm
Tuning File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\Acetamide-Tune-CID-Off_050119.qgt



Quantitation
ID#:1 Mass:239.10
Type:Target
Name:Acetamride

R.T:13.875
Area:15857
SI:100
Conc:0.00000ppm

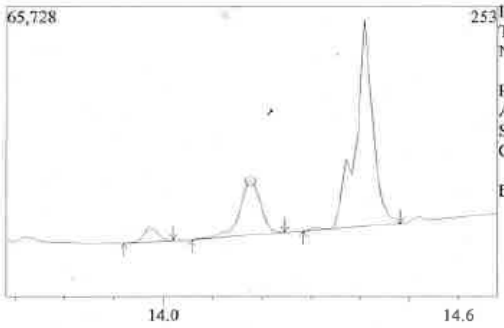
Event:1:Q3 SIM



Quantitation
ID#:2 Mass:242.10
Type:Target
Name:Acetamride d3

R.T:0.000
Area:0
SI:0
Conc: N.D.(W/B)

Event:2:Q3 SIM



Quantitation
ID#:3 Mass:253.10
Type:Target
Name:Internal Standard

R.T:14.176
Area:36510
SI:100
Conc:0.00000ppm

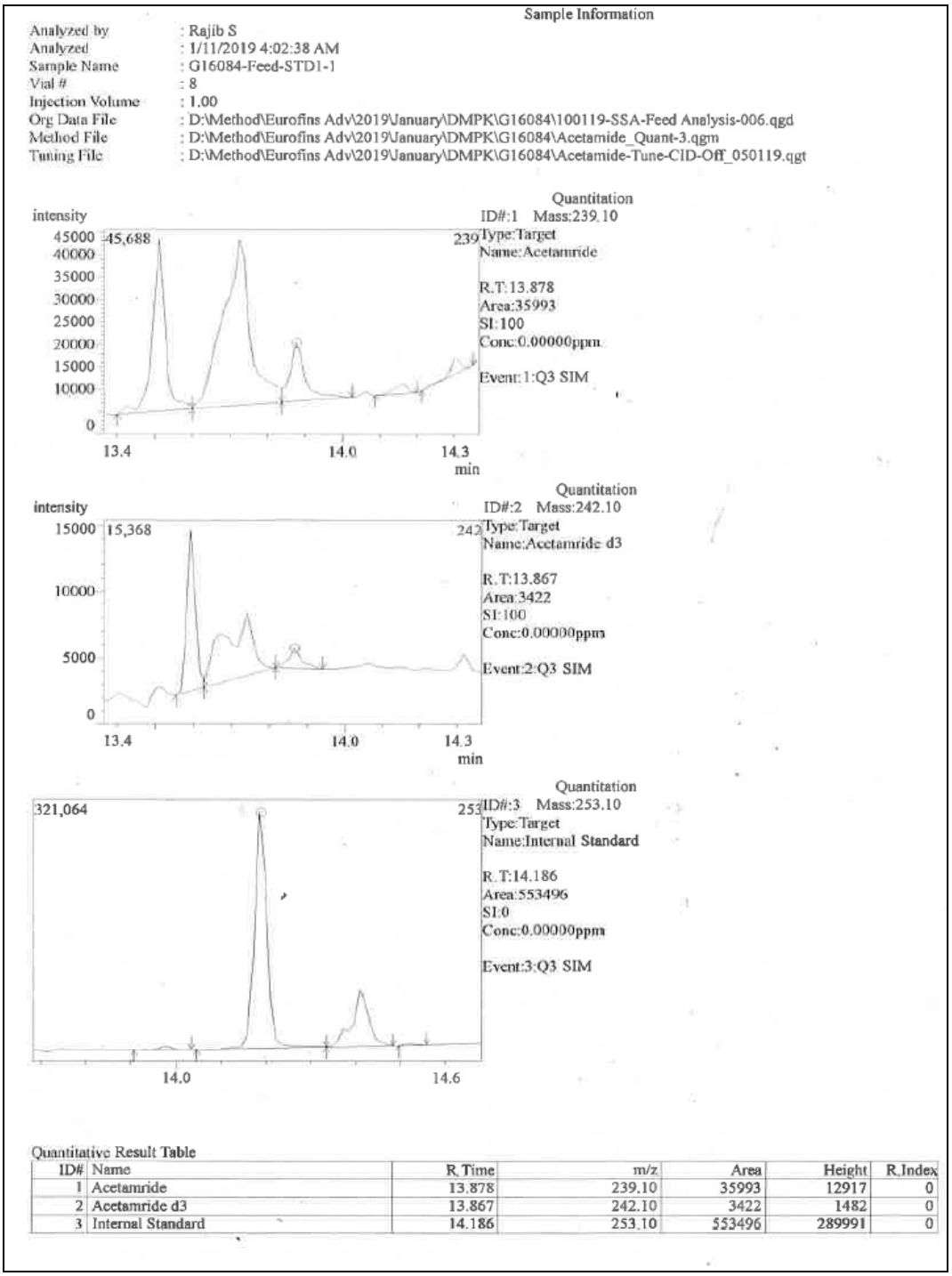
Event:3:Q3 SIM

Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.875	239.10	15857	8349	0
2	Acetamride d3	-	242.10	---	---	0
3	Internal Standard	14.176	253.10	36510	12291	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 31. Chromatogram of Acetamide, Acetamide D3 and IS in LLOQ standard in feed



Quantitative Result Table

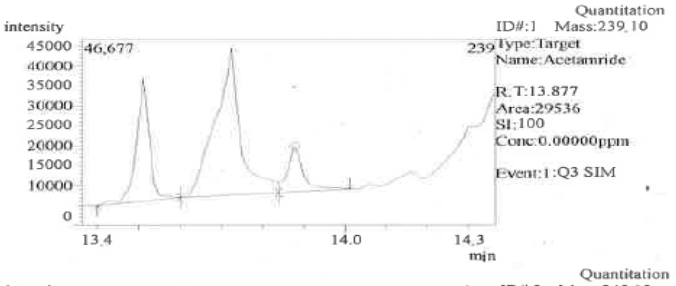
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.878	239.10	35993	12917	0
2	Acetamride d3	13.867	242.10	3422	1482	0
3	Internal Standard	14.186	253.10	553496	289991	0

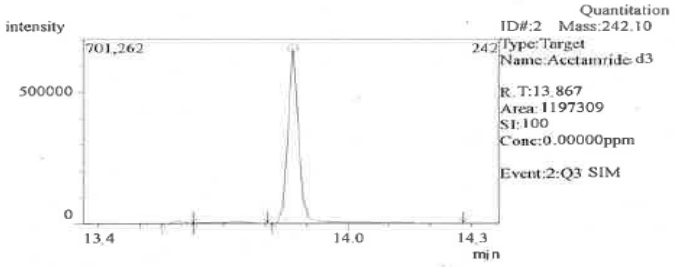
APPENDIX 8 contd. Bioanalytical Report

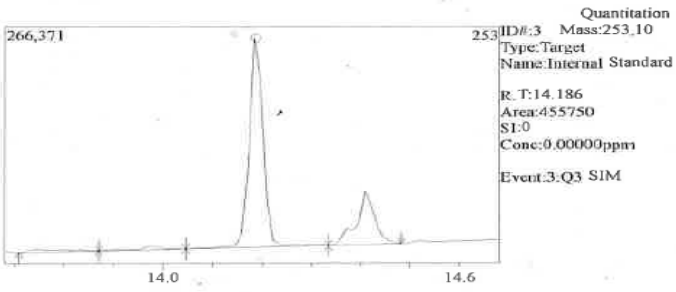
FIGURE 32. Chromatogram of Acetamide, Acetamide D3 and IS in ULOQ standard in feed

Sample Information

Analyzed by	: Rajib S
Analyzed	: 1/11/2019 12:01:57 PM
Sample Name	: G16084-Feed-STD8-1
Vial #	: 22
Injection Volume	: 1.00
Org Data File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\100119-SSA-Feed Analysis-020.qgd
Method File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\Acetamide_Quant-3.qgm
Tuning File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\Acetamide-Tune-CID-Off_050119.qgt







Quantitative Result Table

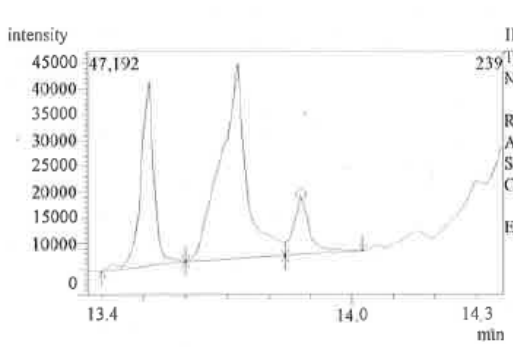
ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.877	239.10	29536	11349	0
2	Acetamride d3	13.867	242.10	1197309	663560	0
3	Internal Standard	14.186	253.10	455750	235228	0

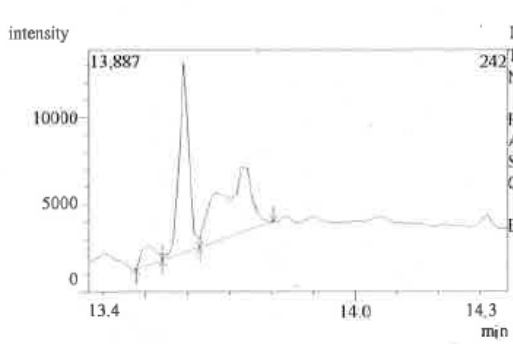
APPENDIX 8 contd. Bioanalytical Report

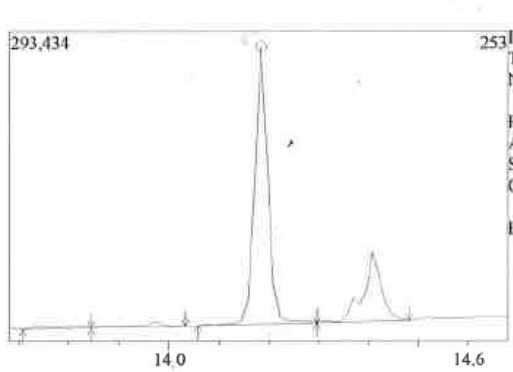
FIGURE 33. Chromatogram of Acetamide, Acetamide D3 and IS in feed sample

Sample Information

Analyzed by	: Rajib S
Analyzed	: 1/11/2019 6:53:28 AM
Sample Name	: G16084-Feed sample-1
Vial #	: 13
Injection Volume	: 1.00
Org Data File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\100119-SSA-Feed Analysis-011.qgd
Method File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\Acetamide_Quant-3.qgm
Tuning File	: D:\Method\Eurofins Adv\2019\January\DMPK\G16084\Acetamide-Tune-CID-Off_050119.qgt





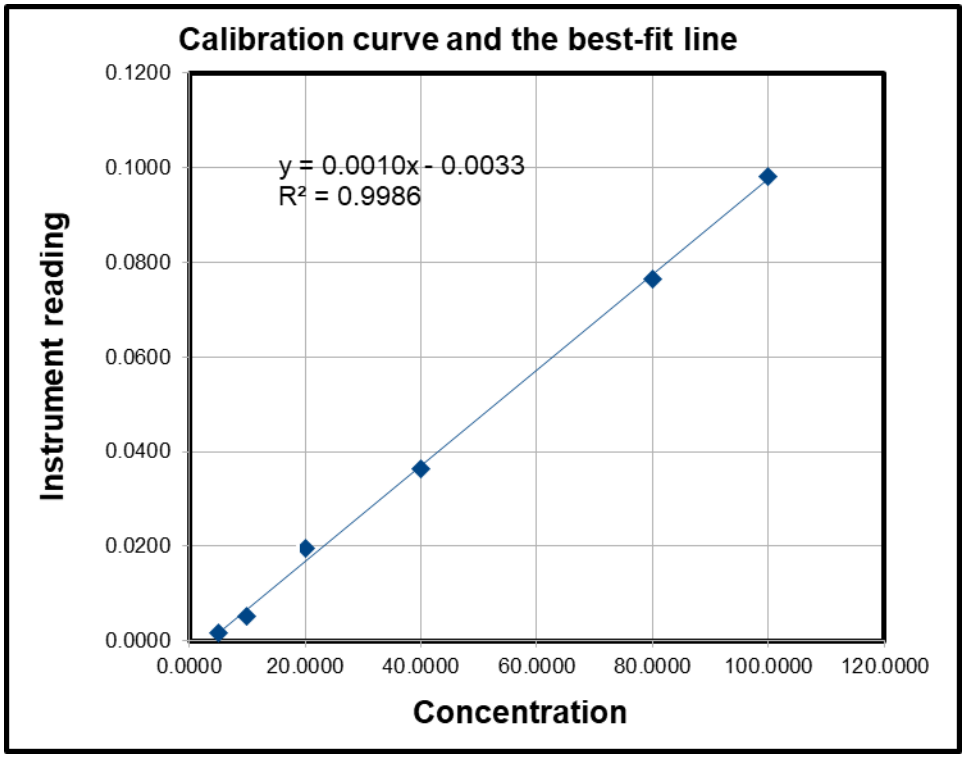


Quantitative Result Table

ID#	Name	R.Time	m/z	Area	Height	R.Index
1	Acetamride	13.876	239.10	29818	11659	0
2	Acetamride d3	-	242.10	---	---	0
3	Internal Standard	14.184	253.10	474930	263179	0

APPENDIX 8 contd. Bioanalytical Report

FIGURE 34. Calibration curve of N-hydroxyacetamide in rat plasma



11. APPENDICES

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 1. Certificate of analysis of Analyte: Acetamide D3

Clearsynth
Inspiring Research

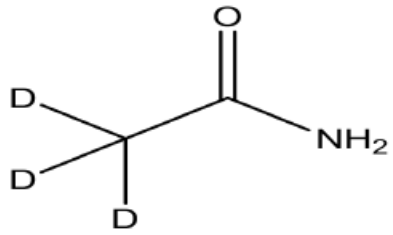
ASIA OFFICE:
Clearsynth Labs Ltd.
17, Lotus Business Park,
Off New Link Rd, Andheri (W)
Mumbai – 400053 India
Tel: +91.22.45045945

ASIA R&D CENTRE:
Clearsynth Research Centre
Plot No. 177, IDA,
Mallapur, Hyderabad - 500076
Telangana, India
T: +91.40.27155481

NORTH AMERICAN R&D CENTRE
Clearsynth Canada Inc.
2395 Speakman Drive,
Lab, Suite :1001, Mississauga,
Ontario L5K 1B3, Canada
Tel: +1.289.729.0060

CERTIFICATE OF ANALYSIS

Product Name	**Acetamide-2,2,2-D3**		
Chemical Name	Acetamide-2,2,2-D3		
Synonyms	NA		
CAS #	23724-60-9		
Catalogue #	CS-C-00204	**Batch #**	CS/SI/AAA-0680/01
Molecular formula	CD_3CONH_2	**Molecular weight**	62.09
Date of Analysis	22/08/2017 (dd/mm/yyyy)	**Retest Date**	22/08/2019 (dd/mm/yyyy)
Storage conditions	For long term usage store in a Refrigerator		



Test	Specification	Result
Appearance	NA	White crystals
Solubility	NA	In DMSO
1H NMR spectrum	Confirm Structure	Confirms
Mass Spectrum	Confirm molecular weight	Confirms
Isotopic enrichment (by MS)	Not less than 98 %	98.97%
Purity by GC	Not less than 98 %	99.77%

Date: 23/08/2017 *Quality Assurance*

Quality Accreditations – ISO 9001:2015|ISO 14001:2015|OHSAS 18001:2007|ISO Guide 34:2009| ISO/IEC 17025:2005
info@clearsynth.com | www.clearsynth.com | Worldwide Helpline No:- +1.800.650.8239 | CIN :- U24100MH2010PLC201433

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APPENDIX 8 contd. Bioanalytical Report

APPENDIX 2. Certificate of Analysis of Test-item: Acetamide

Certificate of Analysis

Dec 19, 2017 (JST)

TOKYO CHEMICAL INDUSTRY CO.,LTD.
4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan

Chemical Name: Acetamide		
Product Number: A0007 CAS: 60-35-5	Lot: XHH4A	

Tests	Results	Specifications
Purity(GC)	100.0 %	min. 98.0 %
Melting point	81.4 deg-C	80.0 to 84.0 deg-C
Solubility in Water	transparency	almost transparency

TCI Lot numbers are 4-5 characters in length. Characters listed after the first 4-5 characters are control numbers for internal purpose only.
The contents of the specifications are subject to change without advance notice. The specification values displayed here are the most up to date values. There may be cases where the product labels display a different specification, however, the product quality still meets the latest specification.

Customer service:

TCI Chemicals (India) Pvt. Ltd.
Tel: 044-2262 0909 / 044-2262 8878
Fax: 044-2262 8902
E-mail: Sales-IN@TCIchemicals.com

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 3. Certificate of analysis of Internal standard

TCI

Certificate of Analysis

Printed on Jan 8, 2018 (JST)

TCI CHEMICALS (INDIA) PRIVATE LTD.
Plot No.B28, Phase II, 5th Cross Street,MEPZ-SEZ, Tambaram,
Chennai - 600 045

Chemical Name: Propionamide

Product Number: P0499 CAS: 79-05-0	Lot: FIL01	Amount :

Tests	Results	Specifications
Purity(GC)	100.0 %	min. 98.0 %
Purity(Total Nitrogen)	100.6 %	min. 98.0 %
Melting point	81.6 deg-C	77.0 to 82.0 deg-C
Solubility in Water	transparency	almost transparency

Customer service:
TCI CHEMICALS (INDIA) PRIVATE LTD.
Tel :91-44-2262 0909
Fax :91-44-2262 8902
Email : sales-IN@TCIchemicals.com

*Suggested Re-test Date : 1 Year from the date of Invoice
*Quality Complaint Deadline : Within one month upon Invoice Date
* Invoice Dated : Dec/11/2017

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APPENDIX 8 contd. Bioanalytical Report

APPENDIX 4. Bioanalytical Method for Quantification of Acetamide in Rat Plasma

Sample preparation and processing

Preparation of CC, QC and study samples:

- Blank plasma and study samples were withdrawn from freezer/ deep freezer and thawed at room temperature.
- Calibration curve standards and quality control samples were prepared by adding 10 µL of spiking solution into 90 µL of interference-free blank plasma in micro centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Standard blank and standard zero samples were prepared by spiking 10 µL of milli-Q water into 90 µL of interference-free blank plasma and vortexed to mix.
- Transferred aliquot of 100 µL study sample into pre-labelled micro centrifuge tubes.

Sample processing procedure

- A volume of 10 µL of internal standard working solution was added to all tubes except for standard blank sample, where 10 µL of 50% methanol was added and vortexed.
- A volume of 40 µL of water was added to all tubes.
- A volume of 300 µL of 0.5 M HCl in methanol was added to each tube.
- All samples were vortexed to mix and stored in deep freezer (at -70 ± 10 °C) for 1h ± 15 min.
- All samples were centrifuged at 14000 rpm for 10 minutes at set temperature of 4 °C.
- Transferred about 250 µL of supernatant to another RIA tube.
- A volume of 200µL of xanthydrol(5%) solution was added to all tubes and incubated in a water bath at 40°C in darkness for about 90 min ± 15 min.
- All the samples were evaporated to dryness at 40 °C under nitrogen.
- A volume of 800 µL of saturated solution of sodium chloride was added to dried sample and vortexed.
- A volume of 60 µLof 0.7M KOH solution was added to all RIA tubes and vortexed to mix.
- A volume of 2mL of ethyl acetate was added to each tube.
- All samples were vortexed for 5 minutes at 2000 RPM followed by sonication.

APPENDIX 8 contd. Bioanalytical Report

- Samples were centrifuged at 4,000 rpm for 10 minutes at set temperature of 4°C.
- Transferred about 1.6 mL of supernatant to pre-labeled RIA vial and evaporated at 40°C under nitrogen gas until dryness.
- All samples were reconstituted with 0.4 mL of ethyl acetate. Samples were sonicated and vortexed.
- Samples were centrifuge at 4000 rpm for 5 minutes at set temperature of 4°C.
- Supernatant was transferred into inserts kept in prelabeled auto sampler vials and analyzed the sample in the GC-MS system.

Chromatographic conditions

The following conditions were used for the analysis:

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.

Carrier Gas: Helium

Flow Rate: 1.2 mL/min

Column Oven Temperature:

40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.

Injector Temperature: 250°C

Injection volume: 1 µL

Mass Spectrometry: SIM Mode
Solvent Delay Time: 10.0 min
Ion source temperature: 200°C
Interface temperatures: 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide), 242.1 (Xanthyl-acetamide D3) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5

Mode: Splitless
Run Time: 25 minutes

Retention times (Approximate) : Xanthyl Acetamide D3 → ~ 13.5 minute
Xanthyl Acetamide → ~ 13.5 minute
Xanthyl Propionamide → ~ 13.8 minute

APPENDIX 8 contd. Bioanalytical Report

G16082: Summary of Bioanalytical Method Validation in Rat Plasma

Experiment	Analyte / IS	Results
Specificity & selectivity	Acetamide D3	No interference observed in the 5 of the 6 tested lots at the retention time of analyte and internal standard
Auto sampler carryover	Acetamide D3	No carryover observed, after the injection of highest concentrated sample
Calibration curve range	Acetamide D3	0.100 to 50 µg/mL
Dilution integrity	Acetamide D3	5 folds
Linearity	Acetamide D3	$r^2 \geq 0.99$
Intra-batch precision and accuracy	Acetamide D3	%CV ranges from 2.36 to 7.88%
		Mean %bias ranges from 13.33 to 18.10 at LLOQQC level. Mean %bias ranges from -4.25 to 2.00% at LQC, MQC and HQC
Inter-batch precision and accuracy	Acetamide D3	%CV ranges from 5.34 to 6.48%
		Mean %bias was 15.24% at LLOQQC level and ranges from -3.33 to 0.50% at LQC, MQC and HQC levels
Recovery (%)	Acetamide	QC mean %recovery: 105.5% %CV: 1.28
Bench top stability ambient temperature	Acetamide D3	Stable for 21 hours and 40 minutes
Autosampler stability	Acetamide D3	Stable for 36 hours and 29 minutes
Dry extract stability at 2 to 8 °C	Acetamide D3	Stable for 48 hours
Freeze-thaw stability in rat plasma at -70 °C	Acetamide D3	5 cycles
Long-term stability in rat plasma at -70 °C	Acetamide D3	70 days

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 5. Bioanalytical Method for Quantification of Acetamide in Rat Liver

Preparation of Liver Homogenate

One part of liver tissue was homogenized with four parts of phosphate buffered saline pH 7.4 to give liver homogenate. Homogenizer assembly was washed with water, followed by methanol and followed by water to remove any contamination or carryover of analyte of interest or matrix component from one sample to the other.

Sample preparation and processing

Preparation of CC, QC and study samples:

- Blank liver homogenate and study samples were withdrawn from freezer/ deep freezer and thawed at room temperature.
- Calibration curve standards and quality control samples were prepared by adding 10 µL of spiking solution into 90 µL of interference-free blank liver homogenate in micro centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Standard blank and standard zero samples were prepared by spiking 10 µL of milli-Q water into 90 µL of interference-free blank liver homogenate and vortexed to mix.
- Transferred aliquot of 100 µL liver homogenate sample into pre-labelled micro centrifuge tubes.

Sample processing procedure

- A volume of 10 µL of internal standard working solution was added to all tubes except for standard blank sample, where 10 µL of 50% methanol was added and vortexed.
- A volume of 40 µL of water was added to all tubes.
- A volume of 300 µL of 0.5 M HCl in methanol was added to each tube.
- All samples were vortexed to mix and stored in deep freezer (at -70 ± 10 °C) for 1h ± 15 min.
- All samples were centrifuged at 14000 rpm for 10 minutes at set temperature of 4 °C.
- Transferred about 250 µL of supernatant to another RIA tube.
- A volume of 200µL of xanthydrol (5%) solution was added to all tubes and incubated in a water bath at 40°C in darkness for about 90 min ± 15 min.
- All the samples were evaporated to dryness at 40 °C under nitrogen.

APPENDIX 8 contd. Bioanalytical Report

- A volume of 800 µL of saturated solution of sodium chloride was added to dried sample and vortexed.
- A volume of 60 µLof 0.7M KOH solution was added to all RIA tubes and vortexed to mix.
- A volume of 2mL of ethyl acetate was added to each tube.
- All samples were vortexed for 5 minutes at 2000 RPM followed by sonication.
- Samples were centrifuged at 4,000 rpm for 10 minutes at set temperature of 4°C.
- Transferred about 1.6 mL of supernatant to pre-labeled RIA vial and evaporated at 40°C under nitrogen gas until dryness.
- All samples were reconstituted with 0.4 mL of ethyl acetate. Samples were sonicated and vortexed.
- Samples were centrifuge at 4000 rpm for 5 minutes at set temperature of 4°C.
- Supernatant was transferred into inserts kept in prelabeled auto sampler vials and analyzed the sample in the GC-MS system.

Chromatographic conditions

The following conditions were used for the analysis:

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.

Carrier Gas: Helium

Flow Rate: 1.2 mL/min

Column Oven Temperature:

40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.

Injector Temperature: 250°C

Injection volume: 1 µL

Mass Spectrometry: SIM Mode
Solvent Delay Time: 10.0 min
Ion source temperature: 200°C
Interface temperature: 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide), 242.1 (Xanthyl-acetamide D3) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5

Mode: Splitless

APPENDIX 8 contd. Bioanalytical Report

Run Time: 25 minutes

Retention times (Approximate) : Xanthyl Acetamide D3 → ~ 13.5 minute
Xanthyl Acetamide → ~ 13.5 minute
Xanthyl Propionamide → ~ 13.8 minute

G16083: Summary of Bioanalytical Method Validation in Rat Liver

Experiment	Analyte / IS	Results
Specificity & selectivity	Acetamide D3	No interference observed in all the 6 tested lots at the retention time of analyte and internal standard
Auto sampler carryover	Acetamide D3	No carryover observed, after the injection of highest concentrated sample
Calibration curve range	Acetamide D3	0.100 to 50 µg/mL
Dilution integrity	Acetamide D3	5 folds
Linearity	Acetamide D3	$r^2 \geq 0.99$
Intra-batch precision and accuracy	Acetamide D3	%CV ranges from 1.60 to 7.68
		Mean %bias ranges from -9.67 to 10.48
Inter-batch precision and accuracy	Acetamide D3	%CV ranges from 4.23 to 8.09
		Mean %bias ranges from -5.33 to 3.81
Recovery (%)	Acetamide	QC mean %recovery: 108.00% %CV: 3.47
Bench top stability ambient temperature	Acetamide D3	Stable for 17 hours and 20 minutes
Autosampler stability	Acetamide D3	Stable for 66 hours and 10 minutes
Dry extract stability at 2 to 8 °C	Acetamide D3	Stable for 70 hours
Freeze-thaw stability in rat liver homogenate at -70 °C	Acetamide D3	5 cycles
Long-term stability in rat liver homogenate at -70 °C	Acetamide D3	67 days

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 6. Bioanalytical method for quantification of Acetamide in urine sample

Sample preparation and processing

Preparation of CC, QC and study samples:

- Calibration curve standards and quality control samples were prepared by adding 5 µL of spiking solution into 45 µL of interference-free blank milli-Q water in micro centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Transferred aliquot of 50 µL water sample into pre-labelled micro centrifuge tubes.

Sample processing procedure

- A volume of 10 µL of internal standard working solution was added to all tubes except for standard blank sample, where 10 µL of 50% methanol was added and vortexed.
- A volume of 40 µL of water was added to all tubes.
- A volume of 300 µL of 0.5 M HCl in methanol was added to each tube.
- All samples were vortexed to mix and centrifuged at 14000 rpm for 10 minutes at set temperature of 4 °C.
- Transferred about 250 µL of supernatant to another RIA tube.
- A volume of 200µL of xanthydrol(5%) solution was added to all tubes and incubated in a water bath at 40°C in darkness for about 90 min ± 15 min.
- All the samples were evaporated to dryness at 40 °C under nitrogen.
- A volume of 800 µL of saturated solution of sodium chloride was added to dried sample and vortexed.
- A volume of 60 µL of 0.7M KOH solution was added to all RIA tubes and vortexed to mix.
- A volume of 2 mL of ethyl acetate was added to each tube.
- All samples were vortexed for 5 minutes at 2000 RPM followed by sonication.
- Samples were centrifuged at 4,000 rpm for 10 minutes at set temperature of 4°C.
- To 100 µL of supernatant, a volume of 900 µL of ethyl acetate was added and mixed well. 200 µL aliquot was transferred into inserts kept in prelabeled auto sampler vials and analyzed the sample in the GC-MS system.

APPENDIX 8 contd. Bioanalytical Report

Chromatographic conditions

The following conditions were used for the analysis:

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.

Carrier Gas: Helium

Flow Rate: 1.2 mL/min

Column Oven Temperature:

40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.

Injector Temperature: 250°C

Injection volume: 1 µL

Mass Spectrometry: SIM Mode
Solvent Delay Time: 10.0 min
Ion source temperature: 200°C
Interface temperature: 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide), 242.1 (Xanthyl-acetamide D3) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5

Mode: Splitless
Run Time: 25 minutes

Retention times (Approximate) : Xanthyl Acetamide D3 → ~ 13.5 minute
Xanthyl Acetamide → ~ 13.5 minute
Xanthyl Propionamide → ~ 13.8 minute

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 7. Bioanalytical method for quantification of Acetamide in water sample

Sample preparation and processing

Preparation of CC, QC and study samples:

- Calibration curve standards and quality control samples were prepared by adding 10 µL of spiking solution into 90 µL of interference-free blank milli-Q water in micro centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Transferred aliquot of 100 µL water sample into pre-labelled micro centrifuge tubes.

Sample processing procedure

- A volume of 10 µL of internal standard working solution was added to all tubes except for standard blank sample, where 10 µL of 50% methanol was added and vortexed.
- A volume of 40 µL of water was added to all tubes.
- A volume of 300 µL of 0.5 M HCl in methanol was added to each tube.
- All samples were centrifuged at 14000 rpm for 10 minutes at set temperature of 4 °C.
- Transferred about 250 µL of supernatant to another RIA tube.
- A volume of 200µL of xanthydrol(5%) solution was added to all tubes and incubated in a water bath at 40°C in darkness for about 90 min ± 15 min.
- All the samples were evaporated to dryness at 40 °C under nitrogen.
- A volume of 800 µL of saturated solution of sodium chloride was added to dried sample and vortexed.
- A volume of 60 µLof 0.7M KOH solution was added to all RIA tubes and vortexed to mix.
- A volume of 2mL of ethyl acetate was added to each tube.
- All samples were vortexed for 5 minutes at 2000 RPM followed by sonication.
- Samples were centrifuged at 4,000 rpm for 10 minutes at set temperature of 4°C.
- Transferred about 1.6 mL of supernatant to pre-labeled RIA vial and evaporated at 40°C under nitrogen gas until dryness.
- All samples were reconstituted with 0.4 mL of ethyl acetate. Samples were sonicated and vortexed.

APPENDIX 8 contd. Bioanalytical Report

- Samples were centrifuge at 4000 rpm for 5 minutes at set temperature of 4°C.
- Supernatant was transferred into inserts kept in prelabeled auto sampler vials and analyzed the sample in the GC-MS system.

Chromatographic conditions

The following conditions were used for the analysis:

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.

Carrier Gas: Helium

Flow Rate: 1.2 mL/min

Column Oven Temperature:

40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.

Injector Temperature: 250°C

Injection volume: 1 µL

Mass Spectrometry: SIM Mode
Solvent Delay Time: 10.0 min
Ion source temperature: 200°C
Interface temperature: 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide), 242.1 (Xanthyl-acetamide D3) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5

Mode: Splitless
Run Time: 25 minutes

Retention times (Approximate) : Xanthyl Acetamide D3 → ~ 13.5 minute
Xanthyl Acetamide → ~ 13.5 minute
Xanthyl Propionamide → ~ 13.8 minute

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 8. Bioanalytical method for quantification of Acetamide in feed sample

Sample preparation and processing

Preparation of CC, QC and study samples:

- Calibration curve standards were prepared by adding 50 µL of spiking solution (Acetamide D3) into 0.5g of grinded feed in centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Recovery quality control samples were prepared by adding 50 µL of spiking solution (Acetamide) into 0.5g of grinded feed in centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Transferred aliquot of 0.5g of feed sample and 50 µL of water into pre-labelled micro centrifuge tubes.

Sample processing procedure

- A volume of 2.5 mL of water was added to all tubes and vortexed for 15 minutes.
- A volume of 50 µL of internal standard working solution was added to all tubes except for standard blank sample, where 50 µL of 50% methanol was added and vortexed.
- A volume of 2.5 mL of 0.5 M HCl in methanol was added to each tube and all samples were vortexed to mix.
- All samples were centrifuged at 4000 rpm for 10 minutes at set temperature of 4 °C.
- A volume of 200µL of xanthydrol(5%) solution was added to all tubes and incubated in a water bath at 40°C in darkness for about 90 min ± 15 min.
- 3g of sodium chloride was added to each tube.
- A volume of 2 mL of 0.7M KOH solution was added to all tubes and vortexed to mix.
- A volume of 3 mL of ethyl acetate was added to each tube.
- All samples were vortexed for 5 minutes at 2000 RPM followed by sonication.
- Samples were centrifuged at 4,000 rpm for 10 minutes at set temperature of 4°C.
- Transferred about 1.6 mL of supernatant to pre-labeled RIA vial and evaporated at 40°C under nitrogen gas until dryness.
- All samples were reconstituted with 0.4 mL of ethyl acetate. Samples were sonicated and vortexed.

APPENDIX 8 contd. Bioanalytical Report

- Samples were centrifuge at 4000 rpm for 5 minutes at set temperature of 4°C.
- Supernatant was transferred into inserts kept in prelabeled auto sampler vials and analyzed the sample in the GC-MS system.

Chromatographic conditions

The following conditions were used for the analysis:

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.

Carrier Gas: Helium

Flow Rate: 1.2 mL/min

Column Oven Temperature:

40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.

Injector Temperature: 250°C

Injection volume: 1 µL

Mass Spectrometry: SIM Mode
Solvent Delay Time: 10.0 min
Ion source temperature: 200°C
Interface temperature: 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide), 242.1 (Xanthyl-acetamide D3) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5

Mode: Splitless
Run Time: 25 minutes

Retention times (Approximate) : Xanthyl Acetamide D3 → ~ 13.5 minute
Xanthyl Acetamide → ~ 13.5 minute
Xanthyl Propionamide → ~ 13.8 minute

APPENDIX 8 contd. Bioanalytical Report

APPENDIX 9. Bioanalytical method for quantification of N-hydroxyacetamide in plasma sample

Sample preparation and processing

Preparation of CC, QC and study samples:

- Stock and working solutions of N-hydroxyacetamide was prepared in water.
- Calibration curve standards and quality control samples were prepared by adding 5 µL of spiking solution into 50 µL of blank plasma in pink micro centrifuge tubes except standard blank and standard zero and vortexed to mix thoroughly.
- Transferred aliquot of 50 µL study sample sample and 5 µL of diluent into pre-labelled pink micro centrifuge tubes.

Sample processing procedure:

- A volume of 0.25 ml ethanol was added to all the tubes and vortexed to mix. All the tubes were stored at -70˚C for one hour.
- All the samples were thawed at room temperature, then centrifuged for 5 min at 14,000 rpm.
- An aliquot of 0.2 ml of the supernatant was transferred to a new pink micro centrifuge tube.
- A volume of 4 µL of 25% HCl solution and 5 µL of ferric chloride solution were added. All samples were vortexed to mix and centrifuged for 2 min at 14,000 rpm.
- All the tubes were kept at room temperature for 30 min for the color to develop. All the samples were passed through a 0.45 µm PTFE syringe filter and transferred to a micro plate.
- The absorption of each sample at 515nm was read using a plate reader. The blank plasma sample was used for background subtraction.

APPENDIX 9. Pathology Report

PATHOLOGY REPORT

STUDY TITLE

ACETAMIDE: 7-DAY REPEAT DOSE ORAL TOXICITY STUDY IN WISTAR RATS WITH TOXICOGENOMICS

STUDY No.: G16084

STUDY PATHOLOGIST

K. C. JAYACHANDRA, M.V.Sc., DABT

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INDIA

APPENDIX 9 contd. Pathology Report

DECLARATION

This is to declare that the pathology work was performed under the supervision of study pathologist and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the pathology work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The study pathologist accepts overall responsibility for the technical conduct of the pathology work as well as the interpretation, analysis, documentation and reporting of the results.

Jayachandra, KC

K. C. Jayachandra, M.V.Sc., DABT.
Study Pathologist

23 Dec. 2019
Date

APPENDIX 9 contd. Pathology Report

LIST OF ABBREVIATIONS AND SYMBOLS

A	Absolute
Alb	Albumin
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
APTT	Activated Partial Thromboplastin Time
AST	Aspartate aminotransferase
A/G	Albumin/Globulin
Baso	Basophils
BUN	Blood Urea Nitrogen
BW/Bwt	Body weight
Ca	Calcium
Contd.	Continued
Creat	Creatinine
Cl	Chloride
CK	Creatinine kinase
Diff	Difference
Eosi	Eosinophils
fL	Femtolitre
g	Gram
G	Giga
GGT	Gamma Glutamyl Transpeptidase
Glu	Glucose
Glob	Globulin
Hb	Haemoglobin
Hct	Haematocrit
HDW	Haemoglobin Distribution Width
Hyper	Hyperchromic cells
Hypo	Hypochromic cells
K	Potassium
kg	Kilogram
K_2EDTA	Ethylene diamine tetra acetic acid dipotassium salt
L	Litre
Lymp	Lymphocytes
LDH	Lactate dehydrogenase
M	Male
m	Metre
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
mEq	Milli Equivalent
mg	Milligram
mL	Millilitre
mmol	Millimole
Mono	Monocytes
Macro	Macrocytes
Micro	Microcytes
Na	Sodium
Neut	Neutrophils
No.	Number
N	Number of animals
pg	Picogram
Pi	Inorganic phosphorus
Plat	Platelets
PT	Prothrombin Time
RBC	Red Blood Corpuscles
Retic	Reticulocyte
rpm	Revolutions per minute
RDW	Red Cell Distribution Width
SD	Standard Deviation
Seminal & coagu	Seminal vesicles and coagulating glands
T	Tera
T.Bil	Total Bilirubin
T.Pro	Total plasma protein
T.Chol	Total Cholesterol
Trig	Triglyceride
U	Units
WBC	White Blood Corpuscles
%	Per cent
µmol	Micromole
µL	Microlitre

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LIST OF SCIENTISTS / PROFESSIONALS

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Pathology report review	S. K. Vijayasarathi, M.V.Sc., Ph.D., FIATP Expert Pathologist, Department of Safety Assessment
Necropsy and histotechniques	Lourdappa Shetty, M.Sc. Pathology Department of Safety Assessment
Clinical pathology analysis	M. Y. Sunagar, M.Sc. Pathology Department of Safety Assessment
Report compilation	M. Tejaswini, B.Sc. Electronic Data Processing (EDP) Department of Safety Assessment

APPENDIX 9 contd. Pathology Report

1. OBJECTIVE

The objectives of the study are:

- To assess the toxicological and toxicogenomic profile of the test item Acetamide when administered by oral gavage to Wistar rats for a minimum period of 7 consecutive days.
- To provide information on major toxic effects, target organs and an estimate of the No Observed Adverse Effect Level (NOAEL).

2. STUDY DESIGN

The selected rats were assigned to vehicle control and treatment groups as shown below:

Toxicity Groups

a. Acetamide groups:

Group No.	Group	Colour of cage card	Dose (mg/kg /day)	Concent-ration (mg/mL)	Dose volume (mL/kg)	No. of Rats	Sex	Rat Numbers	
								From	To
G1A	Vehicle control	White	0	0	10	6	M	Ru5681	Ru5686
G2A	Low dose	Yellow	30	3	10	6	M	Ru5687	Ru5692
G3A	Mid dose	Green	100	10	10	6	M	Ru5693	Ru5698
G4A	Intermediate dose	Blue	300	30	10	6	M	Ru5699	Ru5704
G5A	High dose	Pink	1000	100	10	6	M	Ru5705	Ru5710

b. Acetamide + BrdU groups:

Group No.	Group	Colour of cage card	Dose (mg/kg/ day)	Concent-ration (mg/mL)	Dose volume (mL/kg)	No. of Rats	Sex	Rat Numbers	
								From	To
G1B	Vehicle control	White	0	0	10	6	M	Ru5711	Ru5716
G2B	Low dose	Yellow	30	3	10	6	M	Ru5717	Ru5722
G3B	Mid dose	Green	100	10	10	6	M	Ru5723	Ru5728
G4B	Intermediate dose	Blue	300	30	10	6	M	Ru5729	Ru5734
G5B	High dose	Pink	1000	100	10	6	M	Ru5735	Ru5740

M = Male

APPENDIX 9 contd. Pathology Report

3. METHODS

3.1. Clinical Pathology Investigations

3.1.1. Blood Collection

Blood was collected at the end of the treatment period (prior to necropsy on Day 8) from all rats. All rats were fasted overnight (water allowed) and approximately 3 mL of blood was collected from each rat (as indicated in the Table below) under isoflurane anesthesia, with the help of a fine capillary tube, by retro-orbital plexus puncture.

Parameter	Approximate volume (mL)	Anticoagulant	
		Name	Concentration per mL of blood
Hematology	0.7	K_2 EDTA	1.6 mg
Clinical chemistry	1.8	Lithium heparin	10 Unit
Coagulation	0.5	Trisodium citrate	3.2 mg

After analysis and data review by the analyst, the residual hematology samples were discarded after data review. The plasma samples used for clinical chemistry analysis were stored frozen. These samples were discarded at the time of final report preparation.

3.1.2. Haematology

The following haematological parameters were determined using ADVIA 2120i haematology system (Siemens Healthcare Diagnostics Inc., NY, USA).

Sl. No.	Parameter	Abbreviations	Units[2]
1	Red Blood Corpuscles	RBC	10^{12}/L
2	Haemoglobin	Hb	g/L
3	Haematocrit	Hct	L/L
4	Mean Corpuscular Volume	MCV	fL
5	Mean Corpuscular Haemoglobin	MCH	pg
6	Mean Corpuscular Haemoglobin Concentration	MCHC	g/L
7	Reticulocytes count	Retic	10^{12}/L
8	White Blood Corpuscles	WBC	10^9/L
9	Differential leukocyte count[1]	DLC	10^9/L
10	Platelets	Plat	10^9/L

APPENDIX 9 contd. Pathology Report

Sl. No.	Parameter	Abbreviations	Units[2]
11	Red Blood Cell Morphology		
	a. Red Cell Distribution Width	RDW	%
	b. Haemoglobin Distribution Width	HDW	g/L
	c. Hyperchromic cells	Hyper	%
	d. Hypochromic cells	Hypo	%
	e. Macrocytes	Macro	%
	f. Microcytes	Micro	%
	g. RBC Fragments	RBC Frag	10^{12}/L
	h. RBC Ghosts	RBC Gh	10^{12}/L

[1]: Differential Leukocyte parameters and their respective abbreviations are: Neutrophils (Neut), Lymphocytes (Lymp), Monocytes (Mono), Eosinophils (Eosi) and Basophils (Baso).
[2]: Expansion of units are as follows: L:Litre, g: Gram, pg: Picogram, fL:Femtolitre,

3.1.3. Coagulation

Blood samples collected for coagulation analysis were centrifuged at 2500 times gravity (x*g*) at 15°C for 10 minutes for separation of plasma and analysed for the following parameters in plasma sample using STart-4 coagulation analyzer (Diagnostica stago, 92600 Asnieres, France):

Sl. No.	Parameter	Abbreviations	Units
1	Prothrombin Time	PT	Sec
2	Activated Partial Thromboplastin Time	APTT	Sec

3.1.4. Clinical Chemistry

Plasma was separated in a refrigerated centrifuge at 5000 rpm, 4° C for 5 minutes and analysed using Dimension RxL MaX Clinical Chemistry System (Dade Behring Inc. Newark, DE 19714, USA) Automatic Analyser for the following parameters:

Serial Number	Parameters	Abbreviations	Units [b]
1	Alkaline Phosphatase	ALP	U/L
2	Alanine aminotransferase	ALT	U/L
3	Albumin	Alb	g/L
4	Albumin/Globulin ratio (calculated)	A/G	-
5	Aspartate aminotransferase	AST	U/L
6	Blood Urea Nitrogen	BUN	mmol/L
7	Calcium	Ca	mmol/L
8	Chloride	Cl	mEq/L
9	Creatinine	Creat	µmol/L
10	Direct Bilirubin [a]	D.Bil	µmol/L

APPENDIX 9 contd. Pathology Report

Serial Number	Parameters	Abbreviations	Units [b]
11	Gamma Glutamyl Transpeptidase	GGT	U/L
12	Globulin (calculated)	Glob	g/L
13	Glucose	Glu	mmol/L
14	Indirect Bilirubin [a] (calculated)	Ind. Bil	µmol/L
15	Inorganic phosphorous	Pi	mmol/L
16	Lactate dehydrogenase	LDH	U/L
17	Potassium	K	mEq/L
18	Sodium	Na	mEq/L
19	Total Cholesterol	T.Chol	mmol/L
20	Total Protein	T.Pro	g/L
21	Total Bilirubin	T.Bil	µmol/L
22	Triglycerides	Trig	mmol/L

Notes:
[a]: *Direct and indirect bilirubin were not determined as total bilirubin values are <10 µmol/L*
[b]: *Expansion of units are as follows: U:Unit, L:Litre, g: Gram, mmol: Millimole, mEq: Milli equivalent, µmol: Micromole*

3.1.5. Urinalysis

Urine was collected in urine collection tubes from all main group rats on Day 7.

Each rat were placed in a specially fabricated cage (water allowed) for about 3-4 hours and the collected urine was sent for analysis. Urine was analyzed for the following parameters:

Sl. No.	Parameters
1	Specific gravity[1]
2	Nitrite[2]
3	pH[2]
4	Proteins[2]
5	Glucose[2]
6	Ketone bodies[2]
7	Urobilinogen[2]
8	Bilirubin[2]
9	Appearance (colour and clarity)[3]
10	Volume (approximate)[3]

[1]: measured using Refractometry method (Refractometer-PAL-10S. Atago Co., Ltd., Japan).
[2]: analyzed using Multistix 10 SG strips, read with Clinitek status analyzer (Bayer Healthcare LLC, UK)
[3]: recorded manually.

Urine was also subjected to microscopic examination for sediments such as crystals, epithelial cells, erythrocytes, leukocytes and casts.

In addition, the urine samples (after urinalysis) from Acetamide groups (G1A, G2A, G3A, G4A and G5A) were analysed for presence of Acetamide or to determine how much Acetamide was present in urine samples to provide an insight into clearance rates. The analysis was performed on a Non-GLP basis using a fit-for-purpose GC-MS method and the results are included to the main toxicology report.

3.2. Anatomic Pathology

3.2.1. Necropsy

All rats in the study were subjected to detailed necropsy (examination of external surfaces of the body, all orifices, cranial, thoracic and abdominal cavities and their contents) and findings were recorded. All rats were subjected to necropsy after overnight fasting (water allowed) at the end of treatment period. Terminal body weights were recorded for all animals immediately prior to sacrifice. Rats sacrificed were euthanized by exsanguination under isoflurane anesthesia. The terminal necropsy was done as per the random numbers generated. Between the different groups and the rats in each group was randomized for each round of sacrifice, one animal in each group were necropsied. To begin with, in the total of 10 groups with 6 rats each, the randomization was done among the 10 groups and among the 6 rats in each group. For the following first round, 10 groups were randomized with the randomization among the remaining 5 rats in each group. This procedure was followed till the last round of rats subjected for terminal necropsy.

3.2.2. Tissue Collection, Weighing and Preservation

On completion of the gross pathology examination, the tissues and organs noted below were collected from all rats. The tissues were weighed from all rats. The organ weight ratios (organ to terminal fasting body weight and organ to brain weight) was determined and presented in the report. Paired organs were weighed together and combined weight was presented. Tissues were preserved in 10% Neutral Buffered Formalin (NBF) unless noted otherwise.

Sl. No.	Tissue	Organ weight	Collection and preservation
1	Adrenals	X	X
2	All gross lesions		X
3	Aorta		X
4	Bone marrow smear		X

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Sl. No.	Tissue	Organ weight	Collection and preservation
5	Brain including cerebrum, cerebellum and medulla/pons	X	X
6	Cecum		X
7	Colon		X
8	Duodenum		X
9	Epididymides	X	X
10	Eyes with optic nerves[1]		X
11	Femur bone with distal joint[6]		X
12	Heart	X	X
13	Ileum (with Peyer's patches)		X
14	Jejunum		X
15	Kidneys	X	X
16	Liver	X	X
17	Larynx		X
18	Lungs[2]	X	X
19	Mandibular lymph nodes		X
20	Mesenteric lymph nodes		X
21	Nose		X
22	Oesophagus		X
23	Pancreas		X
24	Pituitary[3]	X	X
25	Prostate[5]	X	X
26	Rectum		X
27	Salivary glands		X
28	Sciatic nerves		X
29	Seminal vesicles and coagulating glands [5]	X	X
30	Skeletal muscle		X
31	Skin		X
32	Spinal cord at 3 levels (cervical, mid thoracic and lumber)		X
33	Spleen	X	X
34	Sternum[6]		X
35	Stomach		X
36	Tongue		X
37	Testes[4]	X	X
38	Thymus	X	X
39	Thyroid with parathyroids[3]	X	X
40	Trachea		X
41	Urinary bladder		X
42	Ureters		X

[1]: collected in Davidson's fluid
[2]: inflated with 10 % neutral buffered formalin and then immersed in formalin
[3]: weighed after formalin fixation
[4]: collected in modified Davidson's fluid
[5]: Prostate + Seminal vesicles with coagulating glands were weighed as a whole;

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Sl. No.	Tissue	Organ weight	Collection and preservation
subsequently prostate was separated and weighed. The derived weight was presented for the seminal vesicles and coagulating glands. [6]: decalcified prior to sectioning X: Organs weighed, collected and preserved			

Note 1: A section of right caudal liver lobe was preserved for determining the tissue concentration of acetamide.

Note 2: A section of liver specimen from left lobe was snap frozen and shipped to Syngene for toxicogenomics analysis adopting the following procedure:

- To prevent microbial contamination (and subsequent RNAse contamination) disposable gloves was used and good sterile technique was practiced when handling samples and reagents.
- Before starting isolation, work surface area was wiped out with 10% sodium hypochloride followed by 70% ethanol and then by RNAse ZAP RNAse ZAP™ to ensure an RNAse free environment.
- All surgical instruments which were used for dissection were autoclaved. After tissue collection from each animal, surgical instruments were cleaned with RNase Zap.
- Aluminum foil was wiped with RNAse ZAP before placing in balance for weighing.
- 20 to 30 mg of liver was placed into individually labelled sterile / RNAse / DNAse free cryo vials, and was snap frozen in liquid nitrogen container as soon as possible.
- RNaseZAP agent was used to wipe surfaces between samples, as well as cryo vials after labeling. Work surface area was wiped with RNAse ZAP™ after each collection.
- These samples were stored -70° C deep freezer and stored until shipment. The samples were shipped in frozen condition in dry ice to Syngene for toxicogenomics assay.

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Address for shipment:

Seetha K, Ph.D
Lead Investigator, Biology
Syngene International Ltd.
SEZ-16 UNIT-IX,
Plot No.2,3,4 & 5, Phase IV,
Biocon Park, Jigani Link Road
Bengaluru-560099
Telephone: +91 804014 8607
Mobile: +91 99005 80779
Email: seetha.krishnan@syngeneintl.com

The above mentioned samples were collected in duplicate and one set of samples were stored at Eurofins Advinus Limited. The stored second set of samples were discarded prior to finalization of the report as per the request of the Sponsor.

Note 2: A small quantity of liver sample was also collected and stored. The stored liver sample was discarded prior to finalization of the report as per the request of the Sponsor.

3.2.3 Histopathology

Histopathological examination was carried out only for liver of all rats. A section of left lobe of liver was used.

Additionally, histopathological examination was carried out for femur bone with distal joint, sternum and spleen for control and high dose rats as there were changes observed in haematological parameters (increase in RBC counts, haemoglobin concentration, haematocrit values and decrease in reticulocytes). These tissues were not examined in lower dose groups as treatment-related changes were not present in the above tissues.

Tissues were processed for routine paraffin embedding and 4-5 micron sections were stained with Mayer's Haematoxylin and Eosin stain. Unused tissues were archived.

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Mitotic Index:

Calculation of Mitotic Index of Hepatocytes: This was done only for G1A-G5A groups (BrdU minus groups).

Procedure for mitotic index scoring:

1. Haematoxylin and Eosin stained liver slides were placed under a microscope.
2. First the slide was viewed under low power, 10X. Then the slide was viewed under 40X so as to distinguish each cells from one another.
3 Total number of hepatocytes in mitosis and not in mitosis contained within one high power (40X) field was counted.
4. The slide stage was randomly moved around the tissue and 10 different counts of this nature was recorded.
5. 10 counts/tissue slice and >2000 hepatocyte per slide were recorded.
6. Mitotic index of the slide was calculated by dividing the total number of hepatocytes in mitosis by the total number of hepatocytes counted (see below).

$$\text{Mitotic Index \%} = \frac{\text{Total number of hepatocyte in mitosis} \times 100}{\text{Total number of hepatocytes counted}}.$$

3.2.4 Immunohistochemistry

From all rats, paraffin embedded liver sections were subjected for Ki67 immunohistochemistry staining. From all rats administered with BrdU, the immunohistochemistry staining for BrdU was performed. The formalin preserved tissues were processed for paraffin embedding during 72 hours post fixation. The procedure for BrdU and Ki67 immunohistochemistry staining are given below:

Detection of BrdU in Formalin-Fixed Paraffin-Embedded Rat Tissue

Reagent and Antibody Information

- 1x Wash Buffer (PBS)
- 3% Hydrogen Peroxide
- 1% BSA Diluent
- 1x citrate buffer
- DAB chromogen and substrate. Dako Cytomation
- Hematoxylin solution
- Blocking Solution: Dakocytomation protein block (Ready-to-Use)
- Avidin / Biotin Blocking Kit. Vector Labs.
- Primary Antibody: Mouse Monoclonal BrdU Antibody (Clone BU20a) Dako Cytomation.

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- Secondary Antibody: Biotinylated Horse Anti-Mouse IgG (H+L). Vector Labs
- Label complex: R.T.U. Vectastain Elite ABC reagent Vector Labs

Positive Control Tissue: Intestine section for each BrdU incorporated rat
Stain localization: Nuclear

Staining Procedure

1. Slides were deparaffinized and hydrated through the following solutions:

Solution	Repetitions	Time
Xylene	2 times	5 minutes
100% Ethanol	2 times	3 minutes
95% Ethanol	2 times	3 minutes
1X Wash Buffer	2 times	5 minutes

2. Heat-Induced Epitope Retrieval Using the Decloaker :
   500 ml of distilled water was added to the pan inside the decloaker. Full rack of slides were placed into container with 200 ml of 1X citrate buffer. The container was placed stably inside the pan and the slides were decloaked for 15 minutes at 110°C. Pan top was removed and cooled for 10 minutes.
3. Slides were rinsed in 2 changes of distilled water for 3 minutes each time.
4. Endogenous peroxidase was quenched by placing the slides in 3% hydrogen peroxide for 15 minutes.
5. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
6. Non-specific protein binding was blocked with the Dako protein-blocking reagent for 10 minutes at room temperature.
7. Slides were not rinsed and Avidin-Biotin blocking was continued.
8. Avidin block was applied for 15 minutes at room temperature. Slides were quick rinsed in 1X wash buffer. Biotin block was applied for 15 minutes at room temperature. Sections were not rinsed with buffer before adding primary antibody, only excess block was wiped out.
9. Primary antibody was applied at 1:200 dilution and incubated for 1 hour at room temperature.
10. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
11. Horse anti-mouse secondary antibody was applied at a 1:500 dilution and incubated for 30 minutes at room temperature.
12. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each

13. Vectastain R.T.U. Elite Label was applied and incubated for 30 minutes at room temperature.
14. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
15. DAB chromogen was applied and incubated in the dark for 6 minutes at room temperature.
16. Slides were rinsed in tap water 3 minutes.
17. Slides were counterstained with hematoxylin for 20 seconds.
18. Slides were rinsed in running tap water until water was clear.
19. Slides were gently agitated in 1X wash buffer until the tissues turned blue.
20. Slides were dehydrate through the following solutions:

Solutions	Repetitions	Time
95% Ethanol	1 time	3 minutes
100% Ethanol	3 times	3 minutes
Xylene	2 times	5 minutes

21. 1-2 drops of Vecta Mount was applied to each slide and covered with cover glass. Slides were examined under microscope for brown staining of Nucleus.

Detection of Ki 67 proliferation antigen in Formalin-Fixed Paraffin-Embedded Rat Tissue

Reagent and Antibody Information

1. 1X Wash Buffer (PBS)
2. 1X Citrate buffer
3. 3% Hydrogen Peroxide

4 Blocking Solution: Dakocytomation protein block (Ready-to-Use)

5 Anti-Ki67 [SP6] antibody (Abcam – catalog # ab16667)

6 Vectastain Elite ABC Kit Rabbit IgG (Vector Lab – catalog # PK-6101)

a) Blocking goat serum

b) Biotinylated secondary antibody

c) Reagent A (Avidin DH)

d) Reagent B (Biotinylated Horseradish Peroxidase H)

7 DAB Chromogen and substrate buffer (Dako)

8 Hematoxylin solution

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Staining Procedure
Positive Control Tissue: Intestine
Stain Localization: Nuclear (proliferating cells, distinctly stains nucleoli)

1. Slides were deparaffinized and hydrated through the following solutions :

Solution	Repetitions	Time
Xylene	2 times	5 minutes
100% Ethanol	2 times	3 minutes
95% Ethanol	2 times	3 minutes
1X Wash Buffer	2 times	5 minutes

2. Heat-Induced Epitope Retrieval Using The Decloaker:

   500 ml of distilled water was added to the pan inside the decloaker. Full rack of slides were placed into container with 200 ml of 1X citrate buffer. The container was placed stably inside the pan and the slides were decloaked for 15 minutes at 110°C. Pan top was removed and cooled for 10 minutes.
3. Slides were rinsed in 2 changes of distilled water for 3 minutes each time.
4. Then Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
5. Endogenous peroxidase was quenched by placing the slides in 3% hydrogen peroxide for 15 minutes.
6. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each
7. Non-specific protein binding was blocked with the Dako protein-blocking reagent for 10 minutes at room temperature.
8. The slides were not rinsed and continued to next step.
9. Avidin block was applied for 15 minutes at room temperature. Slides were quick rinsed in 1X wash buffer. Then biotin block was applied for 15 minutes at room temperature.
10. Before adding primary antibody slides were not rinsed with buffer, only excess block was wiped.
11. Primary antibody was applied at 1:200 dilution and incubated for 2 hour at room temperature.
12. Slides were rinsed in 2 changes of 1X wash buffer for 5 minutes each.
13. Biotinylated secondary antibody was applied at a 1:1000 dilution and incubated for 1 hour at room temperature.

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14. ABC reagent was prepared 30 minutes before its usage. ABC reagent was prepared by adding 1 drop of reagent A into 5ml of 1X PBS and then add 1drop of B.
15. Slides were rinsed in 3 changes of 1X wash buffer for 5 minutes each
16. Then slides were incubated with ABC reagent for 40 minutes at room temperature.
17. Slides were rinsed in 3 changes of 1X wash buffer for 5 minutes each
18. DAB chromogen was applied and incubated in the dark for 6 minutes at room temperature.
19. Slides were rinsed in tap water 3 minutes.
20. Counterstained with hematoxylin for 20 seconds.
21. Slides were rinsed in running tap water until water is clear.
22. Slides were gently agitated in 1X wash buffer until the tissues turn blue.
23. Slides were dehydrated through the following solutions:

Solutions	Repetitions	Time
95% Ethanol	1 time	3 minutes
100% Ethanol	3 times	3 minutes
Xylene	2 times	5 minutes

24. 1-2 drops of Vecta Mount was applied to each slide and covered with cover glass. Slides were examined under microscope for brown staining of nucleus.

Analysis of BrdU/Ki67 Stained Slides

Procedure for scoring slides:

1. After the preparation of slides by the appropriate staining procedure, slides were placed under microscope.
2. Slides were viewed under low power, 10X. Then slides viewed under 40X so as to distinguish cells from one another.
3. The total number of positively and negatively hepatocytes within one high power (40X) field was counted.
4. Slide stage was randomly moved around the tissue to make 10 different such counts.
5. 10 counts/tissue slice and >2000 nuclei per slide were recorded.
6. Labeling index of the slide was calculated by dividing the total number of positively stained hepatocytes by the total number of hepatocytes counted (see below).

$$\text{BrdU/Ki67 Index \%} = \frac{\text{Total number of BrdU/Ki67 stained cells}}{\text{Total number of hepatocytes counted}} \times 100.$$

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Peer review: Haematoxylin and Eosin stained and Ki-67 immuno-stained liver slides of Acetamide + BrdU groups were shipped for the Peer review to:

Dr. J. E. Klaunig, Ph.D., Fellow ATS, Fellow IATP (Peer Review Pathologist)
To the following address:

Dr. J. E. Klaunig, Ph.D., Fellow ATS, Fellow IATP
Professor, Environmental Health
Professor, School of Public and Environmental Affairs
Innovation Center
Office Room 256
Office Phone 812 856 3957
Laboratory Rooms 230-236
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Bloomington Indiana 47408
Klauniglab.com
jklauni@indiana.edu

The findings from the Peer review were discussed by the study pathologist and reviewer. The consensus diagnoses/findings were included in the report. The review process was indicated in the peer review statement, signed peer review statement was included in the main report. All the slides were shipped back to Eurofins Advinus for archiving.

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4. STATISTICAL ANALYSES

Data captured using Provantis™: Parameters such as terminal fasting body weight, organ weights and their ratios, laboratory Investigations – Haematology, (Coagulation tests PT and APTT data was entered retrospectively in Provantis™) and Clinical Chemistry data analyzed using Provantis built-in statistical tests.

The integrated decision tree of Provantis™:

(i) Variance homogeneity was tested by Levene's method. When variances were heterogeneous, suitable transformation was performed automatically by the software.

(ii) Further one-way analysis of variance (ANOVA) was performed. When ANOVA is significant, Dunnett's control versus treatment group mean comparisons was performed.

Statistical analysis of the experimental data generated outside Provantis™ (Ki 67 proliferation index and Mitotic index) was carried out using licensed copies of SYSTAT Statistical package Version 12.0. The data was tested for homogeneity of variances (Levene's test) within the group before performing One-Way Analysis of Variance (ANOVA). If the data was found non-optimal (non-normal or heteroschedastic), ANOVA was done using suitable transformation. Comparison of means between treatment groups and vehicle control group was be done using Dunnett's test, if the overall treatment 'F' test was found to be significant.

All analyses and comparisons were evaluated at the 5% ($p < 0.05$) level. Statistically significant differences ($p<0.05$), indicated by the aforementioned tests was designated by the following throughout the report:

+/-: Significantly higher (+) /lower (-) than the vehicle control group

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5. RESULTS AND DISCUSSION

5.1 Clinical Pathology Investigations

5.1.1 Haematology

Refer Tables 1 and 2, Appendices 1 and 2

The hematology changes associated with test item administration when compared to vehicle control are tabulated below and the details are presented in Tables 1 and 2:

Treatment	Acetamide groups				Acetamide + BrdU groups			
Group	G2A	G3A	G4A	G5A	G2B	G3B	G4B	G5B
Dose (mg/kg/day)	30	100	300	1000	30	100	300	1000
No. of rats	6	6	6	6	6	6	6	6
Red Blood Corpuscles	—	—	—	↑9%*	—	—	—	↑10%*
Haemoglobin	—	—	—	↑9%*	—	—	—	↑12%*
Haematocrit	—	—	—	↑6%*	—	—	—	↑11%*
Reticulocytes- absolute count	—	—	—	↓48%*	—	—	—	↓40%*

↑: Increase ↓: Decrease *: Statistically significant change
Changes are expressed as mean % change from control values
'-'indicates that values are not considered meaningfully different from controls.

Increase in RBC counts, hemoglobin concentration, hematocrit and decrease reticulocytes in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related non adverse change as they were not associated with histopathological changes in haemopoietic organs (bone marrow and spleen).

Decreased hypochromic RBC in 1000 mg/kg/day acetamide dose group was considered toxicologically insignificant as it was not associated with any significant changes in erythrocyte indices.

Increased MCHC in 1000 mg/kg/day acetamide dose group was considered incidental change and was likely due to random biological variation as the magnitude of change was very minimal (<5%).

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5.1.2 Coagulation

Refer Tables 1 and 2, Appendices 1 and 2

There were no test item-related changes in the coagulation parameters.

Increased prothrombin time in 1000 mg/kg/day acetamide + BrdU dose groups was considered toxicologically insignificant as it was not associated with any significant changes in APTT and platelet counts were within historical control range.

5.1.3 Clinical Chemistry

Refer Tables 3 and 4, Appendices 3 and 4

The clinical chemistry changes associated with test item administration when compared to vehicle control are tabulated below and the details are shown in Tables 3 and 4:

Treatment	**Acetamide groups**				**Acetamide + BrdU groups**			
Group	**G2A**	**G3A**	**G4A**	**G5A**	**G2B**	**G3B**	**G4B**	**G5B**
Dose (mg/kg/day)	**30**	**100**	**300**	**1000**	**30**	**100**	**300**	**1000**
No. of rats	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
AST	—	—	—	↑119%*	—	—	—	↑36%*
ALT	—	—	—	↑101%*	—	—	—	↑25%*
ALP	—	—	—	↑61%*	—	—	—	↑84%*
Triglycerides	—	—	—	↓47%*	—	—	—	↓34%
Total Bilirubin	—	—	—	↑43%	—	—	—	↑91%*

↑: Increase ↓: Decrease *: Statistically significant change
Changes are expressed as mean % change from control values
'-'indicates that values are not considered meaningfully different from controls.

Increase in ALT, AST and ALP activity in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related adverse change as it was associated with single cell necrosis of hepatocytes in liver.

Decreased triglycerides concentration in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change. This change was associated with hepatocytes vacuolation in liver.

Increased total bilirubin concentration in 1000 mg/kg/day acetamide and acetamide + BrdU groups was considered test item-related change.

Decreased creatinine values in all treated acetamide + BrdU groups was considered as toxicologically insignificant as it was not associated with any decreased body weight (muscle mass).

Decreased total bilirubin concentration in 300 mg/kg/day acetamide and acetamide + BrdU groups was considered toxicologically insignificant change as decreased in total bilirubin does not have any biological significance.

Increased total protein and globulins and decreased albumin globulin ratio in 100 and 300 mg/kg/day acetamide dose groups was considered as toxicologically insignificant changes as the change did not show a dose-dependent progression at 1000 mg/kg/day.

All the electrolytes alteration (calcium, phosphorous, sodium and chloride) which reached statistical significance at different acetamide dose levels were likely due to random biological variation as these changes were within historical control range.

5.1.4 Urinalysis

Refer Tables 5 and 6, Appendices 5 and 6

There were no test item-related changes in the urinalysis parameters.

5.2 Anatomic Pathology

5.2.1 Terminal Fasting Body Weights, Organ Weights and Organ Weight Ratio

Refer Tables 7 to 12, Appendices 7 to 12

There were no significant changes in terminal fasting body weights in treated groups when compared to respective controls.

The organ weight changes in liver associated with test item administration when compared to vehicle control are tabulated below and the details are shown in Tables 7 to 12:

Treatment	Acetamide groups				Acetamide + BrdU groups			
Group	G2A	G3A	G4A	G5A	G2B	G3B	G4B	G5B
Dose (mg/kg/day)	30	100	300	1000	30	100	300	1000
No. of rats	6	6	6	6	6	6	6	6
Liver – absolute weights	—	—	—	↓25%*	—	—	—	↓15%*
– relative to body weights	—	—	—	↓20%*	—	—	—	↓10%*
– relative to brain weights	—	—	—	↓22%*	—	—	—	↓16%*

↓: Decrease *: Statistically significant change
Changes are expressed as mean % change from control values
'-'indicates that values are not considered meaningfully different from controls.

Decrease absolute and relative weight of liver in 1000 mg/kg/day acetamide and Acetamide + BrdU groups was considered test item-related change.

Decreased absolute weight and organ-to-brain weight ratios of seminal vesicles and coagulating glands in acetamide + Brdu groups were considered as incidental changes as the values were within historical control range.

5.2.2 Gross Pathology

Refer Table 13, Appendices 13 and 14

There were no test item-related gross changes in all treated groups.

All the gross changes observed in different dose groups were considered as incidental change of spontaneous origin as they were randomly distributed across the groups.

5.2.3 Histopathology

Refer Table 14, Appendices 15 and 16

The incidences and severity of test item-related microscopic changes in liver are tabulated below and the details are presented in Table 15 and Appendices 15 and 16:

Group	G1A	G2A	G3A	G4A	G5A	G1B	G2B	G3B	G4B	G5B
Dose (mg/kg Bwt/day)	**0**	**30**	**100**	**300**	**1000**	**0**	**30**	**100**	**300**	**1000**
No. of rats	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
LIVER;	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
Increased mitotic activity	0	0	0	0	6	0	0	0	0	6
minimal	–	–	–	–	5	–	–	–	–	5
mild	–	–	–	–	1	–	–	–	–	1
Single cell necrosis; hepatocyte	0	0	0	0	5	0	0	0	0	5
minimal	–	–	–	–	3	–	–	–	–	4
mild	–	–	–	–	2	–	–	–	–	1
Vacuolation; hepatocyte	0	0	0	0	5	0	0	0	0	3
minimal	–	–	–	–	5	–	–	–	–	3

Increased mitotic activity, single cell necrosis and vacuolation of hepatocytes in 1000 mg/kg/day acetamide and acetamide + BrdU groups were considered test item-related change. Increased mitotic activity in high dose was associated with high Ki67 proliferation index.

All the microscopic changes observed in different dose groups were considered as incidental change of spontaneous origin as they were randomly distributed across the groups.

5.2.4 Mitotic Index

Refer Table 15, Appendix 17

High hepatocytes mitotic index in 1000 mg/kg/day acetamide was considered test item-related change (refer to table 15). The mitotic index at <1000 mg/kg/day doses was comparable to control.

5.2.5 Immunohistochemistry

Refer Table 16, Appendices 18 to 19

Immuno-histochemical staining was performed for Ki 67 proliferation antigen and BrdU. The optimum immunostaining of Ki 67 proliferation antigen was observed in positive control (duodenum) and Ki 67 proliferation index was calculated for all the groups.

Suboptimal immunostaining of BrdU was noted in the positive control (duodenum). BrdU index of hepatocytes was not calculated by counting BrdU immuno-stained hepatocytes as optimal staining was not observed in positive control tissue (crypt epithelium of duodenum) and BrdU index thus obtained was not considered to be accurate representations of the cell proliferation activity.

Ki67 proliferation index.

High Ki 67 proliferation index in 1000 mg/kg/day acetamide and acetamide + BrdU groups (refer to table 16) was considered test item-related change.

Low Ki 67 proliferation index in 30 and 100 mg/kg/day acetamide dose groups when compared to control was considered as incidental change as low index in these two groups was due to slightly high index in two controls (Ru5682 and Ru5685) resulting in marginally high Ki 67 index of control group. Hence, low Ki 67 proliferation index in 30 and 100 mg/kg/day acetamide dose groups was not considered to be related to test item treatment.

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6. SUMMARY

Oral administration of the test item, (acetamide and acetamide + BrdU), daily at dose levels of 30,100, 300 and 1000 mg/kg/day for seven consecutive days by gavage resulted in test item-related changes in hematology, clinical chemistry parameters, organ weights, organ weights, histopathology, mitotic index and Ki67 proliferation index at 1000 mg/kg/day dose group alone.

The clinical pathology changes observed at 1000 mg/kg/day acetamide and acetamide + BrdU dose groups included: increased RBC counts, hemoglobin concentration and hematocrit, decrease reticulocytes, increased ALT, AST and ALP activity, decreased triglycerides and increased total bilirubin.

The anatomic pathology changes observed at 1000 mg/kg/day acetamide and acetamide + BrdU dose groups included: decrease absolute and relative weight of liver, increased mitotic activity, single cell necrosis and vacuolation of hepatocytes and high mitotic and Ki 67 proliferation indices of hepatocytes.

7. TABLES

TABLE 1. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

Refer Appendix 1

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
RBC (10^12/L)	8	Mean	8.70	8.68	8.80	8.81	9.48+
		SD	0.17	0.26	0.37	0.31	0.40
		N	6	6	6	6	6
		%Diff		--	--	--	9
Hb (g/L)	8	Mean	164	168	166	168	179+
		SD	3	4	7	5	8
		N	6	6	6	6	6
		%Diff		--	--	--	9
Hct (L/L)	8	Mean	0.506	0.509	0.506	0.519	0.535+
		SD	0.011	0.012	0.023	0.008	0.012
		N	6	6	6	6	6
		%Diff		--	--	--	6
MCV (fL)	8	Mean	58.2	58.7	57.5	58.9	56.5
		SD	1.0	1.6	0.4	2.0	1.9
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 1 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

Refer Appendix 1

Sex: Male	Day(s) Relative to Start Date		G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
MCH (pg)	8	Mean	18.8	19.4	18.8	19.2	18.9
		SD	0.4	0.5	0.3	0.7	0.8
		N	6	6	6	6	6
		%Diff		--	--	--	--
MCHC (g/L)	8	Mean	323	330	328	325	334+
		SD	4	3	5	6	9
		N	6	6	6	6	6
		%Diff		--	--	--	3
Retic A (10^12/L)	8	Mean	0.299	0.304	0.245	0.237	0.154–
		SD	0.041	0.054	0.048	0.046	0.035
		N	6	6	6	6	6
		%Diff		--	--	--	48
RDW (%)	8	Mean	12.4	11.9	11.5	11.7	11.7
		SD	0.8	0.6	0.6	0.5	0.7
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

TABLE 1 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

Refer Appendix 1

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
HDW (g/L)	8	Mean	24.5	24.7	23.2	22.8	24.4
		SD	1.8	1.2	1.3	1.0	1.3
		N	6	6	6	6	6
		%Diff		--	--	--	--
Hyper (%)	8	Mean	1.3	1.8	1.2	1.0	1.5
		SD	0.5	0.5	0.6	0.5	0.8
		N	6	6	6	6	6
		%Diff		--	--	--	--
Hypo (%)	8	Mean	0.5	0.3	0.3	0.3	0.3–
		SD	0.1	0.1	0.2	0.1	0.1
		N	6	6	6	6	6
		%Diff		--	--	--	48
Macro (%)	8	Mean	0	0	0	0	0
		SD	0	0	0	0	0
		N	6	6	6	6	6
		%Diff		.	.	.	.

--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 1 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

Refer Appendix 1

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Micro (%)	8	Mean	0	0	0	0	0
		SD	0	0	0	0	0
		N	6	6	6	6	6
		%Diff		.	.	.	.
RBC Frag- ments (10^12/L)	8	Mean	0.11	0.13	0.13	0.13	0.11
		SD	0.01	0.02	0.02	0.02	0.01
		N	6	6	6	6	6
		%Diff		--	--	--	--
RBC Ghosts (10^12/L)	8	Mean	0.07	0.07	0.06	0.07	0.06
		SD	0.01	0.01	0.00	0.01	0.01
		N	6	6	6	6	6
		%Diff		--	--	--	--
Plat (10^9/L)	8	Mean	1515	1710	1660	1701	1481
		SD	75	151	162	205	78
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 1 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

Refer Appendix 1

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
WBC (10^9/L)	8	Mean	5.34	6.93	4.35	5.28	5.90
		SD	1.17	0.88	1.61	1.19	2.26
		N	6	6	6	6	6
		%Diff		--	--	--	--
Neut A (10^9/L)	8	Mean	0.59	0.77	0.57	0.56	0.70
		SD	0.06	0.26	0.24	0.13	0.14
		N	6	6	6	6	6
		%Diff		--	--	--	--
Lymp A (10^9/L)	8	Mean	4.54	5.88	3.63	4.54	4.95
		SD	1.18	0.78	1.39	1.17	2.12
		N	6	6	6	6	6
		%Diff		--	--	--	--
Mono A (10^9/L)	8	Mean	0.13	0.16	0.08	0.10	0.13
		SD	0.04	0.06	0.04	0.02	0.04
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 1 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

Refer Appendix 1

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Baso A (10^9/L)	8	Mean	0.02	0.03	0.01	0.02	0.03
		SD	0.01	0.01	0.01	0.01	0.02
		N	6	6	6	6	6
		%Diff		--	--	--	--
Eosi A (10^9/L)	8	Mean	0.07	0.08	0.04	0.07	0.08
		SD	0.05	0.04	0.02	0.03	0.06
		N	6	6	6	6	6
		%Diff		--	--	--	--
Prothrombin Time (seconds)	8	Mean	15.8	16.4	16.4	15.5	16.9
		SD	0.7	0.6	0.6	1.1	1.3
		N	6	6	6	6	6
		%Diff		--	--	--	--
APTT (seconds)	8	Mean	10.4	11.9	9.7	12.0	11.8
		SD	1.2	2.1	1.0	1.2	2.2
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 2. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 2

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
RBC (10^12/L)	8	Mean	8.79	8.71	8.81	8.76	9.63+
		SD	0.46	0.14	0.35	0.54	0.69
		N	6	6	6	6	6
		%Diff		--	--	--	10
Hb (g/L)	8	Mean	163	164	165	168	182+
		SD	5	2	7	5	8
		N	6	6	6	6	6
		%Diff		--	--	--	12
Hct (L/L)	8	Mean	0.506	0.514	0.520	0.519	0.560+
		SD	0.019	0.009	0.020	0.011	0.031
		N	6	6	6	6	6
		%Diff		--	--	--	11
MCV (fL)	8	Mean	57.6	59.1	59.1	59.4	58.3
		SD	2.3	1.3	2.4	2.6	2.0
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 2 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 2

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
MCH (pg)	8	Mean	18.6	18.9	18.8	19.2	19.0
		SD	0.7	0.2	0.6	0.8	0.9
		N	6	6	6	6	6
		%Diff		--	--	--	--
MCHC (g/L)	8	Mean	323	320	318	324	326
		SD	8	6	13	6	8
		N	6	6	6	6	6
		%Diff		--	--	--	--
Retic A (10^12/L)	8	Mean	0.223	0.220	0.237	0.200	0.133–
		SD	0.022	0.059	0.028	0.037	0.048
		N	6	6	6	6	6
		%Diff		--	--	--	40
RDW (%)	8	Mean	12.0	11.8	11.9	11.9	12.4
		SD	0.5	0.3	0.5	0.4	0.6
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 2 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 2

Sex: Male	Day(s) Relative to Start Date		G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
HDW (g/L)	8	Mean	24.1	23.9	24.2	23.1	23.4
		SD	1.1	2.0	2.1	0.6	0.7
		N	6	6	6	6	6
		%Diff		--	--	--	--
Hyper (%)	8	Mean	0.8	1.0	1.1	0.8	0.8
		SD	0.4	0.7	0.6	0.1	0.2
		N	6	6	6	6	6
		%Diff		--	--	--	--
Hypo (%)	8	Mean	0.7	0.4	0.5	0.3	0.3
		SD	0.3	0.3	0.4	0.1	0.2
		N	6	6	6	6	6
		%Diff		--	--	--	--
Macro (%)	8	Mean	0	0	0	0	0
		SD	0	0	0	0	0
		N	6	6	6	6	6
		%Diff		.	.	.	.

--: Not presented as difference is not statistically significant.

TABLE 2 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 2

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Micro (%)	8	Mean	0	0	0	0	0
		SD	0	0	0	0	0
		N	6	6	6	6	6
		%Diff		.	.	.	.
RBC Frag- ments (10^12/L)	8	Mean	0.08	0.09	0.09	0.10	0.09
		SD	0.02	0.01	0.01	0.01	0.01
		N	6	6	6	6	6
		%Diff		--	--	--	--
RBC Ghosts (10^12/L)	8	Mean	0.05	0.06	0.06	0.07	0.06
		SD	0.01	0.01	0.01	0.01	0.01
		N	6	6	6	6	6
		%Diff		--	--	--	--
Plat (10^9/L)	8	Mean	1106	1186	1094	1211	1087
		SD	217	144	142	76	82
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 2 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 2

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
WBC (10^9/L)	8	Mean	5.51	5.53	5.75	5.39	6.02
		SD	1.39	2.09	1.96	0.54	0.72
		N	6	6	6	6	6
		%Diff		--	--	--	--
Neut A (10^9/L)	8	Mean	0.96	0.61	0.71	0.58	0.57
		SD	0.45	0.17	0.14	0.14	0.16
		N	6	6	6	6	6
		%Diff		--	--	--	--
Lymp A (10^9/L)	8	Mean	4.31	4.63	4.77	4.61	5.24
		SD	1.33	2.03	1.94	0.52	0.58
		N	6	6	6	6	6
		%Diff		--	--	--	--
Mono A (10^9/L)	8	Mean	0.11	0.17	0.11	0.11	0.12
		SD	0.03	0.11	0.03	0.03	0.01
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 2 contd. Summary of Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 2

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Baso A (10^9/L)	8	Mean	0.01	0.01	0.01	0.01	0.02
		SD	0.01	0.01	0.00	0.00	0.01
		N	6	6	6	6	6
		%Diff		--	--	--	--
Eosi A (10^9/L)	8	Mean	0.12	0.10	0.14	0.06	0.06
		SD	0.12	0.08	0.08	0.02	0.03
		N	6	6	6	6	6
		%Diff		--	--	--	--
Prothrombin Time (seconds)	8	Mean	15.8	16.1	16.0	16.8	18.1+
		SD	0.6	0.5	0.7	1.1	1.1
		N	6	6	6	6	6
		%Diff		--	--	--	14
APTT (seconds)	8	Mean	13.9	12.3	12.4	11.6	13.5
		SD	4.2	1.1	3.0	1.2	1.2
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 3. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide Groups

Refer Appendix 3

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Glu (mmol/L)	8	Mean	7.15	7.64	7.45	7.59	6.72
		SD	0.77	0.79	0.47	0.57	0.64
		N	6	6	6	6	6
		%Diff		--	--	--	--
BUN (mmol/L)	8	Mean	6.82	7.52	6.41	7.37	6.40
		SD	0.69	1.08	1.26	1.14	0.61
		N	6	6	6	6	6
		%Diff		--	--	--	--
Creat (µmol/L)	8	Mean	45	46	48	52	48
		SD	5	4	5	7	5
		N	6	6	6	6	6
		%Diff		--	--	--	--
AST (U/L)	8	Mean	77	66	70	79	168+
		SD	10	10	5	17	63
		N	6	6	6	6	6
		%Diff		--	--	--	119

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 3 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide Groups

Refer Appendix 3

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
ALT (U/L)	8	Mean	57	57	53	59	115+
		SD	11	9	10	11	32
		N	6	6	6	6	6
		%Diff		--	--	--	101
GGT (U/L)	8	Mean	5	5	5	4	5
		SD	1	1	1	1	0
		N	6	6	6	6	6
		%Diff		--	--	--	--
Alp (U/L)	8	Mean	167	180	142	189	269+
		SD	26	37	47	39	72
		N	6	6	6	6	6
		%Diff		--	--	--	61
LDH (U/L)	8	Mean	252	251	238	278	338
		SD	58	55	59	53	66
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 3 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide Groups

Refer Appendix 3

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
T.Bil (µmol/L)	8	Mean	3.22	2.89	3.25	2.17–	4.61
		SD	0.53	0.45	0.43	0.87	2.01
		N	6	6	6	6	6
		%Diff		--	--	33	--
T.Chol (mmol/L)	8	Mean	2.15	2.28	1.93	2.25	2.44
		SD	0.27	0.38	0.26	0.32	0.23
		N	6	6	6	6	6
		%Diff		--	--	--	--
Trig (mmol/L)	8	Mean	1.29	1.51	1.13	0.81	0.68–
		SD	0.25	0.40	0.47	0.35	0.30
		N	6	6	6	6	6
		%Diff		--	--	--	47
T.Pro (g/L)	8	Mean	65.1	67.2	68.7+	69.2+	63.9
		SD	2.3	1.8	2.3	2.0	2.5
		N	6	6	6	6	6
		%Diff		--	6	6	--

--: Not presented as difference is not statistically significant.

+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

TABLE 3 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide Groups

Refer Appendix 3

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
ALB (g/L)	8	Mean	33.1	33.3	33.2	33.6	32.1
		SD	0.9	0.7	1.2	1.1	1.1
		N	6	6	6	6	6
		%Diff		--	--	--	--
GLOB (g/L)	8	Mean	32.0	34.0	35.4+	35.6+	31.9
		SD	1.5	1.5	2.0	1.3	1.8
		N	6	6	6	6	6
		%Diff		--	11	11	--
A/G (ratio)	8	Mean	1.04	0.98	0.94–	0.94–	1.01
		SD	0.03	0.04	0.06	0.04	0.05
		N	6	6	6	6	6
		%Diff		--	9	9	--
Pi (mmol/L)	8	Mean	2.61	2.63	2.42	2.40	2.13–
		SD	0.15	0.30	0.26	0.25	0.17
		N	6	6	6	6	6
		%Diff		--	--	--	19

--: Not presented as difference is not statistically significant.

+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

TABLE 3 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide Groups

Refer Appendix 3

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Ca (mmol/L)	8	Mean	2.82	2.66	2.64–	2.59–	2.46–
		SD	0.06	0.44	0.05	0.06	0.08
		N	6	6	6	6	6
		%Diff		--	6	8	13
Na (mEq/L)	8	Mean	137.7	137.8	140.4	141.0+	143.6+
		SD	2.4	1.6	1.3	1.5	2.1
		N	6	6	6	6	6
		%Diff		--	--	2	4
K (mEq/L)	8	Mean	3.37	3.82	3.69	3.55	3.80
		SD	0.30	0.59	0.17	0.13	0.24
		N	6	6	6	6	6
		%Diff		--	--	--	--
Cl (mEq/L)	8	Mean	98.2	95.8–	99.2	98.6	100.5+
		SD	0.9	1.2	0.9	1.1	0.9
		N	6	6	6	6	6
		%Diff		2	--	--	2

--: Not presented as difference is not statistically significant.

+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

TABLE 4. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 4

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Glu (mmol/L)	8	Mean	6.80	6.52	6.12	6.58	5.74
		SD	2.16	0.78	0.54	1.00	0.78
		N	6	6	6	6	6
		%Diff		--	--	--	--
BUN (mmol/L)	8	Mean	7.75	8.02	8.23	8.00	8.35
		SD	1.41	0.50	1.06	1.02	0.91
		N	6	6	6	6	6
		%Diff		--	--	--	--
Creat (µmol/L)	8	Mean	49	44–	43–	40–	39–
		SD	3	4	1	2	4
		N	6	6	6	6	6
		%Diff		11	13	18	20
AST (U/L)	8	Mean	157	77	79	93	214+
		SD	197	6	7	23	42
		N	6	6	6	6	6
		%Diff		--	--	--	36

--: Not presented as difference is not statistically significant.

+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

TABLE 4 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 4

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
ALT (U/L)	8	Mean	122	52	54	62	153+
		SD	164	12	6	9	51
		N	6	6	6	6	6
		%Diff		--	--	--	25
GGT (U/L)	8	Mean	4	4	5	4	5
		SD	0	1	1	1	1
		N	6	6	6	6	6
		%Diff		--	--	--	--
Alp (U/L)	8	Mean	172	163	172	171	316+
		SD	25	36	41	39	60
		N	6	6	6	6	6
		%Diff		--	--	--	84
LDH (U/L)	8	Mean	333	332	339	314	429
		SD	76	36	101	80	62
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 4 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 4

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
T.Bil (µmol/L)	8	Mean	2.11	1.92	2.09	2.00	4.04+
		SD	0.40	0.21	0.27	0.50	0.99
		N	6	6	6	6	6
		%Diff		--	--	--	91
T.Chol (mmol/L)	8	Mean	2.14	2.10	2.06	1.98	2.46
		SD	0.22	0.32	0.33	0.15	0.26
		N	6	6	6	6	6
		%Diff		--	--	--	--
Trig (mmol/L)	8	Mean	1.55	1.28	1.64	1.12	1.02
		SD	0.89	0.26	0.74	0.50	0.43
		N	6	6	6	6	6
		%Diff		--	--	--	--
T.Pro (g/L)	8	Mean	68.8	68.2	68.4	66.8	67.2
		SD	3.1	1.1	1.3	2.7	1.7
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

+: Significantly higher than the vehicle control group at $p < 0.05$

TABLE 4 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 4

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
ALB (g/L)	8	Mean	32.7	32.2	32.4	31.6	32.2
		SD	1.3	1.1	0.6	0.8	0.6
		N	6	6	6	6	6
		%Diff		--	--	--	--
GLOB (g/L)	8	Mean	36.1	35.9	36.0	35.2	35.1
		SD	2.0	1.7	1.0	2.3	1.1
		N	6	6	6	6	6
		%Diff		--	--	--	--
A/G (ratio)	8	Mean	0.91	0.90	0.90	0.90	0.92
		SD	0.03	0.07	0.02	0.06	0.02
		N	6	6	6	6	6
		%Diff		--	--	--	--
Pi (mmol/L)	8	Mean	2.38	2.39	2.37	2.59	2.33
		SD	0.17	0.10	0.23	0.26	0.20
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 4 contd. Summary of Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

Refer Appendix 4

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Ca (mmol/L)	8	Mean	2.47	2.40	2.38	2.37	2.37
		SD	0.06	0.13	0.12	0.07	0.15
		N	6	6	6	6	6
		%Diff		--	--	--	--
Na (mEq/L)	8	Mean	144.1	142.5	144.2	142.4	141.8
		SD	2.3	0.9	1.7	1.9	1.4
		N	6	6	6	6	6
		%Diff		--	--	--	--
K (mEq/L)	8	Mean	3.84	3.49	3.68	3.67	3.90
		SD	0.20	0.37	0.29	0.27	0.26
		N	6	6	6	6	6
		%Diff		--	--	--	--
Cl (mEq/L)	8	Mean	98.9	98.2	99.0	100.5	100.3
		SD	2.1	0.8	1.1	1.1	0.4
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 5. Summary of Clinical Analysis of Urine on Day 8 – Acetamide Groups

Refer Appendix 5

Group No.	G1A	G2A	G3A	G4A	G5A
Dose (mg/kg/day)	0	30	100	300	1000
No. of rats	6	6	6	6	6
Volume (mL) $	3.3	3.8	4.0	5.4	5.1
Glucose*	1	1	0	0	1
Bilirubin*	0	1	0	0	0
Ketone Bodies*	4	2	3	0	1
Specific gravity $	1.024	1.021	1.023	1.014	1.021
pH $	8.8	8.8	8.9	9.0	9.0
Proteins*	6	6	6	5	6
Urobilinogen* #	0	0	0	0	0
Nitrite*	1	0	1	0	0

$: Mean value *: Incidences of findings, Negative incidences not considered
#: values less than or equal to 3.2 are normal and hence not considered as positive findings/incidence.

TABLE 6. Summary of Clinical Analysis of Urine on Day 8 – Acetamide + BrdU Groups

Refer Appendix 6

Group No.	G1B	G2B	G3B	G4B	G5B
Dose (mg/kg/day)	0	30	100	300	1000
No. of rats	6	6	6	6	6
Volume (mL) $	2.3	2.2	6.8	2.7	1.1
Glucose*	4	5	6	6	4
Bilirubin*	2	3	4	1	4
Ketone Bodies*	6	3	4	3	4
Specific gravity $	1.033	1.037	1.036	1.033	1.044
pH $	8.2	8.5	8.9	8.9	8.8
Proteins*	6	6	6	6	6
Urobilinogen* #	0	0	0	0	0
Nitrite*	2	0	1	1	1

$: Mean value *: Incidences of findings, Negative incidences not considered
#: values less than or equal to 3.2 are normal and hence not considered as positive findings/incidence.

TABLE 7. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

Refer Appendix 7

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Terminal Fasting BW (g)	Day 8	Mean	314.94	316.30	313.71	313.00	293.69
		SD	20.09	24.41	23.23	22.77	15.83
		N	6	6	6	6	6
		%Diff		--	--	--	--
Adrenals (g)	Day 8	Mean	0.0798	0.0815	0.0691	0.0732	0.0696
		SD	0.0032	0.0115	0.0083	0.0054	0.0081
		N	6	6	6	6	6
		%Diff		--	--	--	--
Brain (g)	Day 8	Mean	2.1301	2.0583	2.0430	2.0456	2.0282
		SD	0.0753	0.0896	0.0629	0.0961	0.0388
		N	6	6	6	6	6
		%Diff		--	--	--	--
Epididymides (g)	Day 8	Mean	1.0806	1.0639	1.0526	1.0218	1.0301
		SD	0.0750	0.0958	0.1105	0.0807	0.1868
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

APPENDIX 9 contd. Pathology Report

TABLE 7 contd. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

Refer Appendix 7

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Heart (g)	Day 8	Mean SD N %Diff	0.9991 0.0690 6	0.9617 0.0855 6 --	0.9617 0.0838 6 --	0.9716 0.0932 6 --	0.9255 0.0823 6 --
Kidneys (g)	Day 8	Mean SD N %Diff	1.9880 0.1333 6	2.0287 0.2527 6 --	1.9528 0.1928 6 --	2.0450 0.1709 6 --	1.9445 0.1333 6 --
Liver (g)	Day 8	Mean SD N %Diff	9.7686 0.9137 6	10.1685 0.6327 6 --	9.4750 0.8643 6 --	9.7123 1.1901 6 --	7.2866– 0.5794 6 25
Lungs (g)	Day 8	Mean SD N %Diff	1.5075 0.0800 6	1.5428 0.0933 6 --	1.5141 0.1007 6 --	1.5964 0.0935 6 --	1.5327 0.1891 6 --

--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 7 contd. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

Refer Appendix 7

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Pituitary (g)	Day 9*	Mean	0.0101	0.0104	0.0099	0.0103	0.0102
		SD	0.0013	0.0013	0.0006	0.0010	0.0018
		N	6	6	6	6	6
		%Diff		--	--	--	--
Prostate (g)	Day 8	Mean	0.5570	0.6041	0.5384	0.5372	0.5505
		SD	0.1069	0.1068	0.0797	0.1325	0.1121
		N	6	6	6	6	6
		%Diff		--	--	--	--
Seminal vesicles & coagulating glands (g)	Day 8	Mean	0.7999	0.6824	0.8075	0.7239	0.6350
		SD	0.1867	0.2456	0.1225	0.1407	0.0741
		N	6	6	6	6	6
		%Diff		--	--	--	--
Spleen (g)	Day 8	Mean	0.7978	0.6858	0.7608	0.7308	0.6762
		SD	0.1201	0.1379	0.1252	0.1722	0.0733
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 7 contd. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

Refer Appendix 7

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Testes (g)	Day 8	Mean SD N %Diff	3.2814 0.5417 6	3.5055 0.2000 6 --	3.4120 0.3458 6 --	3.3813 0.1717 6 --	2.9718 1.0489 6 --
Thymus (g)	Day 8	Mean SD N %Diff	0.6075 0.0832 6	0.6773 0.2037 6 --	0.5698 0.0915 6 --	0.6881 0.1579 6 --	0.5397 0.0687 6 --
Thyroid with parathyroids (g)	Day 9*	Mean SD N %Diff	0.0248 0.0035 6	0.0230 0.0053 6 --	0.0211 0.0025 6 --	0.0259 0.0014 6 --	0.0226 0.0049 6 --

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 8. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 8

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Terminal Fasting BW (g)	Day 8	Mean	314.94	316.30	313.71	313.00	293.69
		SD	20.09	24.41	23.23	22.77	15.83
		N	6	6	6	6	6
		%Diff		--	--	--	--
Adrenals (%)	Day 8	Mean	0.0255	0.0258	0.0221	0.0234	0.0237
		SD	0.0022	0.0036	0.0028	0.0019	0.0026
		N	6	6	6	6	6
		%Diff		--	--	--	--
Brain. (%)	Day 8	Mean	0.6790	0.6540	0.6535	0.6556	0.6923
		SD	0.0543	0.0586	0.0389	0.0451	0.0396
		N	6	6	6	6	6
		%Diff		--	--	--	--
Epididymides (%)	Day 8	Mean	0.3437	0.3364	0.3363	0.3267	0.3511
		SD	0.0234	0.0160	0.0343	0.0173	0.0639
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 8 contd. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 8

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Heart (%)	Day 8	Mean SD N %Diff	0.3181 0.0268 6	0.3040 0.0111 6 --	0.3064 0.0085 6 --	0.3104 0.0204 6 --	0.3154 0.0258 6 --
Kidneys (%)	Day 8	Mean SD N %Diff	0.6325 0.0461 6	0.6402 0.0447 6 --	0.6223 0.0375 6 --	0.6536 0.0355 6 --	0.6627 0.0425 6 --
Liver (%)	Day 8	Mean SD N %Diff	3.1021 0.2185 6	3.2199 0.1354 6 --	3.0201 0.1616 6 --	3.0986 0.2484 6 --	2.4843– 0.1997 6 20
Lungs (%)	Day 8	Mean SD N %Diff	0.4796 0.0272 6	0.4885 0.0195 6 --	0.4839 0.0349 6 --	0.5113 0.0336 6 --	0.5214 0.0517 6 --

--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

APPENDIX 9 contd. Pathology Report

TABLE 8 contd. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 8

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Pituitary (%)	Day 9*	Mean	0.0032	0.0033	0.0032	0.0033	0.0035
		SD	0.0005	0.0003	0.0002	0.0004	0.0007
		N	6	6	6	6	6
		%Diff		--	--	--	--
Prostate (%)	Day 8	Mean	0.1769	0.1912	0.1717	0.1723	0.1876
		SD	0.0318	0.0302	0.0220	0.0431	0.0387
		N	6	6	6	6	6
		%Diff		--	--	--	--
Seminal vesicles & coagulating glands (%)	Day 8	Mean	0.2524	0.2125	0.2569	0.2296	0.2161
		SD	0.0466	0.0605	0.0276	0.0291	0.0211
		N	6	6	6	6	6
		%Diff		--	--	--	--
Spleen (%)	Day 8	Mean	0.2528	0.2153	0.2422	0.2319	0.2313
		SD	0.0285	0.0302	0.0321	0.0448	0.0332
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation

--: Not presented as difference is not statistically significant.

TABLE 8 contd. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 8

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Testes (%)	Day 8	Mean	1.0439	1.1115	1.0904	1.0828	1.0096
		SD	0.1824	0.0722	0.1100	0.0554	0.3480
		N	6	6	6	6	6
		%Diff		--	--	--	--
Thymus (%)	Day 8	Mean	0.1930	0.2113	0.1821	0.2190	0.1848
		SD	0.0240	0.0473	0.0290	0.0407	0.0289
		N	6	6	6	6	6
		%Diff		--	--	--	--
Thyroid with parathyroid. (%)	Day 9*	Mean	0.0079	0.0072	0.0067	0.0083	0.0077
		SD	0.0014	0.0014	0.0010	0.0009	0.0018
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation

--: Not presented as difference is not statistically significant.

TABLE 9. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 9

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Brain (g)	Day 8	Mean SD N %Diff	2.1301 0.0753 6	2.0583 0.0896 6 --	2.0430 0.0629 6 --	2.0456 0.0961 6 --	2.0282 0.0388 6 --
Adrenals (%)	Day 8	Mean SD N %Diff	3.7538 0.2376 6	3.9672 0.6093 6 --	3.3791 0.3597 6 --	3.5801 0.2623 6 --	3.4341 0.4039 6 --
Epididymides (%)	Day 8	Mean SD N %Diff	50.8566 4.9284 6	51.8550 6.1327 6 --	51.5233 5.1151 6 --	49.9785 3.6018 6 --	50.7240 8.8998 6 --
Heart (%)	Day 8	Mean SD N %Diff	46.9701 3.9515 6	46.8231 4.9984 6 --	47.0419 3.4530 6 --	47.4644 3.5469 6 --	45.6357 4.0507 6 --

--: Not presented as difference is not statistically significant.

TABLE 9 contd. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 9

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Kidneys (%)	Day 8	Mean SD N %Diff	93.5581 8.8957 6	98.7187 13.0499 6 --	95.6192 9.6128 6 --	99.8865 5.5127 6 --	95.8695 6.2854 6 --
Liver (%)	Day 8	Mean SD N %Diff	459.4088 50.2691 6	494.9367 40.0394 6 --	463.4758 36.4396 6 --	474.6142 52.5986 6 --	359.3094– 28.0877 6 22
Lungs (%)	Day 8	Mean SD N %Diff	70.9233 5.8583 6	75.1020 6.0047 6 --	74.0948 4.1012 6 --	78.2483 6.9444 6 --	75.4936 8.3126 6 --
Pituitary (%)	Day 9*	Mean SD N %Diff	0.4731 0.0453 6	0.5067 0.0580 6 --	0.4849 0.0326 6 --	0.5005 0.0345 6 --	0.5040 0.0916 6 --

*: Weighed after fixation
--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 9 contd. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 9

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Prostate (%)	Day 8	Mean	26.2675	29.3819	26.3965	26.2242	27.1244
		SD	5.6767	5.1039	4.1316	6.0154	5.3447
		N	6	6	6	6	6
		%Diff		--	--	--	--
Seminal vesicles & coagulating glands (%)	Day 8	Mean	37.7647	33.1348	39.5289	35.3437	31.3393
		SD	9.8217	11.6225	5.8403	6.3985	3.8984
		N	6	6	6	6	6
		%Diff		--	--	--	--
Spleen (%)	Day 8	Mean	37.5882	33.2941	37.2002	35.6282	33.3100
		SD	6.5796	6.2577	5.6896	7.8197	3.2296
		N	6	6	6	6	6
		%Diff		--	--	--	--
Testes (%)	Day 8	Mean	154.3032	170.5882	167.1958	165.4128	145.9756
		SD	26.3038	12.3557	17.8182	7.2449	51.0534
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 9 contd. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

Refer Appendix 9

Sex: Male Day(s) Relative to Start Date			G1A 0 mg/kg/day	G2A 30 mg/kg/day	G3A 100 mg/kg/day	G4A 300 mg/kg/day	G5A 1000 mg/kg/day
Thymus (%)	Day 8	Mean	28.4778	32.8720	27.8245	33.6865	26.6107
		SD	3.1997	9.5003	3.7935	7.8422	3.3888
		N	6	6	6	6	6
		%Diff		--	--	--	--
Thyroid with parathyroids (%)	Day 9*	Mean	1.1706	1.1217	1.0319	1.2687	1.1143
		SD	0.1956	0.2980	0.1347	0.1066	0.2454
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 10. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

Refer Appendix 10

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Terminal Fasting BW (g)	Day 8	Mean	307.65	315.77	312.55	309.28	293.27
		SD	20.77	13.73	20.99	25.26	22.96
		N	6	6	6	6	6
		%Diff		--	--	--	--
Adrenals (g)	Day 8	Mean	0.0813	0.0783	0.0736	0.0699	0.0697
		SD	0.0111	0.0097	0.0098	0.0088	0.0062
		N	6	6	6	6	6
		%Diff		--	--	--	--
Brain (g)	Day 8	Mean	2.0262	2.0783	2.0350	2.0259	2.0547
		SD	0.0693	0.0440	0.0825	0.1091	0.0661
		N	6	6	6	6	6
		%Diff		--	--	--	--
Epididymides (g)	Day 8	Mean	1.0431	1.0776	0.9853	1.0006	1.0167
		SD	0.0832	0.0856	0.1955	0.0554	0.0621
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 10 contd. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

Refer Appendix 10

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Heart (g)	Day 8	Mean SD N %Diff	0.9418 0.0668 6	0.9623 0.0706 6 --	0.9220 0.0335 6 --	0.9537 0.1213 6 --	0.9127 0.0443 6 --
Kidneys (g)	Day 8	Mean SD N %Diff	2.0709 0.0765 6	2.0311 0.1860 6 --	2.0285 0.1973 6 --	2.0904 0.2178 6 --	2.0773 0.1467 6 --
Liver (g)	Day 8	Mean SD N %Diff	9.8324 1.0771 6	9.9141 0.5669 6 --	9.8523 0.5604 6 --	9.7460 0.5538 6 --	8.3935– 0.6887 6 15
Lungs (g)	Day 8	Mean SD N %Diff	1.5447 0.0892 6	1.5801 0.0603 6 --	1.5992 0.1023 6 --	1.5409 0.1195 6 --	1.4831 0.0756 6 --

--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 10 contd. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

Refer Appendix 10

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Pituitary (g)	Day 9*	Mean	0.0096	0.0100	0.0097	0.0101	0.0089
		SD	0.0008	0.0007	0.0007	0.0018	0.0012
		N	6	6	6	6	6
		%Diff		--	--	--	--
Prostate (g)	Day 8	Mean	0.5738	0.5496	0.5393	0.5511	0.4627
		SD	0.1200	0.0754	0.1480	0.1310	0.1250
		N	6	6	6	6	6
		%Diff		--	--	--	--
Seminal vesicles & coagulating glands (g)	Day 8	Mean	0.7799	0.7693	0.6350	0.8086	0.5274–
		SD	0.1464	0.1794	0.1682	0.1377	0.1413
		N	6	6	6	6	6
		%Diff		--	--	--	32
Spleen (g)	Day 8	Mean	0.6393	0.6524	0.7240	0.6930	0.6065
		SD	0.1174	0.1080	0.0858	0.1491	0.0614
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation
--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

APPENDIX 9 contd. Pathology Report

TABLE 10 contd. Summary of Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

Refer Appendix 10

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Testes (g)	Day 8	Mean SD N %Diff	3.3894 0.1719 6	3.5696 0.2859 6 --	3.4157 0.5658 6 --	3.2734 0.1045 6 --	3.5791 0.1655 6 --
Thymus (g)	Day 8	Mean SD N %Diff	0.6255 0.2472 6	0.5969 0.1518 6 --	0.5364 0.1130 6 --	0.6311 0.1029 6 --	0.5366 0.0713 6 --
Thyroid with parathyroids (g)	Day 9*	Mean SD N %Diff	0.0243 0.0030 6	0.0258 0.0033 6 --	0.0225 0.0022 6 --	0.0251 0.0025 6 --	0.0236 0.0025 6 --

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 11. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 11

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Terminal Fasting BW (g)	Day 8	Mean	307.65	315.77	312.55	309.28	293.27
		SD	20.77	13.73	20.99	25.26	22.96
		N	6	6	6	6	6
		%Diff		--	--	--	--
Adrenals (%)	Day 8	Mean	0.0263	0.0248	0.0236	0.0226	0.0239
		SD	0.0026	0.0033	0.0029	0.0018	0.0029
		N	6	6	6	6	6
		%Diff		--	--	--	--
Brain. (%)	Day 8	Mean	0.6615	0.6590	0.6523	0.6565	0.7044
		SD	0.0555	0.0265	0.0271	0.0266	0.0629
		N	6	6	6	6	6
		%Diff		--	--	--	--
Epididymides (%)	Day 8	Mean	0.3398	0.3416	0.3171	0.3252	0.3484
		SD	0.0278	0.0272	0.0664	0.0313	0.0328
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.

TABLE 11 contd. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 11

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Heart (%)	Day 8	Mean	0.3062	0.3048	0.2960	0.3075	0.3122
		SD	0.0086	0.0195	0.0205	0.0160	0.0193
		N	6	6	6	6	6
		%Diff		--	--	--	--
Kidneys (%)	Day 8	Mean	0.6752	0.6428	0.6486	0.6769	0.7109
		SD	0.0430	0.0447	0.0357	0.0608	0.0591
		N	6	6	6	6	6
		%Diff		--	--	--	--
Liver (%)	Day 8	Mean	3.1902	3.1401	3.1560	3.1584	2.8625–
		SD	0.1676	0.1365	0.1258	0.1442	0.0851
		N	6	6	6	6	6
		%Diff		--	--	--	10
Lungs (%)	Day 8	Mean	0.5031	0.5014	0.5127	0.5001	0.5073
		SD	0.0296	0.0323	0.0332	0.0451	0.0318
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 11 contd. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 11

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Pituitary (%)	Day 9*	Mean	0.0031	0.0032	0.0031	0.0032	0.0030
		SD	0.0001	0.0002	0.0002	0.0004	0.0004
		N	6	6	6	6	6
		%Diff		--	--	--	--
Prostate (%)	Day 8	Mean	0.1872	0.1746	0.1723	0.1792	0.1566
		SD	0.0403	0.0270	0.0435	0.0450	0.0360
		N	6	6	6	6	6
		%Diff		--	--	--	--
Seminal vesicles & coagulating glands (%)	Day 8	Mean	0.2552	0.2441	0.2030	0.2614	0.1799
		SD	0.0542	0.0578	0.0517	0.0390	0.0469
		N	6	6	6	6	6
		%Diff		--	--	--	--
Spleen (%)	Day 8	Mean	0.2077	0.2063	0.2328	0.2229	0.2071
		SD	0.0355	0.0308	0.0361	0.0345	0.0172
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 11 contd. Summary of Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 11

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Testes (%)	Day 8	Mean	1.1044	1.1331	1.1018	1.0637	1.2273
		SD	0.0702	0.1122	0.2165	0.0858	0.1190
		N	6	6	6	6	6
		%Diff		--	--	--	--
Thymus (%)	Day 8	Mean	0.2027	0.1880	0.1709	0.2036	0.1842
		SD	0.0758	0.0416	0.0290	0.0240	0.0309
		N	6	6	6	6	6
		%Diff		--	--	--	--
Thyroid with parathyroid. (%)	Day 9*	Mean	0.0079	0.0082	0.0072	0.0082	0.0081
		SD	0.0011	0.0010	0.0009	0.0011	0.0011
		N	6	6	6	6	6
		%Diff		--	--	--	--

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 12. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 12

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Brain (g)	Day 8	Mean SD N %Diff	2.0262 0.0693 6	2.0783 0.0440 6 --	2.0350 0.0825 6 --	2.0259 0.1091 6 --	2.0547 0.0661 6 --
Adrenals (%)	Day 8	Mean SD N %Diff	4.0170 0.5976 6	3.7684 0.4798 6 --	3.6240 0.5172 6 --	3.4422 0.3173 6 --	3.3980 0.3342 6 --
Epididymides (%)	Day 8	Mean SD N %Diff	51.5720 5.1267 6	51.8667 4.2630 6 --	48.5202 9.7935 6 --	49.5286 4.1711 6 --	49.5422 3.7155 6 --
Heart (%)	Day 8	Mean SD N %Diff	46.5484 4.0657 6	46.2973 3.1995 6 --	45.3888 2.8488 6 --	46.9602 3.9917 6 --	44.4615 2.6554 6 --

--: Not presented as difference is not statistically significant.

TABLE 12 contd. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 12

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Kidneys (%)	Day 8	Mean SD N %Diff	102.3459 6.2208 6	97.6418 7.3154 6 --	99.6822 8.6626 6 --	103.2407 10.1257 6 --	101.1246 6.7437 6 --
Liver (%)	Day 8	Mean SD N %Diff	486.7500 67.0269 6	477.0784 26.8006 6 --	483.9550 11.9946 6 --	481.5384 25.5475 6 --	408.7382– 34.1757 6 16
Lungs (%)	Day 8	Mean SD N %Diff	76.2552 3.9905 6	76.0488 3.0631 6 --	78.6064 4.3378 6 --	76.1115 5.1639 6 --	72.3063 5.4908 6 --
Pituitary (%)	Day 9*	Mean SD N %Diff	0.4761 0.0463 6	0.4806 0.0360 6 --	0.4780 0.0356 6 --	0.4943 0.0671 6 --	0.4357 0.0719 6 --

*: Weighed after fixation
--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 12 contd. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 12

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Prostate (%)	Day 8	Mean	28.4756	26.4724	26.5861	27.3280	22.6600
		SD	6.7677	3.8331	7.3675	7.0298	6.6583
		N	6	6	6	6	6
		%Diff		--	--	--	--
Seminal vesicles & coagulating glands (%)	Day 8	Mean	38.6416	37.0151	31.2146	39.9225	25.8208–
		SD	7.8875	8.4820	8.3545	6.5575	7.5672
		N	6	6	6	6	6
		%Diff		--	--	--	33
Spleen (%)	Day 8	Mean	31.5982	31.3466	35.6863	34.0460	29.4900
		SD	5.9541	4.8204	5.1477	5.8465	2.4151
		N	6	6	6	6	6
		%Diff		--	--	--	--
Testes (%)	Day 8	Mean	167.5037	171.6436	168.2654	161.8980	174.2617
		SD	11.4930	11.4516	29.2574	8.8518	7.9881
		N	6	6	6	6	6
		%Diff		--	--	--	--

--: Not presented as difference is not statistically significant.
–: Significantly lower than the vehicle control group at $p < 0.05$

TABLE 12 contd. Summary of Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

Refer Appendix 12

Sex: Male Day(s) Relative to Start Date			G1B 0 mg/kg/day	G2B 30 mg/kg/day	G3B 100 mg/kg/day	G4B 300 mg/kg/day	G5B 1000 mg/kg/day
Thymus (%)	Day 8	Mean SD N %Diff	30.8629 11.8200 6	28.7552 7.5035 6 --	26.2890 4.9829 6 --	31.0688 4.0983 6 --	26.1485 3.6790 6 --
Thyroid with parathyroids (%)	Day 9*	Mean SD N %Diff	1.1989 0.1606 6	1.2410 0.1749 6 --	1.1051 0.1195 6 --	1.2414 0.1370 6 --	1.1475 0.0914 6 --

*: Weighed after fixation
--: Not presented as difference is not statistically significant.

TABLE 13. Summary of Gross Pathology Findings

Refer Appendices 13 and 14

Removal Reason: TERMINAL SACRIFICE

Group No.:	G1A	G2A	G3A	G4A	G5A	G1B	G2B	G3B	G4B	G5B
Dose (mg/kg/day):	0	30	100	300	1000	0	30	100	300	1000
Number of Animals on Study :	6	6	6	6	6	6	6	6	6	6
Number of Animals Completed:	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
EPIDIDYMIDES;										
Submitted	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No Visible Lesions	6	6	5	6	5	6	6	5	6	6
Small; bilateral	0	0	0	0	1	0	0	0	0	0
Small; unilateral	0	0	1	0	0	0	0	0	0	0
Absent; unilateral	0	0	0	0	0	0	0	1	0	0
LIVER;										
Submitted	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No Visible Lesions	6	6	6	6	6	5	6	6	6	5
Focus (i); white; right lateral lobe	0	0	0	0	0	1	0	0	0	0
Focus (i); white; right medial lobe	0	0	0	0	0	0	0	0	0	1
TESTES;										
Submitted	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
No Visible Lesions	6	6	5	6	5	6	6	5	6	6
Small; bilateral	0	0	0	0	1	0	0	0	0	0
Small; unilateral	0	0	1	0	0	0	0	0	0	0
Absent; unilateral	0	0	0	0	0	0	0	1	0	0

Note: All the other tissues were examined grossly and no gross abnormalities were observed for all the animals.

TABLE 14. Summary of Histopathology Findings

Refer Appendices 15 and 16

Observations: Non Neo-Plastic
Removal Reason: TERMINAL SACRIFICE

Group No.:	G1A	G2A	G3A	G4A	G5A	G1B	G2B	G3B	G4B	G5B
Dose (mg/kg/day):	0	30	100	300	1000	0	30	100	300	1000
Number of Animals on Study :	6	6	6	6	6	6	6	6	6	6
Number of Animals Completed:	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
FEMUR BONE WITH DISTAL JOINT;										
Examined	(6)	(0)	(0)	(0)	(6)	(6)	(0)	(0)	(0)	(6)
Within Normal Limits	6	0	0	0	6	6	0	0	0	6
LIVER;										
Examined	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
Within Normal Limits	6	6	6	6	0	5	6	6	6	0
Increased mitotic activity	(0)	(0)	(0)	(0)	(6)	(0)	(0)	(0)	(0)	(6)
minimal	0	0	0	0	5	0	0	0	0	5
mild	0	0	0	0	1	0	0	0	0	1
Inflammatory focus(i)	(0)	(0)	(0)	(0)	(0)	(1)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0	1	0	0	0	0
Vacuolation; hepatocyte	(0)	(0)	(0)	(0)	(5)	(0)	(0)	(0)	(0)	(3)
minimal	0	0	0	0	5	0	0	0	0	3
Single cell necrosis; hepatocyte	(0)	(0)	(0)	(0)	(5)	(0)	(0)	(0)	(0)	(5)
minimal	0	0	0	0	3	0	0	0	0	4
mild	0	0	0	0	2	0	0	0	0	1
SPLEEN;										
Examined	(6)	(0)	(0)	(0)	(6)	(6)	(0)	(0)	(0)	(6)
Within Normal Limits	6	0	0	0	6	6	0	0	0	6
STERNUM;										
Examined	(6)	(0)	(0)	(0)	(6)	(6)	(0)	(0)	(0)	(6)
Within Normal Limits	6	0	0	0	6	6	0	0	0	6

TABLE 15. Summary of Mitotic Index – Acetamide Groups

Refer Appendix 17

Group No.	Dose mg/kg/day		Mitotic Index
G1A	0	Mean SD N	0.16 0.16 6
G2A	30	Mean SD N % Diff	0.09 0.08 6
G3A	100	Mean SD N % Diff	0.09 0.06 6
G4A	300	Mean SD N % Diff	0.14 0.07 6
G5A	1000	Mean SD N % Diff	1.11 0.49 6

--: Not presented as difference is not statistically significant.
+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

TABLE 16. Summary of Ki 67 Index

Refer Appendices 18 and 19

Acetamide Groups

Group No.	Dose mg/kg/day		Ki67 Labelling Index
			%
G1A	0	Mean SD N	5.89 2.48 6
G2A	30	Mean SD N % Diff	2.93– 1.59 6 50
G3A	100	Mean SD N % Diff	2.56– 1.13 6 57
G4A	300	Mean SD N % Diff	4.31 1.72 6 27
G5A	1000	Mean SD N % Diff	24.62+ 8.77 6 318

Acetamide + BrdU Groups

Group No.	Dose mg/kg/day		Ki67 Labelling Index
			%
G1B	0	Mean SD N	2.78 0.74 6
G2B	30	Mean SD N % Diff	1.99 0.43 6 --
G3B	100	Mean SD N % Diff	3.47 1.13 6 --
G4B	300	Mean SD N % Diff	3.44 1.16 6 --
G5B	1000	Mean SD N % Diff	25.53+ 3.58 6 818

--: Not presented as difference is not statistically significant.
+/–: Significantly higher / lower than the vehicle control group at $p < 0.05$

8. APPENDICES

APPENDIX 1. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5681	8.64	159	0.494	57.1	18.4	322	0.355	12.7	24.4	1.0	0.5	0	0	0.10
Ru5682	8.50	162	0.492	57.9	19.0	329	0.258	12.3	27.7	2.1	0.5	0	0	0.11
Ru5683	8.91	163	0.509	57.1	18.3	320	0.323	13.0	24.8	1.6	0.4	0	0	0.11
Ru5684	8.52	164	0.506	59.4	19.2	324	0.246	11.3	22.0	0.5	0.4	0	0	0.11
Ru5685	8.84	165	0.519	58.7	18.7	318	0.312	13.3	23.8	1.1	0.6	0	0	0.13
Ru5686	8.76	168	0.515	58.8	19.2	326	0.298	11.7	24.5	1.2	0.5	0	0	0.11

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5681	0.06	1462	7.35	0.52	6.57	0.15	0.02	0.07	16.2	10.4
Ru5682	0.06	1642	4.35	0.53	3.58	0.18	0.02	0.04	16.3	9.9
Ru5683	0.06	1474	5.11	0.60	4.33	0.13	0.01	0.04	16.2	8.9
Ru5684	0.07	1495	5.30	0.59	4.41	0.14	0.02	0.15	16.0	12.4
Ru5685	0.07	1568	5.81	0.67	5.03	0.06	0.01	0.03	15.2	10.0
Ru5686	0.07	1448	4.09	0.61	3.29	0.09	0.01	0.09	14.7	10.8

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5687	9.03	169	0.513	56.8	18.7	330	0.211	11.3	25.5	2.0	0.2	0	0	0.13
Ru5688	8.69	172	0.525	60.4	19.8	327	0.350	12.9	23.6	1.3	0.4	0	0	0.14
Ru5689	8.84	171	0.511	57.8	19.3	335	0.355	11.5	25.1	1.8	0.3	0	0	0.10
Ru5690	8.75	169	0.511	58.4	19.3	329	0.326	12.2	26.5	2.7	0.4	0	0	0.10
Ru5691	8.45	160	0.488	57.7	18.9	328	0.305	12.0	23.8	1.4	0.3	0	0	0.14
Ru5692	8.33	168	0.507	60.8	20.1	330	0.277	11.3	23.6	1.4	0.4	0	0	0.14

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5687	0.06	1750	7.83	0.76	6.79	0.19	0.02	0.05	15.3	10.6
Ru5688	0.07	1624	6.28	0.56	5.50	0.11	0.03	0.06	15.9	9.8
Ru5689	0.07	1541	6.06	0.98	4.71	0.19	0.02	0.15	16.7	11.0
Ru5690	0.06	1607	8.02	1.09	6.55	0.24	0.02	0.09	16.5	10.8
Ru5691	0.06	1783	7.22	0.80	6.20	0.12	0.03	0.05	16.9	14.0
Ru5692	0.07	1957	6.14	0.40	5.54	0.10	0.03	0.07	16.9	15.0

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5693	9.45	177	0.547	57.9	18.7	323	0.190	10.7	21.1	0.4	0.3	0	0	0.11
Ru5694	8.31	157	0.478	57.5	18.9	328	0.315	12.1	24.2	1.5	0.3	0	0	0.14
Ru5695	8.77	169	0.508	57.9	19.3	333	0.227	11.2	23.3	1.1	0.2	0	0	0.16
Ru5696	8.86	164	0.507	57.2	18.5	324	0.276	12.2	24.0	1.1	0.5	0	0	0.14
Ru5697	8.73	162	0.499	57.2	18.5	324	0.264	11.6	22.2	0.6	0.5	0	0	0.12
Ru5698	8.66	165	0.494	57.0	19.1	335	0.199	11.3	24.6	2.2	0.1	0	0	0.13

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5693	0.07	1484	3.67	0.44	3.08	0.09	0.01	0.03	16.1	10.9
Ru5694	0.06	1719	6.92	0.70	6.03	0.09	0.02	0.06	15.6	9.7
Ru5695	0.06	1918	3.20	0.53	2.55	0.08	0.01	0.03	16.4	8.4
Ru5696	0.06	1736	5.79	0.94	4.59	0.15	0.01	0.07	16.1	9.1
Ru5697	0.06	1546	3.10	0.22	2.77	0.06	0.02	0.02	17.2	9.2
Ru5698	0.06	1556	3.42	0.61	2.74	0.03	0.01	0.02	16.7	10.8

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5699	8.97	169	0.513	57.1	18.9	331	0.248	12.6	24.3	1.5	0.3	0	0	0.13
Ru5700	8.50	169	0.518	60.9	19.9	327	0.301	11.2	23.5	1.4	0.3	0	0	0.14
Ru5701	8.87	168	0.518	58.4	18.9	324	0.249	11.5	21.8	0.5	0.4	0	0	0.09
Ru5702	8.43	169	0.522	61.9	20.1	324	0.219	11.7	22.7	1.2	0.2	0	0	0.15
Ru5703	9.28	175	0.532	57.4	18.9	329	0.246	11.8	22.8	1.0	0.3	0	0	0.12
Ru5704	8.79	160	0.508	57.8	18.2	315	0.161	11.6	21.9	0.4	0.3	0	0	0.12

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5699	0.07	1710	6.85	0.57	6.05	0.10	0.04	0.07	15.4	10.8
Ru5700	0.07	1916	6.13	0.37	5.58	0.09	0.02	0.06	17.3	12.0
Ru5701	0.06	1378	4.94	0.74	3.97	0.09	0.01	0.13	14.6	13.5
Ru5702	0.08	1926	5.21	0.64	4.44	0.07	0.01	0.04	14.6	11.8
Ru5703	0.07	1659	5.21	0.58	4.42	0.13	0.01	0.05	14.8	10.7
Ru5704	0.06	1615	3.35	0.44	2.76	0.09	0.01	0.04	16.0	13.2

APPENDIX 9 contd. Pathology Report

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5705	9.49	189	0.550	58.0	19.9	343	0.127	10.9	24.9	2.7	0.1	0	0	0.12
Ru5706	9.22	171	0.520	56.4	18.6	329	0.203	11.7	23.8	0.8	0.3	0	0	0.11
Ru5707	9.24	167	0.523	56.6	18.1	319	0.159	11.5	24.5	0.7	0.4	0	0	0.13
Ru5708	9.25	180	0.536	57.9	19.5	336	0.177	11.5	22.6	0.9	0.2	0	0	0.11
Ru5709	9.41	182	0.539	57.3	19.3	338	0.106	11.8	23.8	1.4	0.2	0	0	0.10
Ru5710	10.27	184	0.543	52.9	17.9	339	0.153	12.9	26.6	2.3	0.3	0	0	0.11

APPENDIX 1 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5705	0.07	1462	8.77	0.69	7.65	0.15	0.07	0.20	15.3	8.8
Ru5706	0.05	1419	8.81	0.87	7.68	0.14	0.03	0.06	16.1	9.3
Ru5707	0.06	1467	3.84	0.44	3.23	0.08	0.04	0.02	16.7	14.2
Ru5708	0.07	1624	4.44	0.68	3.55	0.13	0.02	0.06	16.7	13.1
Ru5709	0.06	1504	4.86	0.75	3.82	0.19	0.03	0.06	17.7	12.4
Ru5710	0.05	1410	4.68	0.76	3.75	0.09	0.01	0.06	18.9	12.8

APPENDIX 2. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5711	8.22	158	0.478	58.2	19.3	331	0.197	11.7	23.7	0.7	0.4	0	0	0.08
Ru5712	8.99	171	0.527	58.7	19.0	323	0.249	12.5	23.4	0.9	0.5	0	0	0.06
Ru5713	8.73	162	0.489	56.0	18.5	331	0.217	11.2	24.5	1.1	0.3	0	0	0.10
Ru5714	8.30	160	0.510	61.4	19.3	313	0.200	11.9	22.7	0.3	1.0	0	0	0.11
Ru5715	9.11	164	0.506	55.5	17.9	323	0.247	12.7	25.9	1.2	0.7	0	0	0.07
Ru5716	9.36	165	0.524	56.0	17.6	315	0.227	12.1	24.2	0.4	1.1	0	0	0.08

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5711	0.05	1126	4.52	1.66	2.70	0.09	0.00	0.05	16.3	11.3
Ru5712	0.06	780	6.08	1.25	4.51	0.09	0.01	0.21	15.5	11.6
Ru5713	0.05	1298	7.30	0.78	6.29	0.15	0.02	0.04	16.9	20.3
Ru5714	0.06	1378	4.74	0.53	4.09	0.08	0.00	0.03	15.5	17.9
Ru5715	0.05	976	6.70	1.04	5.19	0.13	0.01	0.31	15.5	12.5
Ru5716	0.04	1075	3.72	0.48	3.10	0.09	0.00	0.05	15.2	9.6

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5717	8.49	163	0.519	61.1	19.2	314	0.212	11.8	22.1	0.3	0.7	0	0	0.11
Ru5718	8.74	163	0.518	59.3	18.7	314	0.312	12.0	24.1	1.2	0.4	0	0	0.09
Ru5719	8.83	166	0.512	58.0	18.8	324	0.200	11.6	24.2	1.1	0.2	0	0	0.07
Ru5720	8.70	165	0.522	60.0	18.9	315	0.250	12.1	24.2	0.6	0.8	0	0	0.09
Ru5721	8.62	163	0.496	57.5	18.9	329	0.211	11.5	27.2	2.1	0.2	0	0	0.10
Ru5722	8.85	166	0.517	58.5	18.8	321	0.134	11.5	21.7	0.4	0.3	0	0	0.08

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5717	0.07	1377	4.61	0.45	4.00	0.11	0.01	0.03	15.9	11.5
Ru5718	0.05	1244	5.11	0.70	4.16	0.17	0.01	0.05	16.8	12.0
Ru5719	0.05	951	4.31	0.75	3.04	0.39	0.00	0.13	16.2	12.2
Ru5720	0.07	1115	5.22	0.65	4.21	0.10	0.01	0.25	16.5	11.4
Ru5721	0.05	1241	9.71	0.76	8.67	0.11	0.03	0.10	15.5	14.5
Ru5722	0.06	1188	4.23	0.35	3.70	0.11	0.01	0.04	15.5	11.9

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5723	9.07	166	0.515	56.7	18.3	322	0.262	11.6	22.3	1.1	0.2	0	0	0.10
Ru5724	8.92	175	0.539	60.5	19.6	324	0.252	11.7	22.4	0.9	0.2	0	0	0.07
Ru5725	8.50	160	0.491	57.7	18.8	326	0.204	11.5	27.5	2.1	0.3	0	0	0.10
Ru5726	8.29	161	0.508	61.3	19.4	317	0.247	12.6	26.2	1.5	0.6	0	0	0.09
Ru5727	9.23	171	0.522	56.5	18.5	328	0.199	12.4	23.8	0.8	0.3	0	0	0.09
Ru5728	8.82	159	0.544	61.7	18.1	293	0.256	11.6	23.2	0.4	1.1	0	0	0.07

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5723	0.06	1222	6.28	0.66	5.35	0.14	0.01	0.10	16.1	15.0
Ru5724	0.07	945	4.78	0.81	3.61	0.09	0.01	0.27	15.9	16.2
Ru5725	0.05	1241	9.39	0.75	8.37	0.10	0.02	0.12	16.2	11.9
Ru5726	0.06	1182	4.43	0.45	3.83	0.09	0.01	0.02	15.3	12.6
Ru5727	0.05	1055	4.03	0.74	3.01	0.08	0.01	0.19	15.4	8.0
Ru5728	0.07	917	5.56	0.86	4.42	0.14	0.01	0.13	17.2	10.4

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5729	8.90	164	0.509	57.2	18.4	321	0.168	12.5	23.7	0.8	0.3	0	0	0.10
Ru5730	8.53	166	0.524	61.4	19.5	318	0.236	12.2	22.8	0.8	0.2	0	0	0.08
Ru5731	8.50	166	0.516	60.8	19.5	321	0.223	11.7	22.8	0.7	0.3	0	0	0.10
Ru5732	9.78	178	0.539	55.1	18.2	330	0.195	11.7	24.1	0.8	0.4	0	0	0.10
Ru5733	8.32	168	0.508	61.0	20.2	331	0.146	11.6	22.6	0.9	0.1	0	0	0.09
Ru5734	8.50	165	0.517	60.8	19.5	320	0.232	11.5	22.6	0.6	0.3	0	0	0.10

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5729	0.05	1246	6.14	0.54	5.47	0.06	0.01	0.06	17.3	12.7
Ru5730	0.07	1143	5.15	0.76	4.13	0.16	0.02	0.08	15.3	11.6
Ru5731	0.07	1304	5.50	0.65	4.66	0.13	0.01	0.05	17.3	13.1
Ru5732	0.06	1150	5.73	0.61	4.88	0.11	0.01	0.10	17.5	11.8
Ru5733	0.08	1137	4.55	0.33	4.08	0.10	0.01	0.03	17.8	10.2
Ru5734	0.06	1284	5.24	0.60	4.46	0.11	0.01	0.05	15.5	10.2

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	RBC (10^12/L)	Hb (g/L)	Hct (L/L)	MCV (fL)	MCH (pg)	MCHC (g/L)	Retic A (10^12/L)	RDW (%)	HDW (g/L)	Hyper (%)	Hypo (%)	Macro (%)	Micro (%)	RBC Fragments (10^12/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5735	8.26	169	0.503	61.0	20.5	337	0.143	11.5	22.6	0.9	0.2	0	0	0.09
Ru5736	10.01	180	0.549	54.9	18.0	328	0.214	11.8	24.3	0.8	0.3	0	0	0.10
Ru5737	9.68	181	0.575	59.5	18.7	315	0.072	12.2	23.3	0.4	0.7	0	0	0.07
Ru5738	10.14	190	0.586	57.9	18.7	323	0.134	12.8	23.6	0.9	0.1	0	0	0.09
Ru5739	9.84	191	0.577	58.7	19.4	331	0.102	13.0	24.0	1.1	0.1	0	0	0.10
Ru5740	9.83	183	0.570	57.9	18.6	320	0.133	12.8	22.5	0.6	0.2	0	0	0.09

APPENDIX 2 contd. Individual Animal Haematology and Coagulation Parameters on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	RBC ghosts (10^12/L)	Plat (10^9/L)	WBC (10^9/L)	Neut A (10^9/L)	Lymp A (10^9/L)	Mono A (10^9/L)	Baso A (10^9/L)	Eosi A (10^9/L)	PT (seconds)	APTT (seconds)
	8	8	8	8	8	8	8	8	8	8
Ru5735	0.07	1118	4.82	0.32	4.33	0.11	0.01	0.04	18.1	14.8
Ru5736	0.05	1188	5.65	0.60	4.80	0.11	0.01	0.11	17.1	14.2
Ru5737	0.06	980	6.81	0.79	5.82	0.13	0.02	0.04	18.1	13.8
Ru5738	0.06	1074	6.32	0.55	5.56	0.13	0.02	0.06	16.8	11.3
Ru5739	0.07	1155	6.58	0.68	5.69	0.10	0.02	0.08	19.6	13.8
Ru5740	0.06	1007	5.93	0.49	5.25	0.11	0.02	0.05	18.9	13.1

APPENDIX 3. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5681	5.94	7.50	41	79	68	5	120	204	3.58	2.23	1.49	65.4	32.7	32.7
Ru5682	7.04	5.64	40	64	47	5	187	245	2.82	1.87	1.12	62.1	32.4	29.7
Ru5683	7.24	7.42	46	76	68	5	188	205	4.01	2.02	1.14	67.8	34.5	33.3
Ru5684	8.12	6.71	46	92	44	4	164	363	2.90	1.86	1.59	62.6	32.1	30.5
Ru5685	6.78	6.58	46	79	64	4	182	245	3.42	2.53	1.44	65.4	33.0	32.4
Ru5686	7.77	7.09	53	70	51	4	161	251	2.61	2.36	0.97	67.2	33.9	33.3

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5681	1.00	2.67	2.85	133.3	3.43	99.2
Ru5682	1.09	2.54	2.73	136.8	3.10	98.5
Ru5683	1.04	2.57	2.83	138.6	2.97	96.8
Ru5684	1.05	2.60	2.83	138.9	3.62	99.0
Ru5685	1.02	2.87	2.91	138.8	3.34	98.0
Ru5686	1.02	2.43	2.76	140.0	3.76	97.6

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5687	8.37	6.03	46	73	54	4	155	257	3.10	2.34	1.24	64.5	32.5	32.0
Ru5688	6.63	8.22	42	49	71	5	182	173	3.27	2.96	1.31	68.6	34.3	34.3
Ru5689	7.47	7.82	49	66	46	4	149	295	2.28	2.05	1.23	68.3	33.7	34.6
Ru5690	8.66	7.78	51	77	58	4	198	269	3.03	1.94	1.24	68.0	33.6	34.4
Ru5691	7.74	6.43	44	65	48	4	150	198	3.29	2.02	1.89	65.3	32.9	32.4
Ru5692	6.96	8.84	43	67	63	6	243	315	2.35	2.34	2.14	68.7	32.5	36.2

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5687	1.02	2.24	2.75	139.4	3.49	97.1
Ru5688	1.00	2.69	2.98	137.7	3.54	95.9
Ru5689	0.97	2.60	2.82	138.9	3.54	95.7
Ru5690	0.98	2.95	2.82	138.3	3.65	96.2
Ru5691	1.02	2.35	1.77	137.4	5.02	96.5
Ru5692	0.90	2.95	2.80	134.8	3.70	93.6

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5693	7.43	5.49	50	68	55	3	168	259	3.02	2.18	0.71	66.8	32.5	34.3
Ru5694	8.02	7.48	53	68	62	5	154	202	3.65	1.97	0.99	71.2	32.6	38.6
Ru5695	7.12	8.08	49	78	54	5	150	324	2.86	1.83	0.83	70.7	33.8	36.9
Ru5696	6.75	5.67	39	71	45	4	142	261	2.73	2.14	2.02	68.3	33.2	35.1
Ru5697	7.53	6.87	46	65	64	5	185	233	3.71	2.02	1.00	65.4	31.9	33.5
Ru5698	7.87	4.88	51	68	37	5	50	151	3.52	1.46	1.25	69.5	35.4	34.1

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5693	0.95	2.19	2.62	141.6	3.56	99.2
Ru5694	0.84	2.53	2.71	139.2	3.65	98.9
Ru5695	0.92	2.12	2.69	139.6	3.86	98.0
Ru5696	0.95	2.34	2.61	141.0	3.46	98.8
Ru5697	0.95	2.82	2.59	139.0	3.91	99.2
Ru5698	1.04	2.54	2.62	142.1	3.72	100.8

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5699	6.77	6.80	43	67	57	4	159	227	2.94	2.44	0.83	70.5	34.7	35.8
Ru5700	7.70	6.92	47	64	42	5	152	270	3.46	1.62	0.88	68.1	32.9	35.2
Ru5701	8.09	9.39	50	70	71	4	153	353	1.24	2.45	1.01	72.0	34.9	37.1
Ru5702	7.80	7.89	56	81	53	4	242	229	2.12	2.30	0.43	69.1	34.0	35.1
Ru5703	7.03	6.15	54	79	60	5	215	260	1.49	2.45	1.29	69.0	32.3	36.7
Ru5704	8.17	7.09	63	111	70	4	213	331	1.74	2.23	0.39	66.2	32.7	33.5

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5699	0.97	2.69	2.62	138.6 [b]	3.53	96.6
Ru5700	0.93	2.03	2.60	140.0	3.68	98.8
Ru5701	0.94	2.56	2.68	141.8	3.62	99.0
Ru5702	0.97	2.42	2.54	141.2	3.32	98.0
Ru5703	0.88	2.54	2.59	142.8	3.51	98.9
Ru5704	0.98	2.17	2.51	141.6	3.66	100.0

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5705	6.55	6.04	44	219	152	5	332	401	4.70	2.43	1.13	60.4	31.7	28.7
Ru5706	7.29	5.90	54	128	92	5	270	295	3.21	2.55	0.67	66.5	33.3	33.2
Ru5707	5.72	6.84	45	270	151	5	314	439	8.29	2.60	0.27	62.0	31.1	30.9
Ru5708	7.48	5.62	48	106	73	5	158	273	2.45	2.61	0.48	66.5	33.4	33.1
Ru5709	6.45	6.98	54	139	113	5	208	311	4.61	2.44	0.85	65.2	31.9	33.3
Ru5710	6.85	6.99	45	145	107	5	333	306	4.40	2.00	0.68	62.8	30.9	31.9

APPENDIX 3 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5705	1.10	2.30	2.39	142.5	3.97	99.7
Ru5706	1.00	2.34	2.55	145.8	3.53	100.3
Ru5707	1.01	1.97	2.50	140.8	4.11	102.1
Ru5708	1.01	2.06	2.39	145.1	3.78	100.4
Ru5709	0.96	2.17	2.52	142.0	3.52	99.6
Ru5710	0.97	1.92	2.38	145.3	3.91	100.8

APPENDIX 4. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5711	4.70	5.23	52	559	456	4	155	351	2.74	1.98	0.86	65.7	31.3	34.4
Ru5712	6.16	7.61	46	79	55	4	171	268	2.19	2.09	1.40	68.5	32.8	35.7
Ru5713	5.82	7.54	47	76	68	5	169	267	1.59	2.08	0.89	68.3	31.6	36.7
Ru5714	10.58	8.29	52	79	62	4	137	383	2.30	1.99	3.29	66.9	32.4	34.5
Ru5715	8.05	9.44	46	67	38	4	211	276	1.87	2.58	1.51	74.6	34.9	39.7
Ru5716	5.48	8.40	51	82	55	4	186	452	1.97	2.11	1.35	69.0	33.4	35.6

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5711	0.91	2.23	2.52	145.2	3.96	101.2
Ru5712	0.92	2.67	2.46	141.4	4.12	96.1
Ru5713	0.86	2.51	2.43	143.8	3.67	98.0
Ru5714	0.94	2.31	2.39	142.5	3.91	97.5
Ru5715	0.88	2.27	2.47	143.8	3.75	99.5
Ru5716	0.94	2.28	2.56	148.1	3.60	101.3

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5717	5.89	7.47	43	77	43	4	175	285	2.28	1.70	1.48	67.6	33.9	33.7
Ru5718	6.20	8.39	44	80	65	4	148	293	1.83	2.25	1.03	68.1	32.4	35.7
Ru5719	5.79	7.65	42	79	51	4	133	337	1.85	1.76	0.93	66.7	31.8	34.9
Ru5720	6.41	8.30	47	84	66	4	116	339	1.81	2.05	1.48	68.2	32.7	35.5
Ru5721	6.91	8.69	49	75	48	5	197	375	1.71	2.31	1.22	69.9	31.9	38.0
Ru5722	7.89	7.62	38	65	37	3	206	361	2.06	2.51	1.56	68.5	30.7	37.8

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5717	1.01	2.41	2.56	143.7	3.16	98.9
Ru5718	0.91	2.32	2.40	142.5	3.28	98.2
Ru5719	0.91	2.38	2.37	142.8	3.36	99.2
Ru5720	0.92	2.47	2.43	143.1	3.22	97.1
Ru5721	0.84	2.24	2.45	141.8	3.89	97.6
Ru5722	0.81	2.53	2.17	141.1	4.03	98.4

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5723	6.62	7.77	43	87	58	5	146	542	1.60	1.97	2.05	68.8	32.9	35.9
Ru5724	6.59	6.92	43	80	54	5	170	271	2.21	1.90	1.49	67.2	32.2	35.0
Ru5725	6.07	9.40	43	72	53	5	126	301	2.26	2.33	0.95	69.6	32.3	37.3
Ru5726	5.78	7.56	40	75	55	4	148	331	2.06	1.87	1.30	69.0	33.1	35.9
Ru5727	5.24	8.11	44	73	42	4	229	299	2.35	2.58	2.94	66.3	31.4	34.9
Ru5728	6.44	9.59	44	89	61	5	213	292	2.03	1.69	1.11	69.5	32.7	36.8

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5723	0.92	2.23	2.54	142.0	4.03	97.4
Ru5724	0.92	2.78	2.23	142.3	4.07	99.1
Ru5725	0.87	2.48	2.47	144.1	3.56	98.3
Ru5726	0.92	2.19	2.34	146.1	3.57	99.5
Ru5727	0.90	2.18	2.43	145.6	3.45	99.2
Ru5728	0.89	2.36	2.27	145.2	3.42	100.6

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5729	6.80	8.40	42	71	65	6	137	212	1.97	1.94	2.05	68.3	30.9	37.4
Ru5730	5.79	6.46	38	82	54	3	148	312	2.23	1.96	0.73	64.6	31.6	33.0
Ru5731	6.76	9.21	39	91	67	3	190	393	1.32	2.23	0.78	70.2	32.6	37.6
Ru5732	5.47	7.47	43	134	76	4	213	361	2.81	1.77	0.81	65.2	32.1	33.1
Ru5733	6.34	8.87	38	80	55	4	213	223	1.70	1.94	1.16	68.8	32.1	36.7
Ru5734	8.30	7.60	42	102	54	5	126	384	1.98	2.06	1.16	63.5	30.4	33.1

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5729	0.83	2.19	2.33	142.9	3.50	99.3
Ru5730	0.96	2.41	2.25	143.7	3.76	101.3
Ru5731	0.87	2.58	2.39	139.0	3.80	101.6
Ru5732	0.97	2.92	2.43	144.2	3.23	101.5
Ru5733	0.87	2.72	2.44	141.3	3.71	99.8
Ru5734	0.92	2.74	2.35	143.4	4.00	99.4

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Glu (mmol/L)	BUN (mmol/L)	Creat (µmol/L)	AST (U/L)	ALT (U/L)	GGT (U/L)	Alp (U/L)	LDH (U/L)	T.Bil (µmol/L)	T.Chol (mmol/L)	Trig (mmol/L)	T.Pro (g/L)	ALB (g/L)	GLOB (g/L)
	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Ru5735	4.33	8.45	43	154	105	4	276	437	3.51	2.35	0.76	66.7	32.2	34.5
Ru5736	6.24	8.62	40	206	132	5	354	348	4.73	2.60	0.58	67.1	32.3	34.8
Ru5737	5.79	6.88	37	179	102	6	361	450	4.56	2.02	1.29	64.3	30.9	33.4
Ru5738	6.47	9.40	43	238	182	5	279	528	2.38	2.51	0.91	67.7	32.2	35.5
Ru5739	6.18	7.73	33	262	161	6	390	433	5.08	2.51	1.75	68.1	32.6	35.5
Ru5740	5.42	9.00	39	245	234	4	237	380	3.96	2.79	0.84	69.4	32.7	36.7

APPENDIX 4 contd. Individual Animal Clinical Chemistry Parameters on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	A/G (ratio)	Pi (mmol/L)	Ca (mmol/L)	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)
	8	8	8	8	8	8
Ru5735	0.93	2.07	2.48	140.6	3.68	100.6
Ru5736	0.93	2.46	2.36	142.3	3.56	100.5
Ru5737	0.93	2.21	2.40	142.5	3.91	100.6
Ru5738	0.91	2.17	2.41	140.6	4.03	99.6
Ru5739	0.92	2.46	2.49	140.9	3.96	99.9
Ru5740	0.89	2.59	2.08	144.0	4.28	100.3

APPENDIX 5. Individual Animal Clinical Analysis of Urine on Day 8 – Acetamide Groups

Group No. Dose (mg/kg/day)	Animal No.	Volume (mL)	Appearance		Glucose mmol/L	Bilirubin	Ketone Bodies mmol/L	Specific gravity	pH	Proteins g/L
			Colour	Clarity						
G1A	Ru5681	5.0	Light yellow	Clear	Negative	Negative	1.5	1.016	8.5	0.3
0	Ru5682	2.0	Light yellow	Clear	Negative	Negative	Trace	1.025	>=9.0	1.0
	Ru5683	3.0	Yellow	Clear	5.5	Negative	1.5	1.038	8.5	1.0
	Ru5684	4.0	Light yellow	Clear	Negative	Negative	1.5	1.024	>=9.0	1.0
	Ru5685	3.0	Yellow	Clear	Negative	Negative	Trace	1.023	8.5	1.0
	Ru5686	3.0	Light yellow	Clear	Negative	Negative	1.5	1.020	>=9.0	1.0
G2A	Ru5687	5.0	Light yellow	Clear	Negative	Negative	Negative	1.018	>=9.0	1.0
30	Ru5688	3.0	Yellow	Clear	5.5	Small	1.5	1.029	8.5	1.0
	Ru5689	3.0	Light yellow	Clear	Negative	Negative	Trace	1.021	>=9.0	0.3
	Ru5690	3.0	Light yellow	Clear	Negative	Negative	Trace	1.021	8.5	0.3
	Ru5691	4.0	Yellow	Clear	Negative	Negative	1.5	1.020	>=9.0	1.0
	Ru5692	4.5	Yellow	Clear	Negative	Negative	Negative	1.016	>=9.0	1.0
G3A	Ru5693	5.0	Light yellow	Clear	Negative	Negative	1.5	1.029	>=9.0	1.0
100	Ru5694	4.0	Light yellow	Clear	Negative	Negative	Trace	1.028	8.5	0.3
	Ru5695	3.5	Yellow	Clear	Negative	Negative	1.5	1.021	>=9.0	1.0
	Ru5696	4.0	Light yellow	Clear	Negative	Negative	Trace	1.020	>=9.0	>=3.0
	Ru5697	3.5	Light yellow	Clear	Negative	Negative	Trace	1.020	>=9.0	1.0
	Ru5698	4.0	Light yellow	Clear	Negative	Negative	1.5	1.021	>=9.0	1.0
G4A	Ru5699	4.5	Yellow	Clear	Negative	Negative	Negative	1.013	>=9.0	Trace
300	Ru5700	6.5	Light yellow	Clear	Negative	Negative	Negative	1.015	>=9.0	1.0
	Ru5701	4.5	Light yellow	Clear	Negative	Negative	Trace	1.009	>=9.0	1.0
	Ru5702	5.0	Light yellow	Clear	Negative	Negative	Trace	1.019	>=9.0	1.0
	Ru5703	5.0	Light yellow	Clear	Negative	Negative	Negative	1.015	>=9.0	>=3.0
	Ru5704	7.0	Light yellow	Clear	Negative	Negative	Negative	1.013	>=9.0	0.3

APPENDIX 5 contd. Individual Animal Clinical Analysis of Urine on Day 8 – Acetamide Groups

Group No. Dose (mg/kg/day)	Animal No.	Urobilinogen µmol/L	Nitrite	Microscopic Findings							
				Erythrocytes	Leukocytes	Crystals				Epithelial Cells	Casts
						a	b	c	d		
G1A	Ru5681	3.2	Negative	-	-	3+	-	-	-	-	-
0	Ru5682	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5683	3.2	Positive	-	-	2+	-	-	-	-	-
	Ru5684	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5685	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5686	3.2	Negative	-	-	1+	-	-	-	-	-
G2A	Ru5687	3.2	Negative	-	-	3+	-	-	-	-	-
30	Ru5688	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5689	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5690	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5691	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5692	3.2	Negative	-	-	1+	-	-	-	-	-
G3A	Ru5693	3.2	Negative	-	-	3+	-	-	-	-	-
100	Ru5694	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5695	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5696	3.2	Negative	-	-	1+	-	-	-	-	-
	Ru5697	3.2	Positive	-	-	2+	-	-	-	-	-
	Ru5698	3.2	Negative	-	-	2+	-	-	-	-	-
G4A	Ru5699	3.2	Negative	-	-	3+	-	-	-	-	-
300	Ru5700	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5701	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5702	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5703	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5704	3.2	Negative	-	-	3+	-	-	-	-	-

a : Phosphates b: Calcium Carbonate/Oxalate c: Urates/Uric acid d: Hippuric Acid
1+: Occasional/Few in entire slide 2+: Moderate/Few in each field 3+: Large number in each field

APPENDIX 5 contd. Individual Animal Clinical Analysis of Urine on Day 8 – Acetamide Groups

Group No. Dose (mg/kg/day)	Animal No.	Volume (mL)	Appearance		Glucose mmol/L	Bilirubin	Ketone Bodies mmol/L	Specific gravity	pH	Proteins g/L
			Colour	Clarity						
G5A	Ru5705	3.0	Light yellow	Clear	5.5	Negative	1.5	1.030	>=9.0	0.3
1000	Ru5706	4.0	Light yellow	Clear	Negative	Negative	Trace	1.024	>=9.0	1.0
	Ru5707	4.0	Light yellow	Clear	Negative	Negative	Negative	1.021	>=9.0	1.0
	Ru5708	4.5	Light yellow	Clear	Negative	Negative	Negative	1.015	>=9.0	0.3
	Ru5709	7.0	Light yellow	Clear	Negative	Negative	Trace	1.021	>=9.0	0.3
	Ru5710	8.0	Light yellow	Clear	Negative	Negative	Negative	1.015	>=9.0	0.3

Group No. Dose (mg/kg/day)	Animal No.	Urobilinogen µmol/L	Nitrite	Microscopic Findings							
				Erythrocytes	Leukocytes	Crystals				Epithelial Cells	Casts
						a	b	c	d		
G5A	Ru5705	3.2	Negative	-	-	2+	-	-	-	-	-
1000	Ru5706	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5707	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5708	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5709	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5710	3.2	Negative	-	-	2+	-	-	-	-	-

a : Phosphates b: Calcium Carbonate/Oxalate c: Urates/Uric acid d: Hippuric Acid
1+: Occasional/Few in entire slide 2+: Moderate/Few in each field 3+: Large number in each field

APPENDIX 6. Individual Animal Clinical Analysis of Urine on Day 8 – Acetamide + BrdU Groups

Group No. Dose (mg/kg/day)	Animal No.	Volume (mL)	Appearance		Glucose mmol/L	Bilirubin	Ketone Bodies mmol/L	Specific gravity	pH	Proteins g/L
			Colour	Clarity						
G1B	Ru5711	1.5	Yellow	Clear	28	Negative	1.5	1.025	>=9.0	1.0
0	Ru5712	2.0	Yellow	Clear	Negative	Negative	1.5	1.013	>=9.0	0.3
	Ru5713	4.0	Light yellow	Clear	Negative	Negative	1.5	1.022	>=9.0	1.0
	Ru5714	4.0	Yellow	Clear	5.5	Small	3.9	1.053	7.0	>=3.0
	Ru5715	1.5	Light yellow	Clear	14	Negative	1.5	1.035	6.0	0.3
	Ru5716	1.0	Yellow	Clear	14	Small	1.5	1.050	>=9.0	>=3.0
G2B	Ru5717	3.0	Yellow	Clear	14	Small	1.5	1.047	>=9.0	>=3.0
30	Ru5718	4.0	Light yellow	Clear	>=55	Negative	Negative	1.019	>=9.0	0.3
	Ru5719	3.0	Yellow	Clear	28	Negative	Trace	1.023	>=9.0	0.3
	Ru5720	1.0	Yellow	Clear	Negative	Small	3.9	1.056	6.5	1.0
	Ru5721	1.1	Yellow	Clear	28	Negative	Trace	1.032	8.5	>=3.0
	Ru5722	1.2	Brown	Clear	14	Small	1.5	1.042	>=9.0	>=3.0
G3B	Ru5723	1.5	Brown	Clear	28	Small	1.5	1.035	>=9.0	>=3.0
100	Ru5724	1.2	Yellow	Clear	14	Small	1.5	1.039	>=9.0	>=3.0
	Ru5725	33.0	Yellow	Clear	14	Small	1.5	1.033	8.5	>=3.0
	Ru5726	1.5	Yellow	Clear	5.5	Negative	Trace	1.030	>=9.0	>=3.0
	Ru5727	2.0	Yellow	Clear	28	Negative	Trace	1.031	>=9.0	>=3.0
	Ru5728	1.3	Yellow	Clear	14	Small	1.5	1.050	>=9.0	>=3.0
G4B	Ru5729	1.0	Yellow	Clear	14	Negative	1.5	1.035	>=9.0	>=3.0
300	Ru5730	1.5	Yellow	Clear	14	Small	1.5	1.041	>=9.0	>=3.0
	Ru5731	3.5	Yellow	Clear	>=55	Negative	Negative	1.034	>=9.0	0.3
	Ru5732	5.0	Light yellow	Clear	>=55	Negative	Negative	1.021	>=9.0	0.3
	Ru5733	3.5	Light yellow	Clear	28	Negative	Trace	1.027	>=9.0	1.0
	Ru5734	1.5	Light yellow	Clear	28	Negative	1.5	1.038	8.5	1.0

APPENDIX 6 contd. Individual Animal Clinical Analysis of Urine on Day 8 – Acetamide + BrdU Groups

Group No. Dose (mg/kg/day)	Animal No.	Urobilinogen µmol/L	Nitrite	Microscopic Findings							
				Erythrocytes	Leukocytes	Crystals				Epithelial Cells	Casts
						a	b	c	d		
G1B	Ru5711	3.2	Positive	-	-	3+	-	-	-	-	-
0	Ru5712	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5713	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5714	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5715	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5716	3.2	Positive	-	-	2+	-	-	-	-	-
G2B	Ru5717	3.2	Negative	-	-	2+	-	-	-	-	-
30	Ru5718	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5719	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5720	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5721	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5722	3.2	Negative	-	-	3+	-	-	-	-	-
G3B	Ru5723	3.2	Positive	-	-	3+	-	-	-	-	-
100	Ru5724	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5725	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5726	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5727	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5728	3.2	Negative	-	-	2+	-	-	-	-	-
G4B	Ru5729	3.2	Negative	-	-	3+	-	-	-	-	-
300	Ru5730	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5731	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5732	3.2	Negative	-	-	1+	-	-	-	-	-
	Ru5733	3.2	Negative	-	-	2+	-	-	-	-	-
	Ru5734	3.2	Positive	-	-	3+	-	-	-	-	-

a : Phosphates b: Calcium Carbonate/Oxalate c: Urates/Uric acid d: Hippuric Acid
1+: Occasional/Few in entire slide 2+: Moderate/Few in each field 3+: Large number in each field

APPENDIX 6 contd. Individual Animal Clinical Analysis of Urine on Day 8 – Acetamide + BrdU Groups

Group No. Dose (mg/kg/day)	Animal No.	Volume (mL)	Appearance		Glucose mmol/L	Bilirubin	Ketone Bodies mmol/L	Specific gravity	pH	Proteins g/L
			Colour	Clarity						
G5B	Ru5735	1.5	Yellow	Clear	5.5	Negative	1.5	1.048	8.5	1.0
1000	Ru5736	1.2	Yellow	Clear	5.5	Small	3.9	1.039	8.5	>=3.0
	Ru5737	1.1	Yellow	Clear	14	Small	Trace	1.038	>=9.0	>=3.0
	Ru5738	1.0	Yellow	Clear	Negative	Negative	1.5	1.050	8.5	>=3.0
	Ru5739	1.0	Yellow	Clear	Negative	Small	1.5	1.048	>=9.0	1.0
	Ru5740	1.0	Yellow	Clear	14	Small	Trace	1.042	>=9.0	>=3.0

Group No. Dose (mg/kg/day)	Animal No.	Urobilinogen µmol/L	Nitrite	Microscopic Findings							
				Erythrocytes	Leukocytes	Crystals				Epithelial Cells	Casts
						a	b	c	d		
G5B	Ru5735	3.2	Negative	-	-	3+	-	-	-	-	-
1000	Ru5736	3.2	Positive	-	-	2+	-	-	-	-	-
	Ru5737	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5738	3.2	Negative	-	-	3+	-	-	-	-	-
	Ru5739	3.2	Negative	-	-	1+	-	-	-	-	-
	Ru5740	3.2	Negative	-	-	3+	-	-	-	-	-

a : Phosphates b: Calcium Carbonate/Oxalate c: Urates/Uric acid d: Hippuric Acid
1+: Occasional/Few in entire slide 2+: Moderate/Few in each field 3+: Large number in each field

APPENDIX 7. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5681	340.14	0.0770	2.0676	1.1931	1.0596	2.0665	11.2603	1.5159	0.0085	0.6160	1.0560
Ru5682	306.96	0.0819	2.0858	1.0205	1.0860	2.1836	8.9446	1.5241	0.0095	0.4444	0.7029
Ru5683	287.20	0.0797	2.1455	1.0614	0.9948	1.8983	9.8419	1.4406	0.0107	0.5692	0.7205
Ru5684	329.45	0.0826	2.0575	1.1369	0.9363	2.0478	9.9387	1.6544	0.0096	0.7222	1.0102
Ru5685	325.84	0.0751	2.2544	0.9893	1.0107	1.9049	9.9535	1.4495	0.0123	0.4408	0.7126
Ru5686	300.07	0.0827	2.1696	1.0826	0.9069	1.8270	8.6725	1.4602	0.0100	0.5494	0.5972

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5681	1.0223	3.2976	0.5991	0.0243
Ru5682	0.8370	3.5897	0.6098	0.0304
Ru5683	0.7104	3.6184	0.5678	0.0254
Ru5684	0.7738	3.4003	0.5600	0.0259
Ru5685	0.7342	3.5766	0.7689	0.0195
Ru5686	0.7088	2.2057	0.5395	0.0235

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5687	328.87	0.0809	1.9082	1.1551	1.0471	2.1812	10.7251	1.5840	0.0098	0.5440	0.7163
Ru5688	325.87	0.0823	2.1737	1.0085	1.0322	2.1138	10.3985	1.5686	0.0119	0.4799	0.7530
Ru5689	310.49	0.0978	2.0146	1.0950	0.9366	1.8457	9.6402	1.5773	0.0108	0.5950	0.5584
Ru5690	297.90	0.0624	2.0737	0.9791	0.8792	1.7151	9.4209	1.5358	0.0090	0.6566	0.4142
Ru5691	351.65	0.0862	2.1001	1.1874	1.0260	2.4067	10.9968	1.6289	0.0120	0.7869	1.1149
Ru5692	283.03	0.0792	2.0793	0.9584	0.8491	1.9096	9.8296	1.3624	0.0091	0.5622	0.5375

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5687	0.6800	3.4281	0.6644	0.0322
Ru5688	0.8456	3.5292	0.7656	0.0252
Ru5689	0.6739	3.8069	0.6212	0.0196
Ru5690	0.5863	3.3671	0.5395	0.0194
Ru5691	0.8354	3.6476	1.0282	0.0233
Ru5692	0.4933	3.2540	0.4446	0.0180

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5693	277.17	0.0637	1.9668	1.0477	0.8358	1.6998	8.0750	1.3447	0.0092	0.4893	0.7102
Ru5694	327.60	0.0843	2.0987	1.2483	0.9600	1.9223	9.7334	1.6296	0.0096	0.5822	0.8871
Ru5695	294.81	0.0721	2.0088	0.9271	0.8970	1.8273	8.9007	1.5827	0.0096	0.5049	0.7538
Ru5696	321.48	0.0681	2.1301	0.9725	1.0157	1.9166	10.2102	1.5444	0.0097	0.4228	0.6858
Ru5697	321.06	0.0633	1.9985	1.0454	0.9945	2.2250	10.3881	1.5238	0.0106	0.6386	0.7970
Ru5698	340.12	0.0630	2.0553	1.0744	1.0670	2.1256	9.5427	1.4595	0.0107	0.5924	1.0111

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5693	0.6683	3.2759	0.4856	0.0237
Ru5694	0.9766	3.8631	0.6243	0.0237
Ru5695	0.6335	3.3079	0.6014	0.0181
Ru5696	0.7199	2.8590	0.7122	0.0181
Ru5697	0.8301	3.6015	0.5070	0.0209
Ru5698	0.7362	3.5648	0.4885	0.0218

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5699	304.49	0.0672	2.0772	1.0391	1.0556	2.0578	10.3857	1.5064	0.0112	0.5849	0.7130
Ru5700	337.62	0.0799	2.0279	1.0013	1.0512	2.2177	11.4621	1.6523	0.0105	0.5574	0.9004
Ru5701	282.48	0.0769	2.0450	0.8909	0.8643	1.9561	8.4827	1.4591	0.0108	0.4804	0.5244
Ru5702	314.24	0.0703	2.1087	1.0578	0.9804	2.0790	9.4858	1.6767	0.0098	0.7664	0.7178
Ru5703	340.72	0.0769	2.1451	1.1364	1.0292	2.2015	10.1052	1.6026	0.0107	0.4018	0.8602
Ru5704	298.46	0.0678	1.8696	1.0052	0.8487	1.7578	8.3525	1.6811	0.0085	0.4325	0.6273

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5699	0.8740	3.2880	0.7300	0.0277
Ru5700	0.9146	3.3119	0.9893	0.0244
Ru5701	0.4888	3.2437	0.5637	0.0252
Ru5702	0.7405	3.4570	0.6195	0.0265
Ru5703	0.8077	3.6986	0.6323	0.0245
Ru5704	0.5594	3.2885	0.5940	0.0270

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5705	268.51	0.0677	2.0379	1.1537	0.9287	1.9689	7.1108	1.4481	0.0117	0.6228	0.5962
Ru5706	301.28	0.0856	2.0246	1.0054	1.0077	1.9551	7.5451	1.4628	0.0069	0.5745	0.6712
Ru5707	287.12	0.0663	2.0403	1.0186	0.8994	1.8609	6.3650	1.4093	0.0118	0.3484	0.5031
Ru5708	288.67	0.0633	1.9647	0.7075	0.8157	1.7671	7.5106	1.3521	0.0108	0.5030	0.6825
Ru5709	302.45	0.0652	2.0841	1.0319	0.8697	1.9476	8.0900	1.8446	0.0095	0.6602	0.6545
Ru5710	314.11	0.0697	2.0174	1.2634	1.0317	2.1674	7.0980	1.6795	0.0106	0.5942	0.7026

*: Weighed after fixation

APPENDIX 7 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5705	0.7588	3.2540	0.5659	0.0257
Ru5706	0.7172	3.3508	0.5493	0.0302
Ru5707	0.7066	3.1496	0.5283	0.0209
Ru5708	0.5603	0.8978	0.5878	0.0229
Ru5709	0.6979	3.2876	0.5976	0.0168
Ru5710	0.6166	3.8908	0.4091	0.0189

*: Weighed after fixation

APPENDIX 8. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5681	340.14	0.0226	0.6079	0.3508	0.3115	0.6075	3.3105	0.4457	0.0025	0.1811	0.3105	0.3006	0.9695	0.1761
Ru5682	306.96	0.0267	0.6795	0.3325	0.3538	0.7114	2.9139	0.4965	0.0031	0.1448	0.2290	0.2727	1.1694	0.1987
Ru5683	287.20	0.0278	0.7470	0.3696	0.3464	0.6610	3.4268	0.5016	0.0037	0.1982	0.2509	0.2474	1.2599	0.1977
Ru5684	329.45	0.0251	0.6245	0.3451	0.2842	0.6216	3.0168	0.5022	0.0029	0.2192	0.3066	0.2349	1.0321	0.1700
Ru5685	325.84	0.0230	0.6919	0.3036	0.3102	0.5846	3.0547	0.4449	0.0038	0.1353	0.2187	0.2253	1.0977	0.2360
Ru5686	300.07	0.0276	0.7230	0.3608	0.3022	0.6089	2.8902	0.4866	0.0033	0.1831	0.1990	0.2362	0.7351	0.1798

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5681	0.0071
Ru5682	0.0099
Ru5683	0.0088
Ru5684	0.0079
Ru5685	0.0060
Ru5686	0.0078

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5687	328.87	0.0246	0.5802	0.3512	0.3184	0.6632	3.2612	0.4816	0.0030	0.1654	0.2178	0.2068	1.0424	0.2020
Ru5688	325.87	0.0253	0.6670	0.3095	0.3168	0.6487	3.1910	0.4814	0.0037	0.1473	0.2311	0.2595	1.0830	0.2349
Ru5689	310.49	0.0315	0.6488	0.3527	0.3017	0.5944	3.1048	0.5080	0.0035	0.1916	0.1798	0.2170	1.2261	0.2001
Ru5690	297.90	0.0209	0.6961	0.3287	0.2951	0.5757	3.1624	0.5155	0.0030	0.2204	0.1390	0.1968	1.1303	0.1811
Ru5691	351.65	0.0245	0.5972	0.3377	0.2918	0.6844	3.1272	0.4632	0.0034	0.2238	0.3170	0.2376	1.0373	0.2924
Ru5692	283.03	0.0280	0.7347	0.3386	0.3000	0.6747	3.4730	0.4814	0.0032	0.1986	0.1899	0.1743	1.1497	0.1571

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5687	0.0098
Ru5688	0.0077
Ru5689	0.0063
Ru5690	0.0065
Ru5691	0.0066
Ru5692	0.0064

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5693	277.17	0.0230	0.7096	0.3780	0.3015	0.6133	2.9134	0.4852	0.0033	0.1765	0.2562	0.2411	1.1819	0.1752
Ru5694	327.60	0.0257	0.6406	0.3810	0.2930	0.5868	2.9711	0.4974	0.0029	0.1777	0.2708	0.2981	1.1792	0.1906
Ru5695	294.81	0.0245	0.6814	0.3145	0.3043	0.6198	3.0191	0.5369	0.0033	0.1713	0.2557	0.2149	1.1220	0.2040
Ru5696	321.48	0.0212	0.6626	0.3025	0.3159	0.5962	3.1760	0.4804	0.0030	0.1315	0.2133	0.2239	0.8893	0.2215
Ru5697	321.06	0.0197	0.6225	0.3256	0.3098	0.6930	3.2356	0.4746	0.0033	0.1989	0.2482	0.2585	1.1218	0.1579
Ru5698	340.12	0.0185	0.6043	0.3159	0.3137	0.6250	2.8057	0.4291	0.0031	0.1742	0.2973	0.2165	1.0481	0.1436

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5693	0.0086
Ru5694	0.0072
Ru5695	0.0061
Ru5696	0.0056
Ru5697	0.0065
Ru5698	0.0064

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5699	304.49	0.0221	0.6822	0.3413	0.3467	0.6758	3.4109	0.4947	0.0037	0.1921	0.2342	0.2870	1.0798	0.2397
Ru5700	337.62	0.0237	0.6006	0.2966	0.3114	0.6569	3.3950	0.4894	0.0031	0.1651	0.2667	0.2709	0.9810	0.2930
Ru5701	282.48	0.0272	0.7239	0.3154	0.3060	0.6925	3.0029	0.5165	0.0038	0.1701	0.1856	0.1730	1.1483	0.1996
Ru5702	314.24	0.0224	0.6710	0.3366	0.3120	0.6616	3.0186	0.5336	0.0031	0.2439	0.2284	0.2356	1.1001	0.1971
Ru5703	340.72	0.0226	0.6296	0.3335	0.3021	0.6461	2.9658	0.4704	0.0031	0.1179	0.2525	0.2371	1.0855	0.1856
Ru5704	298.46	0.0227	0.6264	0.3368	0.2844	0.5890	2.7985	0.5633	0.0028	0.1449	0.2102	0.1874	1.1018	0.1990

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5699	0.0091
Ru5700	0.0072
Ru5701	0.0089
Ru5702	0.0084
Ru5703	0.0072
Ru5704	0.0090

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5705	268.51	0.0252	0.7590	0.4297	0.3459	0.7333	2.6482	0.5393	0.0044	0.2319	0.2220	0.2826	1.2119	0.2108
Ru5706	301.28	0.0284	0.6720	0.3337	0.3345	0.6489	2.5043	0.4855	0.0023	0.1907	0.2228	0.2381	1.1122	0.1823
Ru5707	287.12	0.0231	0.7106	0.3548	0.3132	0.6481	2.2168	0.4908	0.0041	0.1213	0.1752	0.2461	1.0970	0.1840
Ru5708	288.67	0.0219	0.6806	0.2451	0.2826	0.6122	2.6018	0.4684	0.0037	0.1742	0.2364	0.1941	0.3110	0.2036
Ru5709	302.45	0.0216	0.6891	0.3412	0.2876	0.6439	2.6748	0.6099	0.0031	0.2183	0.2164	0.2307	1.0870	0.1976
Ru5710	314.11	0.0222	0.6423	0.4022	0.3285	0.6900	2.2597	0.5347	0.0034	0.1892	0.2237	0.1963	1.2387	0.1302

*: Weighed after fixation

APPENDIX 8 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5705	0.0096
Ru5706	0.0100
Ru5707	0.0073
Ru5708	0.0079
Ru5709	0.0056
Ru5710	0.0060

*: Weighed after fixation

APPENDIX 9. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5681	2.0676	3.7241	57.7046	51.2478	99.9468	544.6073	73.3169	0.4111	29.7930	51.0737	49.4438
Ru5682	2.0858	3.9266	48.9261	52.0664	104.6888	428.8331	73.0703	0.4555	21.3060	33.6993	40.1285
Ru5683	2.1455	3.7148	49.4710	46.3668	88.4782	458.7229	67.1452	0.4987	26.5299	33.5819	33.1112
Ru5684	2.0575	4.0146	55.2564	45.5067	99.5286	483.0474	80.4083	0.4666	35.1009	49.0984	37.6087
Ru5685	2.2544	3.3313	43.8831	44.8323	84.4970	441.5144	64.2965	0.5456	19.5529	31.6093	32.5674
Ru5686	2.1696	3.8118	49.8986	41.8003	84.2091	399.7281	67.3027	0.4609	25.3226	27.5258	32.6696

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G1A 0 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5681	159.4893	28.9756	1.1753
Ru5682	172.1018	29.2358	1.4575
Ru5683	168.6507	26.4647	1.1839
Ru5684	165.2637	27.2175	1.2588
Ru5685	158.6498	34.1066	0.8650
Ru5686	101.6639	24.8663	1.0831

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5687	1.9082	4.2396	60.5335	54.8737	114.3067	562.0532	83.0102	0.5136	28.5085	37.5380	35.6357
Ru5688	2.1737	3.7862	46.3955	47.4859	97.2443	478.3779	72.1627	0.5475	22.0776	34.6414	38.9014
Ru5689	2.0146	4.8546	54.3532	46.4906	91.6162	478.5168	78.2935	0.5361	29.5344	27.7177	33.4508
Ru5690	2.0737	3.0091	47.2151	42.3976	82.7072	454.3039	74.0609	0.4340	31.6632	19.9740	28.2731
Ru5691	2.1001	4.1046	56.5402	48.8548	114.5993	523.6322	77.5630	0.5714	37.4696	53.0879	39.7791
Ru5692	2.0793	3.8090	46.0924	40.8359	91.8386	472.7360	65.5221	0.4376	27.0379	25.8500	23.7243

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G2A 30 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5687	179.6510	34.8182	1.6875
Ru5688	162.3591	35.2211	1.1593
Ru5689	188.9656	30.8349	0.9729
Ru5690	162.3716	26.0163	0.9355
Ru5691	173.6870	48.9596	1.1095
Ru5692	156.4950	21.3822	0.8657

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5693	1.9668	3.2388	53.2693	42.4954	86.4246	410.5654	68.3699	0.4678	24.8780	36.1094	33.9791
Ru5694	2.0987	4.0168	59.4797	45.7426	91.5948	463.7823	77.6481	0.4574	27.7410	42.2690	46.5336
Ru5695	2.0088	3.5892	46.1519	44.6535	90.9648	443.0854	78.7883	0.4779	25.1344	37.5249	31.5362
Ru5696	2.1301	3.1970	45.6551	47.6832	89.9770	479.3296	72.5036	0.4554	19.8488	32.1957	33.7965
Ru5697	1.9985	3.1674	52.3092	49.7623	111.3335	519.7948	76.2472	0.5304	31.9540	39.8799	41.5362
Ru5698	2.0553	3.0652	52.2746	51.9146	103.4204	464.2972	71.0115	0.5206	28.8230	49.1948	35.8196

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G3A 100 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5693	166.5599	24.6899	1.2050
Ru5694	184.0711	29.7470	1.1293
Ru5695	164.6705	29.9383	0.9010
Ru5696	134.2191	33.4350	0.8497
Ru5697	180.2102	25.3690	1.0458
Ru5698	173.4443	23.7678	1.0607

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5699	2.0772	3.2351	50.0241	50.8184	99.0661	499.9856	72.5207	0.5392	28.1581	34.3251	42.0759
Ru5700	2.0279	3.9400	49.3762	51.8369	109.3594	565.2202	81.4784	0.5178	27.4866	44.4006	45.1008
Ru5701	2.0450	3.7604	43.5648	42.2641	95.6528	414.8020	71.3496	0.5281	23.4914	25.6430	23.9022
Ru5702	2.1087	3.3338	50.1636	46.4931	98.5915	449.8411	79.5134	0.4647	36.3447	34.0399	35.1164
Ru5703	2.1451	3.5849	52.9766	47.9791	102.6292	471.0829	74.7098	0.4988	18.7311	40.1007	37.6533
Ru5704	1.8696	3.6264	53.7655	45.3947	94.0201	446.7533	89.9176	0.4546	23.1333	33.5526	29.9208

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G4A 300 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5699	158.2900	35.1435	1.3335
Ru5700	163.3167	48.7845	1.2032
Ru5701	158.6161	27.5648	1.2323
Ru5702	163.9399	29.3783	1.2567
Ru5703	172.4209	29.4765	1.1421
Ru5704	175.8932	31.7715	1.4442

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5705	2.0379	3.3220	56.6122	45.5714	96.6142	348.9278	71.0584	0.5741	30.5609	29.2556	37.2344
Ru5706	2.0246	4.2280	49.6592	49.7728	96.5672	372.6711	72.2513	0.3408	28.3760	33.1522	35.4243
Ru5707	2.0403	3.2495	49.9240	44.0818	91.2072	311.9639	69.0732	0.5783	17.0759	24.6581	34.6322
Ru5708	1.9647	3.2219	36.0106	41.5178	89.9425	382.2772	68.8197	0.5497	25.6019	34.7381	28.5183
Ru5709	2.0841	3.1284	49.5130	41.7302	93.4504	388.1772	88.5082	0.4558	31.6779	31.4044	33.4869
Ru5710	2.0174	3.4549	62.6252	51.1401	107.4353	351.8390	83.2507	0.5254	29.4538	34.8270	30.5641

*: Weighed after fixation

APPENDIX 9 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide Groups

G5A 1000 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5705	159.6742	27.7688	1.2611
Ru5706	165.5043	27.1313	1.4917
Ru5707	154.3695	25.8933	1.0244
Ru5708	45.6965	29.9181	1.1656
Ru5709	157.7467	28.6742	0.8061
Ru5710	192.8621	20.2786	0.9368

*: Weighed after fixation

APPENDIX 10. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5711	285.70	0.0783	2.0318	1.0516	0.8614	2.0751	9.0510	1.4636	0.0090	0.6890	0.7878
Ru5712	317.08	0.0829	2.0897	0.9868	0.9966	2.0788	10.0642	1.5753	0.0095	0.4202	0.4911
Ru5713	279.42	0.0619	2.0229	0.9907	0.8752	2.0285	8.5925	1.4619	0.0084	0.5401	0.8898
Ru5714	333.10	0.0854	1.9178	1.1730	1.0311	2.2010	11.5552	1.5603	0.0101	0.7425	0.8486
Ru5715	318.83	0.0958	1.9873	0.9530	0.9286	1.9685	10.4265	1.5079	0.0105	0.5457	0.8600
Ru5716	311.77	0.0832	2.1077	1.1036	0.9579	2.0733	9.3051	1.6990	0.0103	0.5050	0.8023

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5711	0.7077	3.5057	0.4321	0.0216
Ru5712	0.7962	3.2555	1.0863	0.0276
Ru5713	0.4979	3.1116	0.6192	0.0271
Ru5714	0.7155	3.5413	0.6742	0.0261
Ru5715	0.5819	3.4078	0.5228	0.0222
Ru5716	0.5365	3.5144	0.4185	0.0209

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5717	324.92	0.0829	2.1529	1.1464	0.9452	2.3723	10.4339	1.5882	0.0101	0.5869	0.9940
Ru5718	310.90	0.0688	2.1050	1.0403	1.0499	1.9786	9.6018	1.6620	0.0101	0.4504	0.4932
Ru5719	296.61	0.0721	2.0502	0.9909	0.9215	1.8208	8.9236	1.5470	0.0090	0.5722	0.7974
Ru5720	317.92	0.0763	2.0340	1.2081	0.9433	2.0385	10.3154	1.6236	0.0111	0.6154	0.8280
Ru5721	335.97	0.0740	2.0750	1.0006	1.0430	2.0417	9.9948	1.4889	0.0096	0.4589	0.6304
Ru5722	308.32	0.0956	2.0528	1.0794	0.8707	1.9345	10.2148	1.5711	0.0100	0.6137	0.8726

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5717	0.7446	3.9323	0.5444	0.0220
Ru5718	0.7622	3.7179	0.5166	0.0228
Ru5719	0.5506	3.6021	0.5080	0.0254
Ru5720	0.6883	3.1693	0.7474	0.0249
Ru5721	0.6764	3.2935	0.8234	0.0304
Ru5722	0.4925	3.7023	0.4413	0.0290

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5723	336.76	0.0843	2.0757	1.1415	0.9581	2.3917	10.0045	1.5389	0.0096	0.7894	0.8382
Ru5724	298.83	0.0812	1.9590	1.0477	0.9323	1.9720	9.4067	1.4864	0.0102	0.5676	0.8275
Ru5725	309.98	0.0718	2.0453	1.1538	0.9004	1.9870	9.5901	1.6156	0.0101	0.4960	0.6207
Ru5726	283.08	0.0692	1.9118	0.9286	0.9333	1.8436	9.1587	1.5692	0.0090	0.5426	0.4305
Ru5727	335.98	0.0777	2.0954	0.6245	0.9424	2.0891	10.5722	1.7857	0.0105	0.5088	0.4947
Ru5728	310.67	0.0573	2.1227	1.0159	0.8654	1.8876	10.3817	1.5991	0.0089	0.3311	0.5984

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5723	0.8062	3.5326	0.5700	0.0196
Ru5724	0.6457	3.5931	0.3946	0.0218
Ru5725	0.6801	3.7755	0.4643	0.0262
Ru5726	0.8567	3.4784	0.5138	0.0226
Ru5727	0.6846	2.2940	0.7281	0.0230
Ru5728	0.6704	3.8203	0.5478	0.0215

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5729	324.34	0.0700	2.0590	0.9964	1.0545	1.9111	10.3951	1.4362	0.0089	0.3880	0.9779
Ru5730	276.43	0.0569	1.9433	0.9228	0.8097	1.7789	8.8150	1.5379	0.0079	0.4457	0.7067
Ru5731	305.91	0.0776	2.0120	0.9846	0.8784	2.0506	9.6215	1.3968	0.0103	0.5215	0.5928
Ru5732	347.42	0.0814	2.2276	0.9843	1.1261	2.3174	10.0172	1.7360	0.0130	0.5502	0.8566
Ru5733	289.11	0.0677	1.9331	1.0911	0.8730	2.1759	9.5422	1.5702	0.0094	0.6648	0.8281
Ru5734	312.49	0.0656	1.9804	1.0244	0.9805	2.3087	10.0851	1.5684	0.0108	0.7363	0.8896

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5729	0.7096	3.1366	0.7970	0.0230
Ru5730	0.5651			
				iffer

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Terminal Fasting BW (g)	Adrenals (g)	Brain (g)	Epididymides (g)	Heart (g)	Kidneys (g)	Liver (g)	Lungs (g)	Pituitary (g)	Prostate (g)	Seminal vesicles & coagulating glands (g)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8
Ru5735	299.53	0.0790	2.0283	1.1027	0.8937	2.0548	8.1282	1.4830	0.0099	0.5669	0.6532
Ru5736	291.42	0.0692	1.9576	1.0286	0.9507	2.1524	8.3862	1.5352	0.0107	0.5736	0.7217
Ru5737	277.15	0.0756	2.1077	1.0723	0.9339	2.1006	8.0698	1.4737	0.0077	0.3088	0.3286
Ru5738	321.36	0.0656	2.0245	0.9845	0.9185	1.9319	9.1170	1.5978	0.0091	0.5749	0.4743
Ru5739	311.61	0.0641	2.1433	0.9677	0.9466	2.3088	9.2421	1.4152	0.0082	0.4189	0.5212
Ru5740	258.53	0.0649	2.0668	0.9443	0.8329	1.9155	7.4174	1.3936	0.0079	0.3331	0.4654

*: Weighed after fixation

APPENDIX 10 contd. Individual Animal Terminal Fasting Body Weights and Organ Weights on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Spleen (g)	Testes (g)	Thymus (g)	Thyroid with parathyroids (g)
	Day 8	Day 8	Day 8	Day 9*
Ru5735	0.5584	3.8165	0.4318	0.0217
Ru5736	0.5636	3.3528	0.5329	0.0214
Ru5737	0.6306	3.7220	0.5445	0.0241
Ru5738	0.6371	3.5152	0.6290	0.0217
Ru5739	0.7037	3.5147	0.4869	0.0279
Ru5740	0.5456	3.5534	0.5944	0.0249

*: Weighed after fixation

APPENDIX 11. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5711	285.70	0.0274	0.7112	0.3681	0.3015	0.7263	3.1680	0.5123	0.0032	0.2412	0.2757	0.2477	1.2271	0.1512
Ru5712	317.08	0.0261	0.6590	0.3112	0.3143	0.6556	3.1740	0.4968	0.0030	0.1325	0.1549	0.2511	1.0267	0.3426
Ru5713	279.42	0.0222	0.7240	0.3546	0.3132	0.7260	3.0751	0.5232	0.0030	0.1933	0.3184	0.1782	1.1136	0.2216
Ru5714	333.10	0.0256	0.5757	0.3521	0.3095	0.6608	3.4690	0.4684	0.0030	0.2229	0.2548	0.2148	1.0631	0.2024
Ru5715	318.83	0.0300	0.6233	0.2989	0.2913	0.6174	3.2702	0.4729	0.0033	0.1712	0.2697	0.1825	1.0688	0.1640
Ru5716	311.77	0.0267	0.6760	0.3540	0.3072	0.6650	2.9846	0.5450	0.0033	0.1620	0.2573	0.1721	1.1272	0.1342

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5711	0.0076
Ru5712	0.0087
Ru5713	0.0097
Ru5714	0.0078
Ru5715	0.0070
Ru5716	0.0067

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5717	324.92	0.0255	0.6626	0.3528	0.2909	0.7301	3.2112	0.4888	0.0031	0.1806	0.3059	0.2292	1.2102	0.1675
Ru5718	310.90	0.0221	0.6771	0.3346	0.3377	0.6364	3.0884	0.5346	0.0032	0.1449	0.1586	0.2452	1.1959	0.1662
Ru5719	296.61	0.0243	0.6912	0.3341	0.3107	0.6139	3.0085	0.5216	0.0030	0.1929	0.2688	0.1856	1.2144	0.1713
Ru5720	317.92	0.0240	0.6398	0.3800	0.2967	0.6412	3.2447	0.5107	0.0035	0.1936	0.2604	0.2165	0.9969	0.2351
Ru5721	335.97	0.0220	0.6176	0.2978	0.3104	0.6077	2.9749	0.4432	0.0029	0.1366	0.1876	0.2013	0.9803	0.2451
Ru5722	308.32	0.0310	0.6658	0.3501	0.2824	0.6274	3.3131	0.5096	0.0032	0.1990	0.2830	0.1597	1.2008	0.1431

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5717	0.0068
Ru5718	0.0073
Ru5719	0.0086
Ru5720	0.0078
Ru5721	0.0090
Ru5722	0.0094

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5723	336.76	0.0250	0.6164	0.3390	0.2845	0.7102	2.9708	0.4570	0.0029	0.2344	0.2489	0.2394	1.0490	0.1693
Ru5724	298.83	0.0272	0.6556	0.3506	0.3120	0.6599	3.1478	0.4974	0.0034	0.1899	0.2769	0.2161	1.2024	0.1320
Ru5725	309.98	0.0232	0.6598	0.3722	0.2905	0.6410	3.0938	0.5212	0.0033	0.1600	0.2002	0.2194	1.2180	0.1498
Ru5726	283.08	0.0244	0.6754	0.3280	0.3297	0.6513	3.2354	0.5543	0.0032	0.1917	0.1521	0.3026	1.2288	0.1815
Ru5727	335.98	0.0231	0.6237	0.1859	0.2805	0.6218	3.1467	0.5315	0.0031	0.1514	0.1472	0.2038	0.6828	0.2167
Ru5728	310.67	0.0184	0.6833	0.3270	0.2786	0.6076	3.3417	0.5147	0.0029	0.1066	0.1926	0.2158	1.2297	0.1763

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5723	0.0058
Ru5724	0.0073
Ru5725	0.0085
Ru5726	0.0080
Ru5727	0.0068
Ru5728	0.0069

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5729	324.34	0.0216	0.6348	0.3072	0.3251	0.5892	3.2050	0.4428	0.0027	0.1196	0.3015	0.2188	0.9671	0.2457
Ru5730	276.43	0.0206	0.7030	0.3338	0.2929	0.6435	3.1889	0.5563	0.0029	0.1612	0.2557	0.2044	1.1474	0.2064
Ru5731	305.91	0.0254	0.6577	0.3219	0.2871	0.6703	3.1452	0.4566	0.0034	0.1705	0.1938	0.1709	1.0738	0.2042
Ru5732	347.42	0.0234	0.6412	0.2833	0.3241	0.6670	2.8833	0.4997	0.0037	0.1584	0.2466	0.2729	0.9733	0.2040
Ru5733	289.11	0.0234	0.6686	0.3774	0.3020	0.7526	3.3005	0.5431	0.0033	0.2299	0.2864	0.2419	1.1728	0.1853
Ru5734	312.49	0.0210	0.6337	0.3278	0.3138	0.7388	3.2273	0.5019	0.0035	0.2356	0.2847	0.2282	1.0481	0.1760

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5729	0.0071
Ru5730	0.0089
Ru5731	0.0096
Ru5732	0.0074
Ru5733	0.0090
Ru5734	0.0071

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Terminal Fasting BW (g)	Adrenals (%)	Brain. (%)	Epididymi-des (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal & coagu (%)	Spleen (%)	Testes (%)	Thymus (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8	Day 8	Day 8
Ru5735	299.53	0.0264	0.6772	0.3681	0.2984	0.6860	2.7137	0.4951	0.0033	0.1893	0.2181	0.1864	1.2742	0.1442
Ru5736	291.42	0.0237	0.6717	0.3530	0.3262	0.7386	2.8777	0.5268	0.0037	0.1968	0.2476	0.1934	1.1505	0.1829
Ru5737	277.15	0.0273	0.7605	0.3869	0.3370	0.7579	2.9117	0.5317	0.0028	0.1114	0.1186	0.2275	1.3430	0.1965
Ru5738	321.36	0.0204	0.6300	0.3064	0.2858	0.6012	2.8370	0.4972	0.0028	0.1789	0.1476	0.1983	1.0939	0.1957
Ru5739	311.61	0.0206	0.6878	0.3105	0.3038	0.7409	2.9659	0.4542	0.0026	0.1344	0.1673	0.2258	1.1279	0.1563
Ru5740	258.53	0.0251	0.7994	0.3653	0.3222	0.7409	2.8691	0.5390	0.0031	0.1288	0.1800	0.2110	1.3745	0.2299

*: Weighed after fixation

APPENDIX 11 contd. Individual Animal Terminal Fasting Body Weights and Organ to Body Weight Ratio on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Thyroid with parathyroids (%)
	Day 9*
Ru5735	0.0072
Ru5736	0.0073
Ru5737	0.0087
Ru5738	0.0068
Ru5739	0.0090
Ru5740	0.0096

*: Weighed after fixation

APPENDIX 12. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5711	2.0318	3.8537	51.7571	42.3959	102.1311	445.4671	72.0346	0.4430	33.9108	38.7735	34.8312
Ru5712	2.0897	3.9671	47.2221	47.6911	99.4784	481.6098	75.3840	0.4546	20.1081	23.5010	38.1012
Ru5713	2.0229	3.0600	48.9742	43.2646	100.2768	424.7615	72.2675	0.4152	26.6993	43.9864	24.6132
Ru5714	1.9178	4.4530	61.1638	53.7647	114.7669	602.5237	81.3588	0.5266	38.7162	44.2486	37.3084
Ru5715	1.9873	4.8206	47.9545	46.7267	99.0540	524.6566	75.8768	0.5284	27.4594	43.2748	29.2809
Ru5716	2.1077	3.9474	52.3604	45.4476	98.3679	441.4812	80.6092	0.4887	23.9598	38.0652	25.4543

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G1B 0 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5711	172.5416	21.2669	1.0631
Ru5712	155.7879	51.9835	1.3208
Ru5713	153.8188	30.6095	1.3397
Ru5714	184.6543	35.1549	1.3609
Ru5715	171.4789	26.3070	1.1171
Ru5716	166.7410	19.8558	0.9916

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5717	2.1529	3.8506	53.2491	43.9036	110.1909	484.6440	73.7703	0.4691	27.2609	46.1703	34.5859
Ru5718	2.1050	3.2684	49.4204	49.8765	93.9952	456.1425	78.9549	0.4798	21.3967	23.4299	36.2090
Ru5719	2.0502	3.5167	48.3319	44.9468	88.8108	435.2551	75.4561	0.4390	27.9095	38.8938	26.8559
Ru5720	2.0340	3.7512	59.3953	46.3766	100.2212	507.1485	79.8230	0.5457	30.2557	40.7080	33.8397
Ru5721	2.0750	3.5663	48.2217	50.2651	98.3952	481.6771	71.7542	0.4627	22.1157	30.3807	32.5976
Ru5722	2.0528	4.6571	52.5818	42.4152	94.2371	497.6033	76.5345	0.4871	29.8958	42.5078	23.9916

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G2B 30 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5717	182.6513	25.2868	1.0219
Ru5718	176.6223	24.5416	1.0831
Ru5719	175.6951	24.7781	1.2389
Ru5720	155.8161	36.7453	1.2242
Ru5721	158.7229	39.6819	1.4651
Ru5722	180.3537	21.4975	1.4127

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5723	2.0757	4.0613	54.9935	46.1579	115.2238	481.9820	74.1388	0.4625	38.0305	40.3816	38.8399
Ru5724	1.9590	4.1450	53.4814	47.5906	100.6636	480.1787	75.8754	0.5207	28.9740	42.2409	32.9607
Ru5725	2.0453	3.5105	56.4123	44.0229	97.1496	468.8848	78.9909	0.4938	24.2507	30.3476	33.2518
Ru5726	1.9118	3.6196	48.5720	48.8179	96.4327	479.0616	82.0797	0.4708	28.3816	22.5180	44.8112
Ru5727	2.0954	3.7081	29.8034	44.9747	99.6993	504.5433	85.2200	0.5011	24.2818	23.6089	32.6716
Ru5728	2.1227	2.6994	47.8589	40.7688	88.9245	489.0799	75.3333	0.4193	15.5981	28.1905	31.5824

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G3B 100 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5723	170.1884	27.4606	0.9443
Ru5724	183.4150	20.1429	1.1128
Ru5725	184.5939	22.7008	1.2810
Ru5726	181.9437	26.8752	1.1821
Ru5727	109.4779	34.7475	1.0976
Ru5728	179.9736	25.8068	1.0129

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5729	2.0590	3.3997	48.3924	51.2142	92.8169	504.8616	69.7523	0.4322	18.8441	47.4939	34.4633
Ru5730	1.9433	2.9280	47.4862	41.6662	91.5402	453.6098	79.1386	0.4065	22.9352	36.3660	29.0794
Ru5731	2.0120	3.8569	48.9364	43.6581	101.9185	478.2058	69.4235	0.5119	25.9195	29.4632	25.9791
Ru5732	2.2276	3.6542	44.1866	50.5522	104.0312	449.6858	77.9314	0.5836	24.6992	38.4539	42.5660
Ru5733	1.9331	3.5021	56.4430	45.1606	112.5601	493.6216	81.2270	0.4863	34.3904	42.8379	36.1751
Ru5734	1.9804	3.3125	51.7269	49.5102	116.5775	509.2456	79.1961	0.5453	37.1794	44.9202	36.0129

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G4B 300 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5729	152.3361	38.7081	1.1170
Ru5730	163.2121	29.3573	1.2607
Ru5731	163.2654	31.0487	1.4563
Ru5732	151.7912	31.8145	1.1537
Ru5733	175.4074	27.7120	1.3398
Ru5734	165.3757	27.7722	1.1210

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Brain (g)	Adrenals (%)	Epididymides (%)	Heart (%)	Kidneys (%)	Liver (%)	Lungs (%)	Pituitary (%)	Prostate (%)	Seminal vesicles & coagulating glands (%)	Spleen (%)
	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 8	Day 9*	Day 8	Day 8	Day 8
Ru5735	2.0283	3.8949	54.3657	44.0615	101.3065	400.7395	73.1154	0.4881	27.9495	32.2043	27.5304
Ru5736	1.9576	3.5349	52.5439	48.5646	109.9510	428.3919	78.4226	0.5466	29.3012	36.8666	28.7904
Ru5737	2.1077	3.5868	50.8754	44.3090	99.6631	382.8723	69.9198	0.3653	14.6510	15.5905	29.9189
Ru5738	2.0245	3.2403	48.6293	45.3692	95.4260	450.3334	78.9232	0.4495	28.3971	23.4280	31.4695
Ru5739	2.1433	2.9907	45.1500	44.1655	107.7217	431.2089	66.0290	0.3826	19.5446	24.3176	32.8325
Ru5740	2.0668	3.1401	45.6890	40.2990	92.6795	358.8833	67.4279	0.3822	16.1167	22.5179	26.3983

*: Weighed after fixation

APPENDIX 12 contd. Individual Animal Brain Weights and Organ to Brain Weight Ratio on Day 8 – Acetamide + BrdU Groups

G5B 1000 mg/kg/day	Testes (%)	Thymus (%)	Thyroid with parathyroids (%)
	Day 8	Day 8	Day 9*
Ru5735	188.1625	21.2888	1.0699
Ru5736	171.2709	27.2221	1.0932
Ru5737	176.5906	25.8338	1.1434
Ru5738	173.6330	31.0694	1.0719
Ru5739	163.9854	22.7173	1.3017
Ru5740	171.9276	28.7594	1.2048

*: Weighed after fixation

APPENDIX 13. Individual Animal Gross Pathology Findings – Acetamide Groups

Group No.: G1A Dose: 0 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5681	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5682	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5683	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5684	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5685	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5686	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

Group No.: G2A Dose: 30 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5687	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5688	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5689	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5690	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5691	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5692	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 13 contd. Individual Animal Gross Pathology Findings – Acetamide Groups

Group No.: G3A Dose: 100 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5693	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5694	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5695	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5696	TERMINAL SACRIFICE	8	(2)	EPIDIDYMIDES; Small; unilateral:3.1 cm: TESTES; Small; unilateral:1.6 cm: Any remaining protocol required tissues, which have been examined, have no visible lesions
Ru5697	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5698	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 13 contd. Individual Animal Gross Pathology Findings – Acetamide Groups

Group No.: G4A Dose: 300 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	(Week)	Observation(s)
Ru5699	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5700	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5701	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5702	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5703	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5704	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 13 contd. Individual Animal Gross Pathology Findings – Acetamide Groups

Group No.: G5A Dose: 1000 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5705	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5706	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5707	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5708	TERMINAL SACRIFICE	8	(2)	EPIDIDYMIDES; Small; bilateral:3.3-3.4 cm: TESTES; Small; bilateral:1.3-1.4 cm: Any remaining protocol required tissues, which have been examined, have no visible lesions
Ru5709	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5710	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 14. Individual Animal Gross Pathology Findings – Acetamide + BrdU Groups

Group No.: G1B Dose: 0 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5711	TERMINAL SACRIFICE	8	(2)	LIVER; right lateral lobe; Focus (i); white:0.9 cm: Any remaining protocol required tissues, which have been examined, have no visible lesions
Ru5712	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5713	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5714	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5715	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5716	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 14 contd. Individual Animal Gross Pathology Findings – Acetamide + BrdU Groups

Group No.: G2B Dose: 30 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	(Week)	Observation(s)
Ru5717	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5718	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5719	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5720	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5721	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5722	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 14 contd. Individual Animal Gross Pathology Findings – Acetamide + BrdU Groups

Group No.: G3B Dose: 100 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5723	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5724	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5725	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5726	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5727	TERMINAL SACRIFICE	8	(2)	EPIDIDYMIDES; Absent; unilateral TESTES; Absent; unilateral Any remaining protocol required tissues, which have been examined, have no visible lesions
Ru5728	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 14 contd. Individual Animal Gross Pathology Findings – Acetamide + BrdU Groups

Group No.: G4B Dose: 300 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	(Week)	Observation(s)
Ru5729	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5730	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5731	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5732	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5733	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5734	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 14 contd. Individual Animal Gross Pathology Findings – Acetamide + BrdU Groups

Group No.: G5B Dose: 1000 mg/kg/day Sex: Male

Animal Ref.	Mode Of Death	Death Day	Death (Week)	Observation(s)
Ru5735	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5736	TERMINAL SACRIFICE	8	(2)	LIVER; right medial lobe; Focus (i); white:1.1 cm: Any remaining protocol required tissues, which have been examined, have no visible lesions
Ru5737	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5738	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5739	TERMINAL SACRIFICE	8	(2)	No Visible Lesions
Ru5740	TERMINAL SACRIFICE	8	(2)	No Visible Lesions

APPENDIX 15. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5681 Group No.: G1A Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

Animal Ref.: Ru5682 Group No.: G1A Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5683 Group No.: G1A Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

Animal Ref.: Ru5684 Group No.: G1A Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5685 Group No.: G1A Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

Animal Ref.: Ru5686 Group No.: G1A Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5687 Group No.: G2A Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5688 Group No.: G2A Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5689 Group No.: G2A Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5690 Group No.: G2A Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5691 Group No.: G2A Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5692 Group No.: G2A Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5693 Group No.: G3A Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5694 Group No.: G3A Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5695 Group No.: G3A Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5696 Group No.: G3A Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5697 Group No.: G3A Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5698 Group No.: G3A Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5699 Group No.: G4A Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5700 Group No.: G4A Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5701 Group No.: G4A Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5702 Group No.: G4A Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5703 Group No.: G4A Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5704 Group No.: G4A Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5705 Group No.: G5A Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Single cell necrosis; hepatocyte; minimal
Vacuolation; hepatocyte; minimal
Increased mitotic activity; mild

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5706 Group No.: G5A Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Vacuolation; hepatocyte; minimal
Single cell necrosis; hepatocyte; minimal
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5707 Group No.: G5A Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Vacuolation; hepatocyte; minimal
Single cell necrosis; hepatocyte; mild
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

Animal Ref.: Ru5708 Group No.: G5A Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5709 Group No.: G5A Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Vacuolation; hepatocyte; minimal
Single cell necrosis; hepatocyte; minimal
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 15 contd. Individual Animal Histopathology Findings – Acetamide Groups

Animal Ref.: Ru5710 Group No.: G5A Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Vacuolation; hepatocyte; minimal
Single cell necrosis; hepatocyte; mild
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 16. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5711 Group No.: G1B Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

Animal Ref.: Ru5712 Group No.: G1B Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5713 Group No.: G1B Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

Animal Ref.: Ru5714 Group No.: G1B Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Inflammatory focus(i); minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5715 Group No.: G1B Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

Animal Ref.: Ru5716 Group No.: G1B Sex: Male Species: Rat Strain: Wistar

Dose: 0 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT LIVER SPLEEN STERNUM

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5717 Group No.: G2B Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5718 Group No.: G2B Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5719 Group No.: G2B Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5720 Group No.: G2B Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5721 Group No.: G2B Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5722 Group No.: G2B Sex: Male Species: Rat Strain: Wistar

Dose: 30 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5723 Group No.: G3B Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5724 Group No.: G3B Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5725 Group No.: G3B Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5726 Group No.: G3B Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5727 Group No.: G3B Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5728 Group No.: G3B Sex: Male Species: Rat Strain: Wistar

Dose: 100 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5729 Group No.: G4B Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5730 Group No.: G4B Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5731 Group No.: G4B Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5732 Group No.: G4B Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5733 Group No.: G4B Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

Animal Ref.: Ru5734 Group No.: G4B Sex: Male Species: Rat Strain: Wistar

Dose: 300 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations: None

The following tissues were within normal limits:

LIVER

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5735 Group No.: G5B Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

Animal Ref.: Ru5736 Group No.: G5B Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Single cell necrosis; hepatocyte; mild
Vacuolation; hepatocyte; minimal
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5737 Group No.: G5B Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Single cell necrosis; hepatocyte; minimal
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

Animal Ref.: Ru5738 Group No.: G5B Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Vacuolation; hepatocyte; minimal
Single cell necrosis; hepatocyte; minimal
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 16 contd. Individual Animal Histopathology Findings – Acetamide + BrdU Groups

Animal Ref.: Ru5739 Group No.: G5B Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Vacuolation; hepatocyte; minimal
Single cell necrosis; hepatocyte; minimal
Increased mitotic activity; mild

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

Animal Ref.: Ru5740 Group No.: G5B Sex: Male Species: Rat Strain: Wistar

Dose: 1000 mg/kg/day Study Type: 7 Day Repeated Dose
Date of Death : 06/02/2018 Study Day No. (Week): 8 (2) Mode of Death: TERMINAL SACRIFICE
Date of Necropsy: 06/02/2018 ** NECROPSY COMPLETE **

** EXAMINATION COMPLETE **

Histo Pathology Observations:

LIVER;
Single cell necrosis; hepatocyte; minimal
Increased mitotic activity; minimal

The following tissues were within normal limits:

FEMUR BONE WITH DISTAL JOINT SPLEEN STERNUM

APPENDIX 17. Individual Animal Data of Mitotic Index – Acetamide Groups

Group No.: G1A Dose: 0 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Mitotic Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5681	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	2	2998	3000	0.07
	2	0	100	1	99	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5682	1	0	100	1	99	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	3	2997	3000	0.10
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
Ru5683	1	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5684	1	0	100	1	99	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	9	2991	3000	0.30
	2	0	100	1	99	0	100	1	99	1	99	0	100	1	99	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	1	99	0	100	1	99	1	99	0	100	0	100				
Ru5685	1	1	99	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	12	2988	3000	0.40
	2	0	100	1	99	0	100	1	99	1	99	0	100	0	100	1	99	1	99	0	100				
	3	1	99	1	99	0	100	1	99	0	100	0	100	1	99	0	100	1	99	0	100				
Ru5686	1	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 17 contd. Individual Animal Data of Mitotic Index – Acetamide Groups

Group No.: G2A Dose: 30 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Mitotic Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5687	1	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	3	2997	3000	0.10
	2	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100				
Ru5688	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5689	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5690	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	2	2998	3000	0.07
	2	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100				
Ru5691	1	0	100	1	99	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100	7	2993	3000	0.23
	2	1	99	0	100	0	100	0	100	1	99	0	100	0	100	1	99	0	100	0	100				
	3	0	100	0	100	1	99	0	100	0	100	0	100	1	99	0	100	0	100	0	100				
Ru5692	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	2	2998	3000	0.07
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	1	99	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 17 contd. Individual Animal Data of Mitotic Index – Acetamide Groups

Group No.: G3A Dose: 100 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Mitotic Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5693	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	2	2998	3000	0.07
	2	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
Ru5694	1	0	100	1	99	0	100	1	99	0	100	0	100	0	100	0	100	1	99	0	100	5	2995	3000	0.17
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100				
	3	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5695	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5696	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
Ru5697	1	0	100	1	99	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	4	2996	3000	0.13
	2	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100				
Ru5698	1	1	99	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	4	2996	3000	0.13
	2	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 17 contd. Individual Animal Data of Mitotic Index – Acetamide Groups

Group No.: G4A Dose: 300 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Mitotic Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5699	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	2999	3000	0.03
	2	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100				
Ru5700	1	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	6	2994	3000	0.20
	2	0	100	1	99	0	100	0	100	1	99	0	100	0	100	1	99	1	99	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100				
Ru5701	1	0	100	1	99	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100	4	2996	3000	0.13
	2	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100				
Ru5702	1	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99	0	100	0	100	3	2997	3000	0.10
	2	0	100	0	100	0	100	1	99	0	100	1	99	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
Ru5703	1	0	100	1	99	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	7	2993	3000	0.23
	2	1	99	0	100	0	100	0	100	1	99	0	100	0	100	1	99	0	100	0	100				
	3	1	99	0	100	0	100	0	100	0	100	1	99	0	100	0	100	0	100	0	100				
Ru5704	1	0	100	1	99	0	100	0	100	1	99	0	100	0	100	1	99	0	100	0	100	5	2995	3000	0.17
	2	0	100	0	100	1	99	0	100	0	100	0	100	0	100	0	100	0	100	0	100				
	3	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	1	99				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 17 contd. Individual Animal Data of Mitotic Index – Acetamide Groups

Group No.: G5A Dose: 1000 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Mitotic Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5705	1	3	97	2	98	1	99	2	98	1	99	0	100	3	97	1	99	1	99	1	99	50	2950	3000	1.67
	2	3	97	0	100	0	100	3	97	2	98	0	100	4	96	1	99	1	99	2	98				
	3	3	97	2	98	1	99	4	96	2	98	1	99	3	97	2	98	0	100	1	99				
Ru5706	1	3	97	1	99	1	99	3	97	0	100	1	99	3	97	1	99	0	100	1	99	40	2960	3000	1.33
	2	3	97	0	100	1	99	2	98	1	99	1	99	2	98	1	99	1	99	1	99				
	3	1	99	1	99	1	99	3	97	1	99	1	99	4	96	0	100	1	99	0	100				
Ru5707	1	2	98	0	100	0	100	2	98	0	100	2	98	1	99	1	99	0	100	0	100	17	2983	3000	0.57
	2	2	98	0	100	0	100	2	98	0	100	0	100	0	100	0	100	0	100	0	100				
	3	2	98	0	100	0	100	2	98	0	100	0	100	1	99	0	100	0	100	0	100				
Ru5708	1	1	99	0	100	0	100	2	98	0	100	0	100	1	99	0	100	0	100	0	100	16	2984	3000	0.53
	2	1	99	0	100	0	100	1	99	0	100	0	100	2	98	0	100	0	100	0	100				
	3	0	100	0	100	0	100	1	99	1	99	0	100	3	97	2	98	0	100	1	99				
Ru5709	1	3	97	1	99	1	99	3	97	1	99	0	100	3	97	1	99	0	100	1	99	47	2953	3000	1.57
	2	4	96	2	98	1	99	3	97	2	98	0	100	2	98	1	99	0	100	1	99				
	3	4	96	2	98	1	99	3	97	1	99	0	100	4	96	1	99	0	100	1	99				
Ru5710	1	2	98	0	100	0	100	2	98	1	99	1	99	3	97	0	100	0	100	0	100	30	2970	3000	1.00
	2	3	97	0	100	0	100	3	97	1	99	0	100	2	98	0	100	0	100	1	99				
	3	4	96	1	99	1	99	2	98	0	100	0	100	2	98	0	100	0	100	1	99				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 18. Individual Animal Data of Ki 67 Index – Acetamide Groups

Group No.: G1A Dose: 0 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5681	1	3	97	5	95	0	100	0	100	3	97	2	98	2	98	3	97	0	100	0	100	67	2933	3000	2.23
	2	0	100	4	96	0	100	2	98	5	95	1	99	3	97	5	95	1	99	1	99				
	3	1	99	4	96	2	98	4	96	3	97	0	100	4	96	6	94	0	100	3	97				
Ru5682	1	7	93	10	90	6	94	11	89	10	90	10	90	13	87	6	94	9	91	7	93	257	2743	3000	8.57
	2	6	94	10	90	10	90	8	92	11	89	10	90	6	94	7	93	7	93	9	91				
	3	8	92	7	93	8	92	5	95	13	87	9	91	8	92	11	89	8	92	7	93				
Ru5683	1	3	97	6	94	6	94	1	99	6	94	5	95	1	99	3	97	7	93	3	97	138	2862	3000	4.60
	2	5	95	4	96	5	95	3	97	4	96	2	98	6	94	5	95	3	97	4	96				
	3	7	93	8	92	4	96	8	92	8	92	5	95	6	94	3	97	5	95	2	98				
Ru5684	1	5	95	10	90	6	94	5	95	4	96	6	94	3	97	6	94	4	96	5	95	140	2860	3000	4.67
	2	4	96	8	92	5	95	4	96	7	93	3	97	2	98	8	92	4	96	3	97				
	3	3	97	4	96	4	96	2	98	6	94	3	97	4	96	7	93	3	97	2	98				
Ru5685	1	9	91	12	88	8	92	10	90	13	87	8	92	7	93	12	88	9	91	6	94	249	2751	3000	8.30
	2	8	92	13	87	7	93	6	94	10	90	5	95	6	94	11	89	5	95	7	93				
	3	9	91	8	92	7	93	8	92	12	88	7	93	5	95	10	90	3	97	8	92				
Ru5686	1	5	95	10	90	4	96	6	94	11	89	7	93	6	94	12	88	6	94	6	94	209	2791	3000	6.97
	2	4	96	5	95	7	93	8	92	10	90	3	97	4	96	11	89	8	92	9	91				
	3	6	94	9	91	3	97	7	93	8	92	6	94	8	92	5	95	9	91	6	94				

+ : Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 18 contd. Individual Animal Data of Ki 67 Index – Acetamide Groups

Group No.: G2A Dose: 30 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5687	1	0	100	0	100	1	99	2	98	3	97	0	100	0	100	1	99	2	98	0	100	26	2974	3000	0.87
	2	1	99	1	99	1	99	0	100	1	99	0	100	1	99	2	98	1	99	1	99				
	3	1	99	2	98	0	100	1	99	0	100	1	99	0	100	1	99	0	100	2	98				
Ru5688	1	5	95	9	91	6	94	7	93	10	90	6	94	5	95	9	91	4	96	6	94	161	2839	3000	5.37
	2	4	96	8	92	5	95	3	97	8	92	5	95	3	97	8	92	4	96	5	95				
	3	3	97	6	94	3	97	4	96	7	93	4	96	2	98	7	93	2	98	3	97				
Ru5689	1	2	98	4	96	0	100	1	99	3	97	2	98	0	100	1	99	0	100	4	96	72	2928	3000	2.40
	2	4	96	5	95	1	99	2	98	4	96	1	99	3	97	4	96	1	99	2	98				
	3	1	99	6	94	2	98	4	96	5	95	0	100	1	99	6	94	3	97	0	100				
Ru5690	1	2	98	5	95	2	98	3	97	4	96	2	98	5	95	3	97	2	98	1	99	68	2932	3000	2.27
	2	1	99	4	96	1	99	4	96	6	94	1	99	0	100	4	96	1	99	3	97				
	3	0	100	2	98	1	99	2	98	1	99	3	97	0	100	1	99	3	97	1	99				
Ru5691	1	2	98	4	96	3	97	0	100	4	96	1	99	2	98	5	95	2	98	2	98	76	2924	3000	2.53
	2	1	99	5	95	2	98	1	99	3	97	2	98	3	97	4	96	3	97	2	98				
	3	2	98	4	96	1	99	3	97	2	98	5	95	1	99	3	97	0	100	4	96				
Ru5692	1	3	97	7	93	4	96	5	95	8	92	4	96	3	97	5	95	3	97	3	97	125	2875	3000	4.17
	2	4	96	8	92	3	97	6	94	5	95	6	94	2	98	6	94	1	99	3	97				
	3	5	95	3	97	4	96	7	93	1	99	5	95	3	97	2	98	2	98	4	96				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 9 contd. Pathology Report

APPENDIX 18 contd. Individual Animal Data of Ki 67 Index – Acetamide Groups

Group No.: G3A Dose: 100 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5693	1	2	98	6	94	3	97	1	99	5	95	3	97	0	100	3	97	2	98	4	96	102	2898	3000	3.40
	2	4	96	7	93	2	98	3	97	6	94	4	96	1	99	5	95	4	96	2	98				
	3	3	97	6	94	4	96	2	98	4	96	1	99	3	97	7	93	2	98	3	97				
Ru5694	1	1	99	3	97	0	100	2	98	5	95	0	100	2	98	3	97	0	100	1	99	58	2942	3000	1.93
	2	3	97	4	96	1	99	1	99	4	96	2	98	3	97	5	95	1	99	2	98				
	3	0	100	2	98	0	100	3	97	3	97	1	99	2	98	1	99	3	97	0	100				
Ru5695	1	2	98	4	96	1	99	0	100	3	97	2	98	1	99	3	97	0	100	2	98	57	2943	3000	1.90
	2	1	99	3	97	0	100	1	99	4	96	0	100	2	98	4	96	1	99	0	100				
	3	3	97	2	98	2	98	2	98	4	96	1	99	3	97	2	98	3	97	1	99				
Ru5696	1	0	100	3	97	2	98	1	99	1	99	2	98	2	98	3	97	0	100	1	99	49	2951	3000	1.63
	2	2	98	4	96	1	99	0	100	3	97	0	100	1	99	4	96	0	100	1	99				
	3	0	100	2	98	0	100	3	97	4	96	2	98	2	98	0	100	2	98	3	97				
Ru5697	1	4	96	8	92	5	95	4	96	7	93	3	97	5	95	10	90	6	94	4	96	134	2866	3000	4.47
	2	6	94	10	90	3	97	2	98	5	95	1	99	2	98	7	93	4	96	2	98				
	3	5	95	7	93	2	98	3	97	5	95	2	98	3	97	8	92	0	100	1	99				
Ru5698	1	1	99	3	97	4	96	2	98	5	95	0	100	1	99	4	96	0	100	3	97	60	2940	3000	2.00
	2	3	97	5	95	2	98	1	99	3	97	1	99	2	98	5	95	1	99	0	100				
	3	2	98	4	96	1	99	0	100	2	98	0	100	1	99	3	97	0	100	1	99				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 18 contd. Individual Animal Data of Ki 67 Index – Acetamide Groups

Group No.: G4A Dose: 300 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5699	1	2	98	4	96	1	99	0	100	3	97	2	98	1	99	3	97	2	98	3	97	59	2941	3000	1.97
	2	0	100	2	98	3	97	1	99	4	96	5	95	2	98	4	96	1	99	0	100				
	3	1	99	3	97	0	100	1	99	2	98	1	99	2	98	3	97	2	98	1	99				
Ru5700	1	8	92	12	88	7	93	6	94	10	90	5	95	1	99	9	91	6	94	4	96	204	2796	3000	6.80
	2	5	95	11	89	8	92	4	96	8	92	2	98	3	97	10	90	7	93	5	95				
	3	6	94	10	90	7	93	5	95	11	89	6	94	5	95	9	91	6	94	8	92				
Ru5701	1	3	97	9	91	1	99	2	98	5	95	6	94	2	98	5	95	1	99	3	97	105	2895	3000	3.50
	2	0	100	1	99	6	94	4	96	7	93	2	98	3	97	5	95	3	97	2	98				
	3	3	97	6	94	2	98	3	97	6	94	3	97	4	96	6	94	2	98	0	100				
Ru5702	1	4	96	5	95	2	98	4	96	3	97	6	94	2	98	9	91	3	97	4	96	101	2899	3000	3.37
	2	3	97	7	93	5	95	2	98	6	94	3	97	0	100	4	96	1	99	2	98				
	3	5	95	2	98	3	97	1	99	4	96	2	98	1	99	3	97	2	98	3	97				
Ru5703	1	2	98	8	92	4	96	3	97	9	91	1	99	4	96	8	92	5	95	2	98	164	2836	3000	5.47
	2	6	94	10	90	5	95	8	92	10	90	2	98	9	91	2	98	4	96	5	95				
	3	4	96	9	91	6	94	5	95	8	92	3	97	4	96	9	91	6	94	3	97				
Ru5704	1	3	97	8	92	4	96	8	92	11	89	4	96	3	97	5	95	1	99	5	95	143	2857	3000	4.77
	2	4	96	10	90	5	95	6	94	9	91	5	95	1	99	6	94	0	100	4	96				
	3	6	94	7	93	2	98	4	96	7	93	2	98	0	100	8	92	3	97	2	98				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 9 contd. Pathology Report

APPENDIX 18 contd. Individual Animal Data of Ki 67 Index – Acetamide Groups

Group No.: G5A Dose: 1000 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5705	1	36	64	51	49	40	60	42	58	49	51	44	56	38	62	48	52	30	70	35	65	1208	1792	3000	40.27
	2	41	59	39	61	35	65	43	57	50	50	38	62	43	57	36	64	44	56	39	61				
	3	33	67	40	60	31	69	51	49	39	61	41	59	44	56	39	61	29	71	40	60				
Ru5706	1	22	78	30	70	24	76	21	79	36	64	29	71	28	72	41	59	20	80	21	79	861	2139	3000	28.70
	2	24	76	32	68	20	80	19	81	31	69	34	66	30	70	42	58	26	74	29	71				
	3	27	73	36	64	22	78	28	72	37	63	30	70	31	69	40	60	25	75	26	74				
Ru5707	1	20	80	22	78	18	82	21	79	23	77	17	83	19	81	26	74	20	80	17	83	608	2392	3000	20.27
	2	18	82	26	74	21	79	20	80	21	79	16	84	17	83	22	78	24	76	21	79				
	3	17	83	25	75	16	84	21	79	26	74	18	82	19	81	16	84	19	81	22	78				
Ru5708	1	21	79	20	80	18	82	16	84	22	78	19	81	17	83	15	85	16	84	18	82	538	2462	3000	17.93
	2	19	81	22	78	17	83	16	84	20	80	18	82	15	85	21	79	16	84	14	86				
	3	22	78	18	82	16	84	18	82	16	84	17	83	19	81	20	80	17	83	15	85				
Ru5709	1	28	72	30	70	22	78	26	74	28	72	20	80	24	76	31	69	25	75	22	78	707	2293	3000	23.57
	2	23	77	29	71	21	79	19	81	25	75	18	82	20	80	30	70	21	79	15	85				
	3	24	76	28	72	20	80	19	81	29	71	16	84	21	79	29	71	24	76	20	80				
Ru5710	1	17	83	15	85	20	80	19	81	16	84	15	85	17	83	14	86	16	84	19	81	510	2490	3000	17.00
	2	18	82	16	84	17	83	20	80	21	79	14	86	19	81	20	80	14	86	18	82				
	3	16	84	14	86	18	82	16	84	20	80	19	81	15	85	18	82	13	87	16	84				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 19. Individual Animal Data of Ki 67 Index – Acetamide + BrdU Groups

Group No.: G1B Dose: 0 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5711	1	1	99	3	97	0	100	2	98	4	96	3	97	0	100	1	99	2	98	3	97	70	2930	3000	2.33
	2	3	97	2	98	1	99	1	99	4	96	2	98	5	95	0	100	1	99	1	99				
	3	1	99	4	96	3	97	2	98	4	96	3	97	2	98	5	95	3	97	4	96				
Ru5712	1	2	98	5	95	3	97	4	96	6	94	3	97	1	99	4	96	2	98	3	97	81	2919	3000	2.70
	2	0	100	3	97	2	98	2	98	5	95	1	99	2	98	6	94	1	99	2	98				
	3	1	99	4	96	0	100	3	97	4	96	2	98	0	100	5	95	3	97	2	98				
Ru5713	1	2	98	3	97	0	100	1	99	2	98	0	100	2	98	4	96	2	98	3	97	70	2930	3000	2.33
	2	3	97	5	95	1	99	2	98	4	96	1	99	2	98	5	95	3	97	0	100				
	3	2	98	4	96	1	99	1	99	5	95	0	100	4	96	3	97	4	96	1	99				
Ru5714	1	4	96	7	93	2	98	0	100	6	94	1	99	3	97	6	94	1	99	3	97	96	2904	3000	3.20
	2	2	98	5	95	4	96	1	99	7	93	2	98	5	95	4	96	5	95	4	96				
	3	1	99	4	96	3	97	2	98	4	96	3	97	0	100	3	97	4	96	0	100				
Ru5715	1	3	97	5	95	4	96	4	96	6	94	5	95	5	95	5	95	4	96	2	98	122	2878	3000	4.07
	2	5	95	4	96	5	95	5	95	4	96	3	97	4	96	3	97	5	95	5	95				
	3	4	96	5	95	3	97	3	97	4	96	5	95	4	96	5	95	1	99	2	98				
Ru5716	1	2	98	3	97	1	99	0	100	3	97	1	99	2	98	4	96	0	100	1	99	62	2938	3000	2.07
	2	1	99	0	100	2	98	4	96	3	97	4	96	0	100	0	100	4	96	3	97				
	3	2	98	3	97	4	96	2	98	5	95	0	100	1	99	3	97	2	98	2	98				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 19 contd. Individual Animal Data of Ki 67 Index – Acetamide + BrdU Groups

Group No.: G2B Dose: 30 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5717	1	1	99	0	100	2	98	2	98	1	99	1	99	2	98	3	97	4	96	3	97	69	2931	3000	2.30
	2	2	98	5	95	1	99	2	98	4	96	2	98	1	99	3	97	2	98	3	97				
	3	4	96	2	98	1	99	2	98	3	97	2	98	1	99	4	96	2	98	4	96				
Ru5718	1	2	98	3	97	1	99	3	97	4	96	3	97	0	100	3	97	2	98	0	100	66	2934	3000	2.20
	2	1	99	4	96	2	98	4	96	1	99	4	96	1	99	4	96	1	99	1	99				
	3	0	100	3	97	0	100	5	95	1	99	3	97	2	98	4	96	1	99	3	97				
Ru5719	1	1	99	2	98	1	99	2	98	1	99	2	98	2	98	3	97	0	100	2	98	43	2957	3000	1.43
	2	0	100	2	98	1	99	3	97	0	100	2	98	1	99	3	97	1	99	1	99				
	3	0	100	1	99	1	99	3	97	1	99	3	97	0	100	2	98	1	99	1	99				
Ru5720	1	2	98	4	96	1	99	1	99	3	97	2	98	3	97	1	99	2	98	2	98	75	2925	3000	2.50
	2	3	97	4	96	2	98	2	98	4	96	2	98	3	97	2	98	3	97	2	98				
	3	3	97	1	99	2	98	3	97	4	96	2	98	4	96	2	98	3	97	3	97				
Ru5721	1	1	99	2	98	0	100	1	99	3	97	1	99	2	98	1	99	1	99	4	96	46	2954	3000	1.53
	2	0	100	2	98	1	99	2	98	3	97	1	99	3	97	0	100	3	97	1	99				
	3	0	100	3	97	0	100	2	98	2	98	1	99	2	98	0	100	2	98	2	98				
Ru5722	1	2	98	3	97	1	99	3	97	3	97	2	98	2	98	3	97	0	100	1	99	20	980	1000	2.00
	2	2	98	3	97	2	98	1	99	2	98	1	99	2	98	3	97	0	100	1	99				
	3	0	100	3	97	2	98	1	99	2	98	2	98	3	97	3	97	3	97	2	98				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 19 contd. Individual Animal Data of Ki 67 Index – Acetamide + BrdU Groups

Group No.: G3B Dose: 100 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5723	1	1	99	2	98	1	99	2	98	3	97	1	99	0	100	2	98	2	98	3	97	47	2953	3000	1.57
	2	2	98	3	97	1	99	1	99	3	97	1	99	0	100	2	98	1	99	2	98				
	3	0	100	2	98	2	98	1	99	2	98	1	99	1	99	2	98	1	99	2	98				
Ru5724	1	4	96	6	94	2	98	3	97	7	93	2	98	4	96	5	95	4	96	4	96	133	2867	3000	4.43
	2	5	95	7	93	3	97	3	97	5	95	3	97	4	96	6	94	5	95	4	96				
	3	3	97	7	93	4	96	4	96	6	94	3	97	5	95	7	93	3	97	5	95				
Ru5725	1	5	95	8	92	6	94	4	96	7	93	4	96	3	97	5	95	4	96	6	94	138	2862	3000	4.60
	2	5	95	9	91	6	94	5	95	6	94	5	95	4	96	4	96	3	97	3	97				
	3	4	96	7	93	5	95	2	98	5	95	4	96	2	98	4	96	1	99	2	98				
Ru5726	1	5	95	5	95	6	94	4	96	6	94	3	97	4	96	7	93	3	97	4	96	118	2882	3000	3.93
	2	4	96	4	96	5	95	3	97	2	98	5	95	2	98	4	96	1	99	3	97				
	3	4	96	5	95	3	97	2	98	5	95	3	97	4	96	5	95	3	97	4	96				
Ru5727	1	2	98	4	96	3	97	3	97	5	95	2	98	3	97	4	96	4	96	2	98	101	2899	3000	3.37
	2	4	96	4	96	4	96	2	98	4	96	1	99	4	96	5	95	3	97	4	96				
	3	3	97	5	95	2	98	3	97	4	96	2	98	5	95	6	94	3	97	1	99				
Ru5728	1	2	98	4	96	3	97	4	96	5	95	2	98	2	98	5	95	1	99	3	97	88	2912	3000	2.93
	2	1	99	5	95	4	96	2	98	3	97	1	99	3	97	4	96	0	100	2	98				
	3	4	96	6	94	2	98	1	99	4	96	3	97	1	99	5	95	3	97	3	97				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 19 contd. Individual Animal Data of Ki 67 Index – Acetamide + BrdU Groups

Group No.: G4B Dose: 300 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5729	1	2	98	5	95	3	97	1	99	4	96	2	98	3	97	4	96	2	98	3	97	80	2920	3000	2.67
	2	3	97	4	96	0	100	2	98	5	95	1	99	2	98	5	95	1	99	4	96				
	3	1	99	3	97	1	99	2	98	4	96	0	100	1	99	5	95	2	98	5	95				
Ru5730	1	3	97	3	97	4	96	2	98	4	96	4	96	2	98	2	98	1	99	4	96	73	2927	3000	2.43
	2	4	96	2	98	3	97	1	99	2	98	0	100	3	97	4	96	3	97	2	98				
	3	2	98	3	97	0	100	3	97	3	97	1	99	4	96	3	97	0	100	1	99				
Ru5731	1	4	96	8	92	3	97	5	95	9	91	3	97	2	98	6	94	1	99	4	96	144	2856	3000	4.80
	2	5	95	9	91	2	98	6	94	1	99	4	96	4	96	7	93	5	95	3	97				
	3	3	97	7	93	4	96	3	97	8	92	5	95	5	95	9	91	4	96	5	95				
Ru5732	1	4	96	6	94	0	100	4	96	5	95	1	99	4	96	6	94	1	99	3	97	93	2907	3000	3.10
	2	2	98	5	95	1	99	4	96	5	95	0	100	2	98	5	95	2	98	3	97				
	3	3	97	6	94	0	100	3	97	6	94	2	98	1	99	5	95	0	100	4	96				
Ru5733	1	1	99	4	96	2	98	2	98	4	96	2	98	1	99	4	96	2	98	4	96	79	2921	3000	2.63
	2	2	98	5	95	0	100	3	97	4	96	3	97	2	98	4	96	3	97	0	100				
	3	2	98	3	97	1	99	3	97	5	95	3	97	2	98	4	96	3	97	1	99				
Ru5734	1	4	96	8	92	4	96	4	96	5	95	5	95	3	97	5	95	4	96	6	94	151	2849	3000	5.03
	2	5	95	7	93	5	95	2	98	6	94	3	97	5	95	4	96	5	95	5	95				
	3	6	94	9	91	3	97	3	97	7	93	3	97	6	94	9	91	6	94	4	96				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

APPENDIX 19 contd. Individual Animal Data of Ki 67 Index – Acetamide + BrdU Groups

Group No.: G5B Dose: 1000 mg/kg/day

Rat No.	Tissue slice No.	High power field number																				Total +ve counted	Total -ve counted	Sum Total	Ki 67 Index
		1		2		3		4		5		6		7		8		9		10					
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-				
Ru5735	1	21	79	32	68	25	75	24	76	35	65	22	78	26	74	30	70	26	74	23	77	797	2203	3000	26.57
	2	24	76	34	66	20	80	27	73	30	70	26	74	20	80	33	67	21	79	28	72				
	3	27	73	31	69	23	77	21	79	29	71	30	70	25	75	28	72	30	70	26	74				
Ru5736	1	23	77	36	64	29	71	40	60	30	70	25	75	28	72	33	67	22	78	30	70	818	2182	3000	27.27
	2	26	74	29	71	21	79	25	75	33	67	24	76	19	81	22	78	24	76	26	74				
	3	24	76	25	75	22	78	41	59	20	80	31	69	26	74	35	65	28	72	21	79				
Ru5737	1	14	86	20	80	19	81	16	84	23	77	18	82	20	80	23	77	19	81	14	86	569	2431	3000	18.97
	2	13	87	21	79	15	85	18	82	24	76	16	84	21	79	18	82	19	81	16	84				
	3	16	84	26	74	20	80	15	85	29	71	19	81	22	78	16	84	20	80	19	81				
Ru5738	1	23	77	33	67	24	76	20	80	31	69	23	77	25	75	30	70	26	74	28	72	748	2252	3000	24.93
	2	20	80	34	66	21	79	22	78	29	71	25	75	23	77	31	69	22	78	26	74				
	3	24	76	30	70	20	80	18	82	28	72	20	80	24	76	28	72	19	81	21	79				
Ru5739	1	30	70	39	61	31	69	28	72	35	65	24	76	26	74	36	64	24	76	28	72	887	2113	3000	29.57
	2	32	68	36	64	29	71	28	72	33	67	29	71	20	80	30	70	26	74	23	77				
	3	31	69	37	63	30	70	34	66	38	62	22	78	24	76	32	68	28	72	24	76				
Ru5740	1	26	74	31	69	30	70	25	75	30	70	20	80	24	76	31	69	21	79	28	72	777	2223	3000	25.9
	2	25	75	33	67	28	72	26	74	32	68	19	81	23	77	33	67	16	84	23	77				
	3	28	72	32	68	20	80	22	78	29	71	21	79	29	71	29	71	18	82	25	75				

+: Positively stained hepatocyte - : Negatively stained hepatocyte

ANNEXURE 1. Historical Control Data – Clinical Pathology

HISTORICAL DATA – 91

7-DAY REPEATED DOSE ORAL TOXICITY STUDY IN WISTAR RATS

91.4. Hematology Values - Males

Species : Rats
Strain : Wistar
Age : 6-10 weeks (at the start of treatment)
No. of Studies : 10
Time period : 2011 – 2013

	WBC	RBC	Hb	Hct	MCV	MCH	MCHC	MPV	Neut	Lymp	Mono	Baso	Eos	Retic	Retic	RDW	P.T.	APTT
	G/L	T/ L	g/ L	L/L	fL	pg	g/ L	fL	G/L	G/L	G/L	G/L	G/L	T/L	%	%	s	s
N	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
Mean	6.68	8.20	150.07	0.49	59.48	18.32	308.27	10.22	1.17	5.12	0.23	0.03	0.05	0.43	5.24	1127.80	14.49	11.07
SD	1.09	0.33	5.74	0.02	2.52	0.81	12.72	0.64	0.35	0.98	0.14	0.01	0.02	0.05	0.61	83.48	0.84	1.49
Range (Low)	4.54	7.69	140.00	0.45	55.40	16.80	288.00	9.40	0.59	3.50	0.05	0.01	0.03	0.35	4.12	953.00	12.90	8.80
Range (High)	8.42	8.83	161.00	0.52	64.40	19.60	327.00	11.30	1.77	6.99	0.66	0.05	0.09	0.53	6.36	1285.00	16.40	13.40

ANNEXURE 1 contd. Historical Control Data – Clinical Pathology

HISTORICAL DATA – 91

7-DAY REPEATED DOSE ORAL TOXICITY STUDY IN WISTAR RATS

91.6. Clinical Chemistry Values - Males

Species : Rats
Strain : Wistar
Age : 6-10 weeks (at the start of treatment)
No. of Studies : 10
Time period : 2011 – 2013

	Glu	BUN	Creat	AST	ALT	GGT	ALP	LDH	T.Bil	T.Chol	Trig	T.Pro	Alb	Glob
	mmol/L	mmol/L	µmol/L	U/L	U/L	U/L	U/L	U/L	µmol/L	mmol/L	mmol/L	g/L	g/L	g/L
N	15	15	15	15	15	15	15	10	15	15	15	15	15	15
Mean	8.00	5.13	39.33	81.80	40.93	1.87	177.53	266.20	1.73	1.89	1.09	63.66	43.44	20.22
SD	1.35	1.67	4.75	16.08	10.13	0.92	60.24	165.07	0.67	0.31	0.42	3.33	2.79	5.04
Range (Low)	6.64	2.80	28.00	62.00	30.00	1.00	93.00	90.00	0.72	1.36	0.46	58.50	39.40	12.70
Range (High)	11.77	8.32	45.00	122.00	66.00	3.00	290.00	682.00	2.73	2.43	1.96	70.10	49.00	30.40

	A/G	Pi	Ca	Na	K	Cl
	-	mmol/L	mmol/L	mEq/L	mEq/L	mEq/L
N	15	15	15	15	15	15
Mean	2.32	2.63	2.69	150.29	3.77	112.17
SD	0.75	0.47	0.15	1.32	0.33	2.08
Range (Low)	1.31	1.83	2.41	146.90	3.29	107.30
Range (High)	3.63	3.23	2.86	152.20	4.70	115.80

APPENDIX 10. Toxicogenomics Phase Report

TOXICOGENOMICS PHASE REPORT

ACETAMIDE: 7-DAY REPEAT DOSE ORAL TOXICITY STUDY IN WISTAR RATS WITH TOXICOGENOMICS

STUDY No.: G16084

STUDY PHASE IDENTIFICATION No. RP.17.0954

TEST SITE
(TOXICOGENOMICS)

SYNGENE INTERNATIONAL LTD
DISCOVERY BIOLOGY
S-16, PLOT NO. 2&3,
BOMMASANDRA INDUSTRIAL ESTATE, PHASE IV
BOMMASANDRA JIGANI LINK ROAD
BANGALORE-560 099
INDIA

TEST FACILITY

DEPARTMENT OF SAFETY ASSESSMENT
EUROFINS ADVINUS LIMITED
POST BOX No. 5813, PLOT Nos. 21 & 22
PEENYA II PHASE, BENGALURU 560 058
INDIA

SPONSOR
MICHIGAN STATE UNIVERSITY
COLLEGE OF ENGINEERING, MICHIGAN STATE UNIVERSITY
EAST LANSING
MICHIGAN 48824

APPENDIX 10 Contd. Toxicogenomics Phase Report

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APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Toxicogenomics Phase Report
Report Number: RPT-EAL-001

Study Phase Identification No.: RP.17.0954

Study No.: G16084

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Jigani Link Road
Bengaluru-560 099, India

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Study Phase Schedule

Experiment Start Date	:	13 Feb 2019
Experiment Completion Date	:	17 Mar 2018
Draft Report sent to Study Director	:	13 July 2018
Final Report Date	:	02 Aug 2019

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

2. GLP COMPLIANCE STATEMENT

The study phase (Toxicogenomics) was performed at Syngene International Ltd., SEZ-16 UNIT-IX, Plot No.2,3,4 & 5, Phase IV, Biocon Park, Jigani Link Road, Bengaluru-560 099, India (hereinafter called as Test Site). This Test site was selected for the Toxicogenomics as the test site has already developed analytical methods for toxicogenomics analysis. The test site was not under the national GLP compliance monitoring programme, India. The test site was fully responsible for performing the respective phase in accordance with study plan and in-house Procedures.

Study plan was amended once with relevant to study phase and one study phase related deviation is recorded. The Principle Investigator declares that the experiment was performed under her supervision and as per the study plan. All the results given in the report represent the raw data, truly. The Principle Investigator takes the entire responsibility of the conduct of the toxicogenomics phase, documentation of raw data, the interpretation of the results and preparation and finalization of the phase report

Seetha K, Ph.D
Principal Investigator
Toxicogenomics Phase
Date: 2 Aug 2019

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

3. Quality Assurance Statement

Study Title: Acetamide: 7-day Repeat dose oral toxicity study in wistar rats with toxicogenomics

Test Facility (Eurofins Advinus) Study Identification: G16084

Study Director : Dr. D. P Santhosh Kumar

Phase: Toxicocgenomics - 7 day study

Test Site Phase Identification: RP.17.0954

Principal Investigator: Seetha Krishnan

This is to state that the toxicogenomic phase (NonGLP) was inspected for compliance to study plan and work plan by quality assurance unit and the findings were reported to Principal Investigator and Test Site Management as given below:

S. No.	Inspection Phase	Inspection Dates		Reporting Dates	
		From	To	Principal Investigator	Test Site Management
1	Draft Study Report	24 Jun 2019	26 Jun 2019	26 Jun 2019	26 Jun 2019
2	Final Study Report	29 Jul 2019	29 Jul 2019	29 Jul 2019	29 Jul 2019

Response to Quality Assurance audit reports were verified on 29 JUL 2019, for completion of corrective and preventive actions as part of follow up.

Name: Ananta Kumar M
Quality Assurance Unit

02 Aug 2019
(Signature & Date)

Study Number: G16084

Sample receipt

- Rat liver samples (Total number of samples: 60) were shipped from Eurofins Advinus in dry ice and stored in Syngene at appropriate temperature (-80°C freezer)
- Animal numbers used in the study and corresponding treatment are listed in the table below.
- At each step of the study, randomization was incorporated to eliminate potential bias during the steps.

4. Objectives of the study

- To study the effect of acetamide feed on gene expression in Rat liver by RNA seq
- To Identify the potential pathways that are affected by acetamide
- Predict the minimum concentration at which acetamide could be a potential toxic/carcinogenic agent

Experimental Design:

Group No.	Group	Rat Numbers					
		Rep_1	Rep_2	Rep_3	Rep_4	Rep_5	Rep_6
G1A	Vehicle control (VC)	Ru5681	Ru5682	Ru5683	Ru5684	Ru5685	Ru5686
G2A	Low dose (LD)	Ru5687	Ru5688	Ru5689	Ru5690	Ru5691	Ru5692
G3A	Mid dose (MD)	Ru5693	Ru5694	Ru5695	Ru5696	Ru5697	Ru5698
G4A	Intermediate dose (ID)	Ru5699	Ru5700	Ru5701	Ru5702	Ru5703	Ru5704
G5A	High dose (HD)	Ru5705	Ru5706	Ru5707	Ru5708	Ru5709	Ru5710

Group No.	Group	Rat Numbers					
		Rep_1	Rep_2	Rep_3	Rep_4	Rep_5	Rep_6
G1B	Vehicle control (VC)	Ru5711	Ru5712	Ru5713	Ru5714	Ru5715	Ru5716
G2B	Low dose (LD)	Ru5717	Ru5718	Ru5719	Ru5720	Ru5721	Ru5722
G3B	Mid dose (MD)	Ru5723	Ru5724	Ru5725	Ru5726	Ru5727	Ru5728
G4B	Intermediate dose (ID)	Ru5729	Ru5730	Ru5731	Ru5732	Ru5733	Ru5734
G5B	High dose (HD)	Ru5735	Ru5736	Ru5737	Ru5738	Ru5739	Ru5740

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5. Materials and Methods

Consolidated list of equipment's used in the study

Sl No	Equipment description	Manufacturer	Equipment code
1	Fridge 4°C	Hanpro	S16-GDB-REF-29
2	Freezer -20°C	Panasonic	S16-GDB-FZR-29
3	Freezer -80°C	ESCO	S16-GDB-FZR-35
4	Fume hood	Bio Bee	S16-GDB-FH-08
5	Maxwell RSC	Promega	S16-GDB-MWR-01
6	MS3 Plate Vortexer	IKA	S16-GDB-VTM-34
7	NextSeq500	Illumina	S16-GDB-NSQ-01
8	PCR Nexus	Eppendorf	S16-GDB-PCR-16
9	PCR Nexus	Eppendorf	S16-GDB-PCR-17
10	Plate centrifure	VWR	S16-GDB-CFE-53
11	Proflex PCR	Applied Biosystems	S16-GDB-PSM-01
12	Qubit 3 Fluorometer	Invitrogen	S16-GDB-FMR-01
13	Qubit 3 Fluorometer	Invitrogen	S16-GDB-FMR-03
14	Refrigerator	Samsung	S16-GDB-REF-30
15	Table top spinner	Cubee	S16-GDB-CFE-54
16	Table top spinner	Cubee	S16-GDB-CFE-59
17	Tapestation 4200	Agilent	S16-GDB-TSN-01
18	Thermomixer	Eppendorf	S16-GDB-TMX-07
19	Tissue lyser	Qiagen	S16-GDB-QGN-01
20	Vapo.Protect PCR	Eppendorf	S16-GDB-PCR-15
21	Vortexer	Shalom Instruments	S16-GDB-VTM-33
22	Vortexer	Shalom Instruments	S16-GDB-VTM-36
23	Vortexer	Shalom Instruments	S16-GDB-VTM-335

Materials used

- Maxwell® RSC simplyRNA Tissue Kit (Promega, CatNo: AS1340)
- Qubit™ RNA BR Assay Kit (Thermo, CatNo: Q10210)
- Quant-iT™ dsDNA Assay Kit, high sensitivity (Thermo, CatNo : Q33120)
- TapeStation High Sensitivity D1000 ScreenTapes (Agilent, CatNo: 5067-5584)
- TapeStation High Sensitivity D1000 Sample Buffer (Agilent, CatNo: 5067-5603)
- TruSeq Stranded mRNA Library Preparation kit (Illumina, CatNo: RS-122-2103)
- SuperScript™ II Reverse Transcriptase (Thermo, CatNo: 18064014)
- AMPure XP beads (Beckman Coulter, CatNo: A63880)
- Ethanol (Hayman, CatNo: F204325)

Methods

5.1 RNA Isolation from 60 rat liver samples obtained from the 7-day study

Randomization method: Sample names were fed into a Microsoft Excel spreadsheet and each sample was assigned a random number using the function =rand (). The random numbers were sorted in ascending order along with the sample

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names creating a random arrangement of samples names. 10 samples in order were assigned to each batch to get random samples in each batch.
RNA was isolated in 6 batches of 10 samples per batch (Total 60 samples will be processed). Samples processed in each batch are as follows:

Batch1	Animal No.	Sample Name
1	Ru5691	G2A_LD_05
2	Ru5681	G1A_VC_01
3	Ru5713	G1B_VC_03
4	Ru5720	G2B_LD_04
5	Ru5689	G2A_LD_03
6	Ru5687	G2A_LD_01
7	Ru5733	G4B_ID_05
8	Ru5738	G5B_HD_04
9	Ru5723	G3B_MD_01
10	Ru5707	G5A_HD_03

Batch2	Animal No	Sample Name
1	Ru5712	G1B_VC_02
2	Ru5703	G4A_ID_05
3	Ru5724	G3B_MD_02
4	Ru5716	G1B_VC_06
5	Ru5695	G3A_MD_03
6	Ru5692	G2A_LD_06
7	Ru5727	G3B_MD_05
8	Ru5717	G2B_LD_01
9	Ru5737	G5B_HD_03
10	Ru5686	G1A_VC_06

Batch3	Animal No	Sample Name
1	Ru5702	G4A_ID_04
2	Ru5731	G4B_ID_03
3	Ru5726	G3B_MD_04
4	Ru5688	G2A_LD_02
5	Ru5685	G1A_VC_05
6	Ru5700	G4A_ID_02
7	Ru5736	G5B_HD_02
8	Ru5710	G5A_HD_06
9	Ru5693	G3A_MD_01
10	Ru5698	G3A_MD_06

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Batch4	Animal No	Sample Name
1	Ru5708	G5A_HD_04
2	Ru5709	G5A_HD_05
3	Ru5721	G2B_LD_05
4	Ru5683	G1A_VC_03
5	Ru5718	G2B_LD_02
6	Ru5732	G4B_ID_04
7	Ru5699	G4A_ID_01
8	Ru5684	G1A_VC_04
9	Ru5728	G3B_MD_06
10	Ru5682	G1A_VC_02

Batch 5	Animal No	Sample Name
1	Ru5705	G5A_HD_01
2	Ru5715	G1B_VC_05
3	Ru5694	G3A_MD_02
4	Ru5729	G4B_ID_01
5	Ru5739	G5B_HD_05
6	Ru5735	G5B_HD_01
7	Ru5730	G4B_ID_02
8	Ru5697	G3A_MD_05
9	Ru5725	G3B_MD_03
10	Ru5734	G4B_ID_06

Batch 6	Animal No	Sample Name
1	Ru5690	G2A_LD_04
2	Ru5714	G1B_VC_04
3	Ru5722	G2B_LD_06
4	Ru5696	G3A_MD_04
5	Ru5719	G2B_LD_03
6	Ru5740	G5B_HD_06
7	Ru5706	G5A_HD_02
8	Ru5704	G4A_ID_06
9	Ru5701	G4A_ID_03
10	Ru5711	G1B_VC_01

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5.1.1 RNA isolation was done using Maxwell® RSC simplyRNA Tissue Kit:

- Homogenization tubes were prepared by adding one sterile 5 mm bead to a RNase free screw cap tube and placed on dry ice for 15 minutes.
- Working solution of 1-Thioglycerol/Homogenization was prepared by adding 40 µl of 1-Thioglycerol for 2000 µl of homogenization Solution and kept on ice.
- Liver tissue from cryo vial was transferred to the prepared homogenization tube. The tube was again incubated on dry ice for 15 minutes.
- 200 µl of chilled 1-Thioglycerol/Homogenization solution was added to each tube and homogenized on Qiagen TissueLyser LT at 50Hz for 2 minutes at room temperature.
- Sample was heated at 70°C for 2 minutes and allowed to cool for 1 minute on ice.
- 200µl of Lysis Buffer was added to 200µl of tissue homogenate. Vortexed vigorously for 15 seconds. Transferred all 400 µl of lysate to well #1 of the Maxwell® RSC Cartridge.
- 10 µl of DNase I Solution was added to well #4 of the Maxwell® RSC simply RNA Tissue Cartridge (Promega, Cat No: AS1340).
- One plunger was placed in well #8 of each cartridge.
- Empty elution tubes were labelled and placed into the elution tube position for each cartridge in the deck tray. 50 µl of Nuclease-Free Water was added to the bottom of each elution tube.
- RSC simplyRNA Tissue method was selected and run.
- After the extraction process is complete, samples quantity and quality were checked by Qubit and TapeStation. QC processed RNA was transferred to –80°C freezer for storage.

5.1.2 RNA QC

5.1.2.1 Qubit quantification

1. Allow Qubit RNA Broad Range Assay Kit (ThermoFisher, USA. Cat No: Q10210) to equilibrate to room temperature for minimum 30 minutes.
2. Prepare a working solution by adding 1µL assay reagent to 199 µL of dilution buffer.
3. Dilute the sample by adding 9 µL of nuclease free water to 1µL RNA.
4. Add 190 µL of working solution to diluted RNA sample. Vortex briefly, spin down and incubate in dark for 2 minutes.
5. Measure the RNA quantity using Qubit Fluorometer.

5.1.2.2 TapeStation QC

1. Allow RNA Sample buffer to equilibrate at room temperature for 30 minutes.
2. Dilute RNA 1:10 so that the concentration is between 25-500 ng/µL.
3. For each sample, pipette 5 µL RNA Sample Buffer and 1 µL diluted RNA sample in a 96 well plate or an 8 tube strip. Seal the plate/tube strip.
4. Mix the sample and buffer using the IKA vortex at 2000 rpm for 1 min. Spin down the sample.
5. Denature RNA by heating the samples to 72°C for 3 min in a thermal cycler.
6. Incubate the samples on ice for 2 min.
7. Spin down the samples and load on Agilent 4200 TapeStation instrument along with required number of RNA screen tapes and tips.
8. Select and label the samples and run using the 4200 TapeStation Controller Software.

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9. When the run is completed, RNA Integrity Number (RIN) and 28S:18S ratio of the samples are reported in the Agilent TapeStation Analysis Software. RIN>7 and 28S:18S ratio of ~2 is expected from good quality RNA.

5.2. Preparation of transcriptome library of 50 rat liver RNA samples from the 7day study using TruSeq Stranded mRNA (Illumina, Cat No: RS-122-2103) Library Preparation kit

Randomization method: Based on the results of RNA QC, 5 replicates were selected per group. Sample names were fed into an Microsoft Excel spreadsheet and each sample was assigned a random number using the function =rand(). The random numbers were sorted in ascending order along with the sample names creating a random arrangement of samples names. 10 samples in order were assigned to each batch to get random samples in each batch.

Libraries were prepared in 5 batches of 10 samples+ 1NTC per batch. The vehicle groups were spiked with ERCC spike-in Mix 1 and treatment groups were spiked with ERCC Spike-in Mix 2. Samples were processed for library preparation in each batch as follows:

5.2.1 Purification and fragmentation of mRNA:

- Diluted total RNA and ERCC spike-in mix with nuclease-free water was taken as follows:

Sample dilution plan:

Batch 1:

#	Sample Name	RNA 1:10 dilution Qubit (ng/µL)	Vol. of 1:10 diluted RNA to make 1 µg (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 1 (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 2 (µL)	Vol. of NFW to make 50 µL total vol. (µL)
1	G4B_ID_03	95.6	10.5	-	2	37.5
2	G2A_LD_02	111	9	-	2	39
3	G5B_HD_03	152	6.6	-	2	41.4
4	G1A_VC_02	110	9.1	2	-	38.9
5	G2B_LD_05	98.2	10.2	-	2	37.8
6	G2A_LD_03	66.8	15	-	2	33
7	G3A_MD_02	103	9.7	-	2	38.3
8	G1B_VC_05	143	7	2	-	41
9	G3A_MD_06	106	9.4	-	2	38.6
10	G5A_HD_06	169	5.9	-	2	42.1
11	NTC_1	-	-	-	-	50

Batch 2:

#	Sample Name	RNA 1:10 dilution Qubit (ng/µL)	Vol. of 1:10 diluted RNA to make 1 µg (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 1 (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 2 (µL)	Vol. of NFW to make 50 µL total vol. (µL)
1	G3B_MD_02	152	6.5	-	2	41.5
2	G1A_VC_04	126	7.9	2	-	40.1
3	G1B_VC_04	135	7.4	2	-	40.6
4	G2A_LD_05	52	19.2	-	2	28.8
5	G5B_HD_05	60.4	16.5	-	2	31.5
6	G2B_LD_02	145	6.8	-	2	41.2
7	G4A_ID_06	50	20	-	2	28
8	G1A_VC_03	110	9	2	-	39

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9	G4A_ID_05	144	6.9	-	2	41.1
10	G5B_HD_02	110	9	-	2	39
11	NTC_2	-	-	-	-	50

Batch 3:

#	Sample Name	RNA 1:10 dilution Qubit (ng/µL)	Vol. of 1:10 diluted RNA to make 1 µg (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 1 (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 2 (µL)	Vol. of NFW to make 50 µL total vol. (µL)
1	G2B_LD_03	41.2	24.4	-	2	23.6
2	G3A_MD_03	138	7.4	-	2	40.6
3	G2B_LD_04	62.6	16	-	2	32
4	G2A_LD_01	23.2	43	-	2	5
5	G4B_ID_04	135	7.4	-	2	40.6
6	G1B_VC_06	128	7.8	2	-	40.2
7	G1A_VC_05	65.2	15.4	2	-	32.6
8	G3A_MD_04	63.4	15.8	-	2	32.2
9	G4B_ID_02	108	9.4	-	2	38.6
10	G5A_HD_03	106	9.4	-	2	38.6
11	NTC_3	-	-	-	-	50

Batch 4:

#	Sample Name	RNA 1:10 dilution Qubit (ng/µL)	Vol. of 1:10 diluted RNA to make 1 µg (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 1 (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 2 (µL)	Vol. of NFW to make 50 µL total vol. (µL)
1	G3B_MD_05	136	7.3	-	2	40.7
2	G2A_LD_04	96	10.4	-	2	37.6
3	G4B_ID_06	56.4	17.7	-	2	30.3
4	G1B_VC_02	111	9	2	-	39
5	G2B_LD_01	106	9.4	-	2	38.6
6	G5B_HD_01	142	7	-	2	41
7	G3B_MD_06	107	9.3	-	2	38.7
8	G5B_HD_04	114	8.7	-	2	39.3
9	G4A_ID_04	120	8.3	-	2	39.7
10	G4B_ID_05	103	9.7	-	2	38.3
11	NTC_4	-	-	-	-	50

Batch 5:

#	Sample Name	RNA 1:10 dilution Qubit (ng/µL)	Vol. of 1:10 diluted RNA to make 1 µg (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 1 (µL)	Vol. of 1:100 diluted ERCC Spike-in Mix 2 (µL)	Vol. of NFW to make 50 µL total vol. (µL)
1	G5A_HD_02	132	7.6	-	2	40.4
2	G1A_VC_01	99.8	10	2	-	38
3	G4A_ID_02	126	7.9	-	2	40.1
4	G3B_MD_04	121	8.2	-	2	39.8
5	G5A_HD_05	95.4	10.5	-	2	37.5
6	G3A_MD_05	95.8	10.4	-	2	37.6
7	G3B_MD_03	105	9.5	-	2	38.5
8	G5A_HD_04	82.8	12	-	2	36

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9	G1B_VC_03	114	8.7	2	-	39.3
10	G4A_ID_01	104	9.6	-	2	38.4
11	NTC_5	-	-	-	-	50

- 50 µl of RNA Purification Beads were added to the sample and mixed by gently pipetting 6 times.
- Tube was placed in a thermal cycler and following program was run to denature the RNA and facilitate binding of the poly A RNA to the beads:
  - Preheat lid set to 100°C
  - 65°C for 5 minutes
  - Hold at 4°C
- When thermal cycler reached 4°C, sample was removed and incubated at room temperature for 5 minutes to allow the mRNA to bind to the beads.
- Tube was placed on the magnetic stand for 5 minutes to separate the poly A RNA bound beads from the solution.
- Supernatant from each tube was removed and discarded.
- Tube was removed from the magnetic stand and 200 µl of Bead Washing Buffer was added and mixed by gently pipetting the entire volume up and down 6 time.
- Tube was placed on the magnetic stand at room temperature for 5 minutes.
- Supernatant from tube was removed and discarded. Tube was removed from the magnetic stand.
- 50 µl of Elution Buffer was added to the tube and mixed gently pipetting 6 times.
- Tube was place in a thermal cycler and the following program was run to elute the mRNA from the beads:
  - Preheat lid set to 100°C
  - 80°C for 2 minutes
  - Hold at 25°C
- When thermal cycler reached 25°C, tube was removed and placed on bench at room temperature.
- 50 µl of Bead Binding Buffer was added to the sample to allow mRNA to rebind to the beads. The entire volume was gently pipetted up and down 6 times to mix. Tube was incubated at room temperature for 5 minutes.
- Tube was transferred to the magnetic stand for 5 minutes.
- Supernatant from tube was removed and discarded. Tube was removed from the magnetic stand.
- Beads were washed by adding 200 µl of Bead Washing Buffer. The entire volume was gently pipetted up and down 6 times to mix.
- Tube was placed on the magnetic stand at room temperature for 5 minutes.
- Supernatant from tube was removed and discarded. Tube was removed from the magnetic stand.
- 19.5 µl of thawed Fragment, Prime, Finish Mix was added to the sample. The entire volume was gently pipetted up and down 6 times to mix.
- Tube was placed on the magnetic stand at room temperature for 5 minutes.
- 1 µl of supernatant was transferred to a TapeStation strip for mRNA QC
- The remaining volume was gently pipetted up and down 6 times to mix.
- Tube was placed in a thermal cycler and the following program was run to elute, fragment, and prime the RNA:
  - Preheat lid set to 100°C
  - 94°C for 12 minutes
  - Hold at 4°C
- When thermal cycler reaches 4°C, tube was removed and centrifuge briefly.

5.2.2 First Strand cDNA synthesis

- Tube was placed on the magnetic stand at room temperature for 5 minutes. 17 µl supernatant was transferred to a new PCR tube.

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- First Strand Synthesis Act D Mix and SuperScript II mix (ThermoFisher, USA. CatNo: 18064014) was prepared by adding 1 µl SuperScript II to 9 µl First Strand Synthesis Act D Mix per sample
- 8 µl of First Strand Synthesis Act D Mix and SuperScript II mix was added to the sample. The entire volume was gently pipetted up and down 6 times to mix.
- Tube was placed in a thermal cycler and the following program was run:
  - pre-heat lid option set to 100°C
  - 25°C for 10 minutes
  - 42°C for 15 minutes
  - 70°C for 15 minutes
  - Hold at 4°C
- When the thermal cycler reached 4°C, tube was removed from the thermal cycler and proceed immediately to next step.

5.2.3 Second Strand cDNA synthesis

- 5 µl of Resuspension buffer was added to each sample.
- 20 µl of thawed Second Strand Marking Master Mix was added to the tube. Entire volume was pipetted up and down 6 times to mix thoroughly.
- Tube was place in a thermal cycler and the following program was run:
  - pre-heat lid option set to 30°C
  - 16°C for 60 minutes
- Tube was removed from thermal cycler and placed on bench to bring it to room temperature.

5.2.4 cDNA Purification

- 90 µl of well-mixed room temperature AMPure XP beads were added (Beckman Coulter, USA, CatNo: A63880) to 50 µl of ds cDNA. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Tube was incubated at room temperature for 15 minutes.
- Tube was centrifuged briefly and place on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells. 135 µl supernatant was removed and discarded.
- With the tube on the magnetic stand, 200 µl freshly prepared 80% EtOH was added to the tube without disturbing the beads. Tube was incubated at room temperature for 30 seconds, and then the supernatant was removed and discarded.
- Above step was repeated one time for a total of two 80% EtOH washes.
- Tube was placed on a pre-heated 37°C heat block for 2 minutes to dry the beads.
- 17.5 µl of resuspension buffer was added to the tube. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Tube was incubated at room temperature for 2 minutes.
- Tube was place on the magnetic stand at room temperature for 5 minutes.
- 15 µl supernatant was transferred (ds cDNA) to a new PCR tube.
- Protocol was stopped at this point and continued the next day. The ds cDNA was stored at -15°C to -25°C.

5.2.5 Adenylation of 3' Ends

- 2.5 µl of Resuspension buffer was added to the purified ds cDNA.
- 12.5 µl of thawed A-Tailing Mix was added to the tube. Gently pipetted the entire volume up and down 10 times to mix thoroughly. Centrifuged briefly.
- Tube was placed in a thermal cycler and the following program was run:
  - pre-heat lid option set to 100°C

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- 37°C for 30 minutes
- 70°C for 5 minutes
- Hold at 4°C

- Tube was removed from thermal cycler when it reaches 4°C and proceed immediately to next step.

5.2.6 Adapter Ligation

- 2.5 µl of Resuspension buffer was added to the sample.
- 2.5 µl of Ligation Mix was added to the sample.
- 2.5 µl of thawed RNA Adapter Index was added to the sample as follows.

Randomization method: Barcode names were fed into an Microsoft Excel spreadsheet and each barcode was assigned a random number using the function =rand(). The random numbers were sorted in ascending order along with the barcode names creating a random arrangement of barcode names. 11 barcodes in order were assigned to each sample that is planned to run in the same batch** as per the randomization plan to get random assignment of barcodes to samples in the batch. The randomization function and sorting were rerun 4 more times to assign random barcodes to all the batches. The barcodes generated were assigned to randomized samples that were assigned to be run in each batch to avoid barcode conflict in the runs.

Batch 1:

#	Sample Name	Index	Index Sequence
1	G4B_ID_03	AR020	GTGGCC(T)
2	G2A_LD_02	AR008	ACTTGA(A)
3	G5B_HD_03	AR008	ACTTGA(A)
4	G1A_VC_02	AR027	ATTCCT(T)
5	G2B_LD_05	AR003	TTAGGC(A)
6	G2A_LD_03	AR001	ATCACG(A)
7	G3A_MD_02	AR020	GTGGCC(T)
8	G1B_VC_05	AR001	ATCACG(A)
9	G3A_MD_06	AR022	CGTACG(T)
10	G5A_HD_06	AR027	ATTCCT(T)
11	NTC_1	AR011	GGCTAC(A)

Batch 2:

#	Sample Name	Index	Index Sequence
1	G3B_MD_02	AR009	GATCAG(A)
2	G1A_VC_04	AR022	CGTACG(T)
3	G1B_VC_04	AR027	ATTCCT(T)
4	G2A_LD_05	AR010	TAGCTT(A)
5	G5B_HD_05	AR021	GTTTCG(G)
6	G2B_LD_02	AR003	TTAGGC(A)
7	G4A_ID_06	AR025	ACTGAT(A)
8	G1A_VC_03	AR009	GATCAG(A)
9	G4A_ID_05	AR010	TAGCTT(A)
10	G5B_HD_02	AR011	GGCTAC(A)
11	NTC_2	AR021	GTTTCG(G)

Batch 3:

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#	Sample Name	Index	Index Sequence
1	G2B_LD_03	AR011	GGCTAC(A)
2	G3A_MD_03	AR001	ATCACG(A)
3	G2B_LD_04	AR010	TAGCTT(A)
4	G2A_LD_01	AR009	GATCAG(A)
5	G4B_ID_04	AR001	ATCACG(A)
6	G1B_VC_06	AR008	ACTTGA(A)
7	G1A_VC_05	AR020	GTGGCC(T)
8	G3A_MD_04	AR010	TAGCTT(A)
9	G4B_ID_02	AR023	GAGTGG(A)
10	G5A_HD_03	AR003	TTAGGC(A)
11	NTC_3	AR023	GAGTGG(A)

Batch 4:

	Sample Name	Index	Index Sequence
1	G3B_MD_05	AR009	GATCAG(A)
2	G2A_LD_04	AR022	CGTACG(T)
3	G4B_ID_06	AR020	GTGGCC(T)
4	G1B_VC_02	AR022	CGTACG(T)
5	G2B_LD_01	AR021	GTTTCG(G)
6	G5B_HD_01	AR023	GAGTGG(A)
7	G3B_MD_06	AR027	ATTCCT(T)
8	G5B_HD_04	AR025	ACTGAT(A)
9	G4A_ID_04	AR020	GTGGCC(T)
10	G4B_ID_05	AR008	ACTTGA(A)
11	NTC_4	AR025	ACTGAT(A)

Batch 5:

	Sample Name	Index	Index Sequence
1	G5A_HD_02	AR010	TAGCTT(A)
2	G1A_VC_01	AR001	ATCACG(A)
3	G4A_ID_02	AR021	GTTTCG(G)
4	G3B_MD_04	AR008	ACTTGA(A)
5	G5A_HD_05	AR023	GAGTGG(A)
6	G3A_MD_05	AR025	ACTGAT(A)
7	G3B_MD_03	AR021	GTTTCG(G)
8	G5A_HD_04	AR009	GATCAG(A)
9	G1B_VC_03	AR003	TTAGGC(A)
10	G4A_ID_01	AR023	GAGTGG(A)
11	NTC_5	AR011	GGCTAC(A)

- Gently pipetted the entire volume up and down 10 times to mix thoroughly. Centrifuged briefly.
- Tube was placed in a thermal cycler and the following program was run:
  - pre-heat lid option set to 100°C
  - 30°C for 10 minutes
- Tube was removed from the thermal cycler and 5 µl of thawed Stop Ligation Buffer was added to inactivate the ligation. Gently pipetted the entire volume up and down 10 times to mix thoroughly.

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5.2.7 Clean Up of Adapter Ligated Library

- 42 µl of well-mixed room temperature AMPure XP beads (Beckman Coulter, USA, CatNo: A63880) were added to the sample. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Incubated at room temperature for 15 minutes.
- Centrifuged the tube briefly and placed on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells. All the supernatant was removed and discarded.
- With the tube on the magnetic stand, 200 µl freshly prepared 80% EtOH was added to the tube without disturbing the beads. Incubated at room temperature for 30 seconds, and then removed and discarded all of the supernatant.
- The above steps were repeat one time for a total of two 80% EtOH washes.
- Tube was placed on a pre-heated 37°C heat block for 2 minutes to dry the beads.
- 52.5 µl room temperature Resuspension Buffer was added to the tube. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Incubated at room temperature for 2 minutes.
- Tube was placed on the magnetic stand at room temperature for 5 minutes.
- 50 µl supernatant was transferred to a new tube.
- 50 µl of well-mixed room temperature AMPure XP beads (Beckman Coulter, USA, CatNo: A63880) were added to the sample. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Incubated at room temperature for 15 minutes.
- Centrifuged the tube briefly and placed on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells. All the supernatant was removed and discarded
- With the tube on the magnetic stand, 200 µl freshly prepared 80% EtOH was added to the tube without disturbing the beads. Incubated at room temperature for 30 seconds, and then removed and discarded all of the supernatant.
- The above steps were repeat one time for a total of two 80% EtOH washes.
- Tube was placed on a pre-heated 37°C heat block for 2 minutes to dry the beads.
- 22.5 µl room temperature Resuspension Buffer was added to the tube. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Incubated at room temperature for 2 minutes.
- Tube was placed on the magnetic stand at room temperature for 5 minutes.
- 20 µl of the supernatant was transferred to a new PCR tube.

5.2.8 Enrichment of DNA Fragments

- 5 µl of thawed PCR Primer Cocktail was added to the sample tube.
- 25 µl of thawed PCR Master Mix was added to the tube. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Tube was placed in a thermal cycler the following program was run:
    a. pre-heat lid option set to 100°C
    b. 98°C for 30 seconds
    c. 12 cycles of:
        - 98°C for 10 seconds
        - 60°C for 30 seconds
        - 72°C for 30 seconds
    d. 72°C for 5 minutes
    e. Hold at 4°C

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- Tube was removed from thermal cycler when it reached 4°C and proceed to clean-up.

5.2.9 Clean Up of PCR Product

- 40 µl of well-mixed room temperature AMPure XP beads (Beckman Coulter, USA, CatNo: A63880) were added to the sample. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Incubated at room temperature for 15 minutes.
- Centrifuged the tube briefly and placed on the magnetic stand at room temperature, for 5 minutes to make sure that all of the beads are bound to the side of the wells. All the supernatant was removed and discarded
- With the tube on the magnetic stand, 200 µl freshly prepared 80% EtOH was added to the tube without disturbing the beads. Incubated at room temperature for 30 seconds, and then removed and discarded all of the supernatant.
- The above steps were repeated one time for a total of two 80% EtOH washes.
- Tube was placed on a pre-heated 37°C heat block for 2 minutes to dry the beads.
- 22.5 µl room temperature Resuspension Buffer was added to the tube. Gently pipetted the entire volume up and down 10 times to mix thoroughly.
- Incubated the tube at room temperature for 2 minutes.
- Tube was placed on the magnetic stand at room temperature for 5 minutes.
- 20 µl of the supernatant was transferred to a new tube.

5.2.10 Sequencing of transcriptome libraries

Randomization method: **Sample replicate names for each control/treatment were fed into an Microsoft Excel spreadsheet and replicates from each treatment/control were assigned a random number using the function =rand(). The random numbers were sorted in ascending order for each control/treatment along with the replicate number creating a random arrangement of replicates. All the randomized sample replicate names were transposed and arranged in order to get 5 batches with 1 sample per batch of biological replicate from each control/treatment and 1 NTC.

All the transcriptome libraries passed QC. Libraries were loaded on an Illumina NextSeq 500 for sequencing in 5 batches as follows:

5.2.10.1 Dilution and pooling of libraries

1. For each batch of sequencing, libraries were diluted to 4nm by taking 2µL of library and adding appropriate amount of nuclease free water
2. Diluted libraries were further quantified by qubit and suitable quantity of diluted libraries were pooled to make final pool for sequencing.
3. 5µL of final pool was mixed with 5µL of 0.2N NaOH and incubated for 5 min at room temperature to denature the library to single strands
4. 5µL of 200mM TrisHCl(Ph 7) was added to the mixture to neutralise the NaOH
5. 985µL of pre-chilled HT1 buffer was added to the mixture to make 20pM of denatured library.
6. 105 µL of denatured 20pM library was taken to a new tube and added with 1395 µL of HT1 buffer to create 1.4 pM library
7. Step 3 to 6 was repeated for 4nM phiX library make 1.4pM phiX library
8. 1.425 ml of 1.4 pM library was mixed with 75 µL of 1.4 pM phiX library to make 1.5 ml library
9. 1.3 ml of 1.4 pM library was loaded to NextSeq500 cartridge and sequenced using 1X75 sequencing chemistry

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Batch 1	Sample Name	Adapter Name	Adapter sequence	Qubit nM	volume of lib	volume of NFW to make 4nM	nM of diluted libraries	volume of 4 nM diluted lib to make 4 nM pool
1	G1A_VC_02	AR027	ATTCCT(T)	154.1	2	75.07	5.2	4.0
2	G2A_LD_04	AR022	CGTACG(T)	118.8	2	57.40	3.7	5.7
3	G3A_MD_02	AR020	GTGGCC(T)	124.3	2	60.17	5.3	4.0
4	G4A_ID_02	AR021	GTTTCG(G)	190.3	2	93.16	3.3	6.4
5	G5A_HD_03	AR003	TTAGGC(A)	227.3	2	111.64	3.5	6.0
6	G1B_VC_06	AR008	ACTTGA(A)	178.3	2	87.13	4.0	5.2
7	G2B_LD_04	AR010	TAGCTT(A)	124.2	2	60.12	4.0	5.3
8	G3B_MD_02	AR009	GATCAG(A)	96.9	2	46.44	5.1	4.1
9	G4B_ID_04	AR001	ATCACG(A)	198.2	2	97.11	3.6	5.9
10	G5B_HD_04	AR025	ACTGAT(A)	178.5	2	87.25	4.1	5.1
11	NTC_3	AR023	GAGTGG(A)	-	5	0		5

Batch 2	Sample Name	Adapter Name	Adapter sequence	Qubit nM	volume of lib	volume of NFW to make 4nM	nM of diluted libraries	volume of 4 nM diluted lib to make 4 nM pool
1	G1A_VC_05	AR020	GTGGCC(T)	194.1	2	95.06	4.9	4.3
2	G2A_LD_05	AR010	TAGCTT(A)	126.0	2	61.00	6.3	3.3
3	G3A_MD_06	AR022	CGTACG(T)	156.8	2	76.41	6.9	3.0
4	G4A_ID_06	AR025	ACTGAT(A)	152.1	2	74.03	5.9	3.5
5	G5A_HD_04	AR009	GATCAG(A)	139.1	2	67.57	5.4	3.9
6	G1B_VC_05	AR001	ATCACG(A)	160.6	2	78.31	5.7	3.7
7	G2B_LD_03	AR011	GGCTAC(A)	140.2	2	68.08	5.0	4.2
8	G3B_MD_06	AR027	ATTCCT(T)	128.2	2	62.12	7.2	2.9
9	G4B_ID_05	AR008	ACTTGA(A)	168.6	2	82.29	6.8	3.1
10	G5B_HD_01	AR023	GAGTGG(A)	149.4	2	72.72	4.3	4.9
11	NTC_2	AR021	GTTTCG(G)	-	5	0		5

Batch 3	Sample Name	Adapter Name	Adapter sequence	Qubit nM	volume of lib	volume of NFW to make 4nM	nM of diluted libraries	volume of 4 nM diluted lib to make 4 nM pool
1	G1A_VC_04	AR022	CGTACG(T)	177.6	2	86.82	4.9	4.3
2	G2A_LD_01	AR009	GATCAG(A)	188.3	2	92.13	4.3	4.9
3	G3A_MD_03	AR001	ATCACG(A)	136.5	2	66.23	4.2	5.0
4	G4A_ID_05	AR010	TAGCTT(A)	127.0	2	61.52	5.7	3.7
5	G5A_HD_05	AR023	GAGTGG(A)	202.0	2	99.01	3.7	5.6
6	G1B_VC_04	AR027	ATTCCT(T)	126.0	2	61.00	6.0	3.5
7	G2B_LD_02	AR003	TTAGGC(A)	91.4	2	43.68	6.1	3.5
8	G3B_MD_03	AR021	GTTTCG(G)	95.6	2	45.81	3.9	5.4
9	G4B_ID_03	AR020	GTGGCC(T)	143.8	2	69.88	6.3	3.3
10	G5B_HD_03	AR008	ACTTGA(A)	169.3	2	82.64	6.6	3.2

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11	NTC_1	AR011	GGCTAC(A)	-	5	0		5

Batch 4	Sample Name	Adapter Name	Adapter sequence	Qubit nM	volume of lib	volume of NFW to make 4nM	nM of diluted libraries	volume of 4 nM diluted lib to make 4 nM pool
1	G1A_VC_03	AR009	GATCAG(A)	102.4	2	49.20	2.9	2.9
2	G2A_LD_03	AR001	ATCACG(A)	151.0	2	73.50	2.4	2.4
3	G3A_MD_04	AR010	TAGCTT(A)	200.6	2	98.28	3.8	3.8
4	G4A_ID_01	AR023	GAGTGG(A)	194.4	2	95.18	3.4	3.4
5	G5A_HD_06	AR027	ATTCCT(T)	175.8	2	85.89	2.9	2.9
6	G1B_VC_03	AR003	TTAGGC(A)	112.2	2	54.10	4.0	4.0
7	G2B_LD_01	AR021	GTTTCG(G)	119.5	2	57.77	3.6	3.6
8	G3B_MD_04	AR008	ACTTGA(A)	121.2	2	58.61	4.4	4.4
9	G4B_ID_06	AR020	GTGGCC(T)	122.7	2	59.35	4.1	4.1
10	G5B_HD_02	AR011	GGCTAC(A)	130.6	2	63.32	3.4	3.4
11	NTC_4	AR025	ACTGAT(A)	-	5	0		5

Batch 5	Sample Name	Adapter Name	Adapter sequence	Qubit nM	volume of lib	volume of NFW to make 4nM	nM of diluted libraries	volume of 4 nM diluted lib to make 4 nM pool
1	G1A_VC_01	AR001	ATCACG(A)	224.5	2	110.25	3.4	6.1
2	G2A_LD_02	AR008	ACTTGA(A)	149.4	2	72.71	5.6	3.7
3	G3A_MD_05	AR025	ACTGAT(A)	168.4	2	82.18	3.4	6.2
4	G4A_ID_04	AR020	GTGGCC(T)	155.1	2	75.56	4.0	5.3
5	G5A_HD_02	AR010	TAGCTT(A)	197.8	2	96.88	3.4	6.2
6	G1B_VC_02	AR022	CGTACG(T)	140.4	2	68.18	4.5	4.7
7	G2B_LD_05	AR003	TTAGGC(A)	144.1	2	70.05	6.3	3.4
8	G3B_MD_05	AR009	GATCAG(A)	139.3	2	67.66	3.7	5.7
9	G4B_ID_02	AR023	GAGTGG(A)	217.4	2	106.68	3.9	5.4
10	G5B_HD_05	AR021	GTTTCG(G)	199.4	2	97.71	3.7	5.7
11	NTC_5	AR011	GGCTAC(A)	-	5	0		5

5.2.11 De-multiplexing and data QC

- The data was demultiplexed using bcl2fastq v2.20 and checked for Quality using software named FASTQC v0.11.5.
- Summary for all samples was created using MultiQC v1.2

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6. Results

6.1 RNA QC Results

6.1.1 Qubit readings and yield of RNA for each sample:

Batch 1:

Maxwell RSC simplyRNA Tissue Kit Lot#151409

#	Animal No.	Tissue Replicate	Tissue Weight (g)	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)
1	Ru5691	a	0.030	G2A_LD_05	728	36.4
2	Ru5681	a	0.022	G1A_VC_01	1120	56
3	Ru5713	a	0.023	G1B_VC_03	1230	61.5
4	Ru5720	a	0.030	G2B_LD_04	784	39.2
5	Ru5689	a	0.030	G2A_LD_03	826	41.3
6	Ru5687	a	0.026	G2A_LD_01	344	17.2
7	Ru5733	a	0.026	G4B_ID_05	1080	54
8	Ru5738	a	0.023	G5B_HD_04	1320	66
9	Ru5723	a	0.028	G3B_MD_01	1280	64
10	Ru5707	a	0.022	G5A_HD_03	1200	60

Batch 2:

Maxwell RSC simplyRNA Tissue Kit Lot#151409

#	Animal No.	Tissue Replicate	Tissue Weight (g)	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)
1	Ru5712	a	0.030	G1B_VC_02	1160	58
2	Ru5703	a	0.029	G4A_ID_05	970	48.5
3	Ru5724	a	0.027	G3B_MD_02	1090	54.5
4	Ru5716	a	0.025	G1B_VC_06	660	33
5	Ru5695	a	0.023	G3A_MD_03	1100	55
6	Ru5692	a	0.030	G2A_LD_06	916	45.8
7	Ru5727	a	0.022	G3B_MD_05	1020	51
8	Ru5717	a	0.028	G2B_LD_01	800	40
9	Ru5737	a	0.025	G5B_HD_03	1190	59.5
10	Ru5686	a	0.025	G1A_VC_06	1170	58.5

Batch 3:

Maxwell RSC simplyRNA Tissue Kit Lot#151409

#	Animal No.	Tissue Replicate	Tissue Weight (g)	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)
1	Ru5702	a	0.026	G4A_ID_04	1280	64
2	Ru5731	a	0.022	G4B_ID_03	834	41.7
3	Ru5726	a	0.029	G3B_MD_04	1240	62
4	Ru5688	a	0.028	G2A_LD_02	998	49.9
5	Ru5685	a	0.030	G1A_VC_05	648	32.4
6	Ru5700	a	0.027	G4A_ID_02	924	46.2
7	Ru5736	a	0.027	G5B_HD_02	872	43.6
8	Ru5710	a	0.026	G5A_HD_06	1320	66

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9	Ru5693	a	0.024	G3A_MD_01	922	46.1
10	Ru5698	a	0.022	G3A_MD_06	750	37.5

Batch 4:
Maxwell RSC simplyRNA Tissue Kit Lot#151409

#	Animal No.	Tissue Replicate	Tissue Weight (g)	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)
1	Ru5708	a	0.029	G5A_HD_04	1030	51.5
2	Ru5709	a	0.024	G5A_HD_05	1060	53
3	Ru5721	a	0.029	G2B_LD_05	1070	53.5
4	Ru5683	a	0.024	G1A_VC_03	1060	53
5	Ru5718	a	0.024	G2B_LD_02	1400	70
6	Ru5732	a	0.023	G4B_ID_04	1220	61
7	Ru5699	a	0.029	G4A_ID_01	1100	55
8	Ru5684	a	0.028	G1A_VC_04	1130	56.5
9	Ru5728	a	0.025	G3B_MD_06	1100	55
10	Ru5682	a	0.030	G1A_VC_02	932	46.6

Batch 5:
Maxwell RSC simplyRNA Tissue Kit Lot#159615

#	Animal No.	Tissue Replicate	Tissue Weight (g)	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)
1	Ru5705	a	0.027	G5A_HD_01	3060	153
2	Ru5715	a	0.024	G1B_VC_05	1960	98
3	Ru5694	a	0.028	G3A_MD_02	2160	108
4	Ru5729	a	0.030	G4B_ID_01	1650	82.5
5	Ru5739	a	0.026	G5B_HD_05	820	41
6	Ru5735	a	0.021	G5B_HD_01	1690	84.5
7	Ru5730	a	0.027	G4B_ID_02	1590	79.5
8	Ru5697	a	0.025	G3A_MD_05	2020	101
9	Ru5725	a	0.023	G3B_MD_03	1530	76.5
10	Ru5734	b	0.030	G4B_ID_06	832	41.6

Batch 6:
Maxwell RSC simplyRNA Tissue Kit Lot#159615

#	Animal No.	Tissue Replicate	Tissue Weight (g)	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)
51	Ru5690	a	0.027	G2A_LD_04	692	34.6
52	Ru5714	a	0.022	G1B_VC_04	1390	69.5
53	Ru5722	a	0.026	G2B_LD_06	1250	62.5
54	Ru5696	a	0.030	G3A_MD_04	888	44.4
55	Ru5719	a	0.030	G2B_LD_03	540	27
56	Ru5740	a	0.026	G5B_HD_06	1280	64
57	Ru5706	a	0.023	G5A_HD_02	1430	71.5
58	Ru5704	a	0.027	G4A_ID_06	340	17
59	Ru5701	a	0.025	G4A_ID_03	1270	63.5
60	Ru5711	a	0.025	G1B_VC_01	1370	68.5

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6.1.2 RNA QC Summary

Out of the 6 replicates in each group, the replicate with lowest RIN in the group was omitted for transcriptome library preparation. The omitted replicate is shaded grey in the summary table below.

#	Animal No.	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)	RIN
1	Ru5681	G1A_VC_01	1120	56	8.9
2	Ru5682	G1A_VC_02	932	46.6	9.3
3	Ru5683	G1A_VC_03	1060	53	9.3
4	Ru5684	G1A_VC_04	1130	56.5	9.4
5	Ru5685	G1A_VC_05	648	32.4	9.2
6	Ru5686	G1A_VC_06	1170	58.5	8.7
1	Ru5687	G2A_LD_01	344	17.2	9.0
2	Ru5688	G2A_LD_02	998	49.9	9.5
3	Ru5689	G2A_LD_03	826	41.3	9.0
4	Ru5690	G2A_LD_04	692	34.6	8.8
5	Ru5691	G2A_LD_05	728	36.4	8.9
6	Ru5692	G2A_LD_06	916	45.8	8.8
1	Ru5693	G3A_MD_01	922	46.1	8.6
2	Ru5694	G3A_MD_02	2160	108	8.7
3	Ru5695	G3A_MD_03	1100	55	8.7
4	Ru5696	G3A_MD_04	888	44.4	8.7
5	Ru5697	G3A_MD_05	2020	101	9.6
6	Ru5698	G3A_MD_06	750	37.5	8.9
1	Ru5699	G4A_ID_01	1100	55	9.4
2	Ru5700	G4A_ID_02	924	46.2	9.2
3	Ru5701	G4A_ID_03	1270	63.5	8.4
4	Ru5702	G4A_ID_04	1280	64	8.8
5	Ru5703	G4A_ID_05	970	48.5	8.5
6	Ru5704	G4A_ID_06	340	17	9
1	Ru5705	G5A_HD_01	3060	153	8.3
2	Ru5706	G5A_HD_02	1430	71.5	8.5
3	Ru5707	G5A_HD_03	1200	60	8.6
4	Ru5708	G5A_HD_04	1030	51.5	9.2
5	Ru5709	G5A_HD_05	1060	53	9.2
6	Ru5710	G5A_HD_06	1320	66	8.8
1	Ru5711	G1B_VC_01	1370	68.5	7.7
2	Ru5712	G1B_VC_02	1160	58	8.8
3	Ru5713	G1B_VC_03	1230	61.5	8.6
4	Ru5714	G1B_VC_04	1390	69.5	8.3
5	Ru5715	G1B_VC_05	1960	98	7.8
6	Ru5716	G1B_VC_06	660	33	8.6
1	Ru5717	G2B_LD_01	800	40	8.6

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#	Animal No.	SAMPLE NAME	QUBIT READING (ng/µL)	Total RNA Yield (µg)	RIN
2	Ru5718	G2B_LD_02	1400	70	8.6
3	Ru5719	G2B_LD_03	540	27	8.7
4	Ru5720	G2B_LD_04	784	39.2	8.8
5	Ru5721	G2B_LD_05	1070	53.5	8.6
6	Ru5722	G2B_LD_06	1250	62.5	8.3
1	Ru5723	G3B_MD_01	1280	64	8.5
2	Ru5724	G3B_MD_02	1090	54.5	8.8
3	Ru5725	G3B_MD_03	1530	76.5	8.7
4	Ru5726	G3B_MD_04	1240	62	8.6
5	Ru5727	G3B_MD_05	1020	51	8.7
6	Ru5728	G3B_MD_06	1100	55	9.1
1	Ru5729	G4B_ID_01	1650	82.5	8.2
2	Ru5730	G4B_ID_02	1590	79.5	8.6
3	Ru5731	G4B_ID_03	834	41.7	8.9
4	Ru5732	G4B_ID_04	1220	61	8.8
5	Ru5733	G4B_ID_05	1080	54	8.9
6	Ru5734	G4B_ID_06	832	41.6	8.7
1	Ru5735	G5B_HD_01	1690	84.5	9.1
2	Ru5736	G5B_HD_02	872	43.6	9.3
3	Ru5737	G5B_HD_03	1190	59.5	9
4	Ru5738	G5B_HD_04	1320	66	9.2
5	Ru5739	G5B_HD_05	820	41	9.3
6	Ru5740	G5B_HD_06	1280	64	8.6

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Study Number: G16084
6.2 Library QC

6.2.1 Library QC Summary

Batch #	Library Name	Fragment analysis- Observed Size Range (bp)	Average Fragment size (bp) based on tapestation profile	Fragment analysis- Presence of adapter dimer	Qubit (High Sensitivity) Reading (ng/µL)	Nanomolarity (nM)
Batch 1	G4B_ID_03	175-686	293	NO	27.8	143.8
	G2A_LD_02	171-677	288	NO	28.4	149.4
	G5B_HD_03	169-676	290	NO	32.4	169.3
	G1A_VC_02	164-677	289	NO	29.4	154.1
	G2B_LD_05	165-652	286	NO	27.2	144.1
	G2A_LD_03	164-651	293	NO	29.2	151.0
	G3A_MD_02	169-590	290	NO	23.8	124.3
	G1B_VC_05	161-594	283	NO	30	160.6
	G3A_MD_06	163-655	286	NO	29.6	156.8
	G5A_HD_06	165-584	281	NO	32.6	175.8
	NTC_1	-	-	YES	Too Low – Out of range	N/A
Batch 2	G3B_MD_02	172-660	294	NO	18.8	96.9
	G1A_VC_04	165-629	290	NO	34	177.6
	G1B_VC_04	172-645	291	NO	24.2	126.0
	G2A_LD_05	163-605	279	NO	23.2	126.0
	G5B_HD_05	166-649	278	NO	20.8	113.4
	G2B_LD_02	167-685	289	NO	21.2	111.1
	G4A_ID_06	167-647	281	NO	28.2	152.1
	G1A_VC_03	171-632	290	NO	19.6	102.4
	G4A_ID_05	169-644	291	NO	24.4	127.0
	G5B_HD_02	165-639	283	NO	24.4	130.6
	NTC_2	-	-	YES	Too Low – Out of range	N/A
Batch 3	G2B_LD_03	170-758	307	NO	28.4	140.2
	G3A_MD_03	170-747	302	NO	27.2	136.5
	G2B_LD_04	165-738	300	NO	24.6	124.2
	G2A_LD_01	167-758	301	NO	37.4	188.3
	G4B_ID_04	154-690	292	NO	38.2	198.2
	G1B_VC_06	165-699	289	NO	34	178.3
	G1A_VC_05	156-685	281	NO	36	194.1
	G3A_MD_04	156-649	275	NO	36.4	200.6
	G4B_ID_02	165-659	283	NO	40.6	217.4
	G5A_HD_03	150-644	276	NO	41.4	227.3
	NTC_3	-		YES	Too Low – Out of range	N/A
Batch 4	G3B_MD_05	171-683	298	NO	27.4	139.3
	G2A_LD_04	180-696	301	NO	23.6	118.8
	G4B_ID_06	178-596	284	NO	23	122.7
	G1B_VC_02	173-602	285	NO	26.4	140.4
	G2B_LD_01	176-641	289	NO	22.8	119.5
	G5B_HD_01	175-671	292	NO	28.8	149.4

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Batch #	Library Name	Fragment analysis- Observed Size Range (bp)	Average Fragment size (bp) based on tapestation profile	Fragment analysis- Presence of adapter dimer	Qubit (High Sensitivity) Reading (ng/µL)	Nanomolarity (nM)
	G3B_MD_06	180-663	293	NO	24.8	128.2
	G5B_HD_04	174-625	292	NO	34.4	178.5
	G4A_ID_04	174-669	295	NO	30.2	155.1
	G4B_ID_05	174-655	293	NO	32.6	168.6
	NTC_4	-	-	YES	Too Low – Out of range	N/A
Batch 5	G5A_HD_02	179-619	285	NO	37.2	197.8
	G1A_VC_01	179-649	301	NO	44.6	224.5
	G4A_ID_02	179-599	285	NO	35.8	190.3
	G3B_MD_04	182-687	310	NO	24.8	121.2
	G5A_HD_05	176-660	306	NO	40.8	202.0
	G3A_MD_05	180-656	306	NO	34	168.4
	G3B_MD_03	178-670	309	NO	19.5	95.6
	G5A_HD_04	176-649	294	NO	27	139.1
	G1B_VC_03	180-589	289	NO	21.4	112.2
	G4A_ID_01	178-600	290	NO	37.2	194.4
	NTC_5	-	-	NO	Too Low – Out of range	N/A

APPENDIX 10 Contd. Toxicogenomics Phase Report

6.3 Sequence results and run summary

Sequencing run summary for all the runs

Sl No	Run name	Raw data folder name	Read Length	Date	Pool Concentration	% phiX	pM Loaded	Cluster Density (k/mm2)	Aligned % (phiX)	Data Obtained (GB)	% Q30
1	Batch 1	180308_NB501695_0015_AHTH3HBGX3	75x1	08/Mar/18	4	5	1.4	197	4.83	39.7	95.5
2	Batch 2	180309_NB501695_0016_AHKV2VBGX5	75x1	09/Mar/18	2	5	1.4	217	0.87	41.5	94.1
3	Batch 3	180313_NB501695_0018_AHTH2JBGX3	75x1	13/Mar/18	4	5	1.4	220	2.99	43.9	95.2
4	Batch 4	180315_NB501695_0019_AHTH3CBGX3	75x1	15/Mar/18	4	5	1.4	183	2.8	36.3	95.3
5	Batch 5	180316_NB501695_0020_AHTHCJBGX3	75x1	16/Mar/18	4	5	1.4	212	2.76	42	95

Lot number of reagents used for sequencing run

Run name	Date	Reagent Cartridge (Ref no 15057934) Lot no	Reagent Cartridge expiry date	Buffer Cartridge (Ref no: 15057941) Lot no	Buffer Cartridge expiry date	Flow cell Lot no	Flow cell expiry date	NaOH 10M Lot No	Tris HCl 1M Lot No
BATCH 1	08/Mar/18	20210128	10/Nov/18	20215267	19/Dec/18	20210383	15/Aug/18	72068	170474
BATCH 2	09/Mar/18	20210128	10/Nov/18	20225711	08/Jan/19	20221641	22/Sept/18	72068	170474
BATCH 3	13/Mar/18	20220890	22/Dec/18	20225711	08/Jan/19	20210383	15/Aug/18	72068	170474
BATCH 4	15/Mar/18	20210128	10/Nov/18	20215267	19/Dec/18	20210383	15/Aug/18	72068	170474
BATCH 5	16/Mar/18	20210128	10/Nov/18	20225711	08/Jan/19	20210383	15/Aug/18	72068	170474

Study Number: G16084

6.3 De-multiplexing and data QC

Batch 1 (Run Name: 180308_NB501695_0015_AHTH3HBGX3)

- Illumina NextSeq500 RNA-Seq Single End data
- Final pool concentration: 4 nM
- Final loading concentration: 1.4 pM
- Run details:
  - Flowcell type : 1x75
  - PhiX loading : 5%
  - Cluster density : 197 K/mm2
  - bases >=Q30 = 95.5%
  - PhiX aligned reads = 4.83%

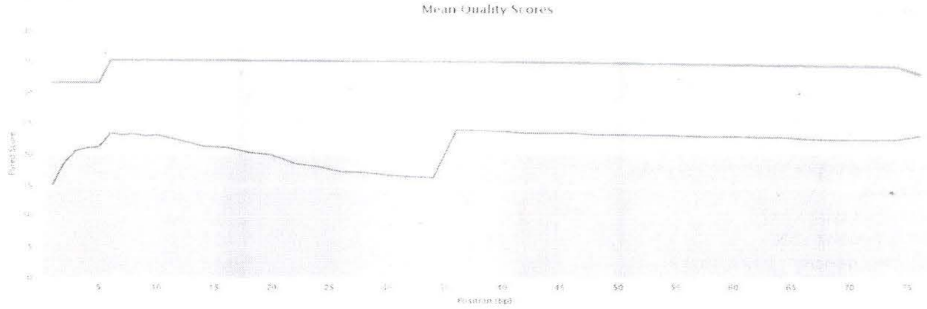
Sample details (10 samples + NTC)

Sample Name	% Duplicates	% GC	Average Read Length	Read Count (in millions)
G1A_VC_02_S1	77.8%	50%	75 bp	51.6
G1B_VC_06_S6	74.8%	50%	75 bp	46.6
G2A_LD_04_S2	76.7%	49%	75 bp	46.2
G2B_LD_04_S7	80.7%	51%	75 bp	45.5
G3A_MD_02_S3	74.2%	49%	75 bp	35.2
G3B_MD_02_S8	75.9%	49%	75 bp	44.9
G4A_ID_02_S4	76.0%	50%	75 bp	42.9
G4B_ID_04_S9	72.1%	50%	75 bp	51.3
G5A_HD_03_S5	67.7%	50%	75 bp	49.6
G5B_HD_04_S10	70.8%	49%	75 bp	40.2
NTC_3_S11	61.9%	72%	61 bp	0.3

Sequence Quality Histogram

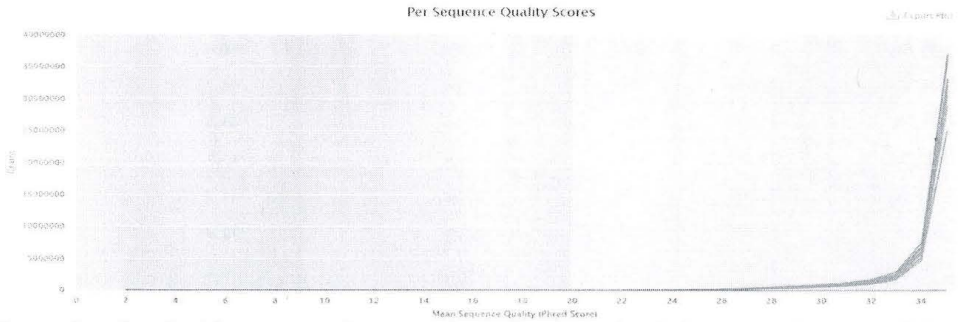
APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084



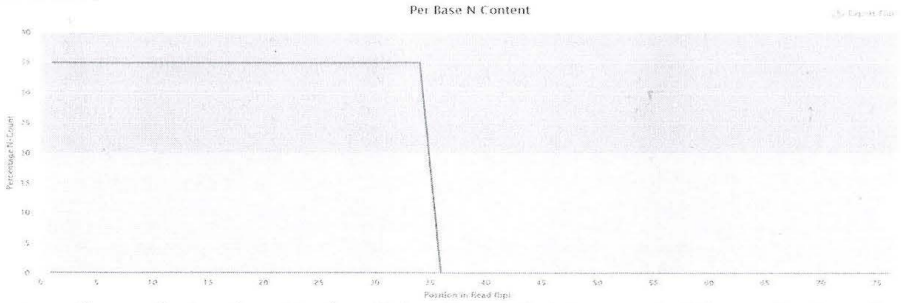
The mean quality value across each base position in the read for all the samples is plotted above. Phred quality score is defined as a property which is logarithmically related to the base calling error probabilities. For example, if Phred assigns a quality score of 20 to a base, the chances that this base is called incorrectly are 1 in 100.

Per sequence quality histograms



The number of reads with average quality scores. Shows if a subset of reads has poor quality. Here, all the samples have a minimum sequence quality of 32, which is good.

Per base N content

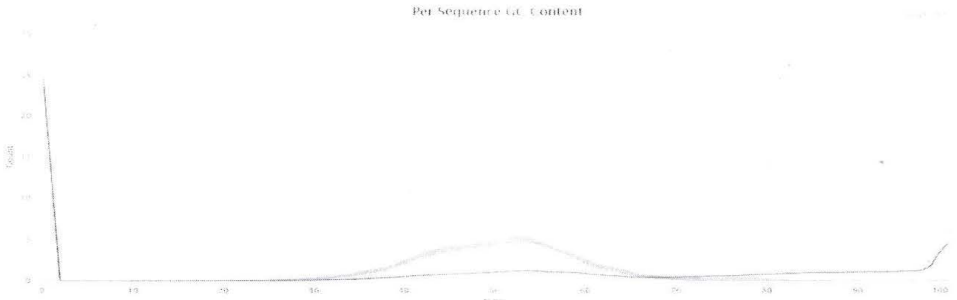


The percentage of base calls at each position for which an N was called. Here, most of the samples do not have N content.

APPENDIX 10 Contd. Toxicogenomics Phase Report

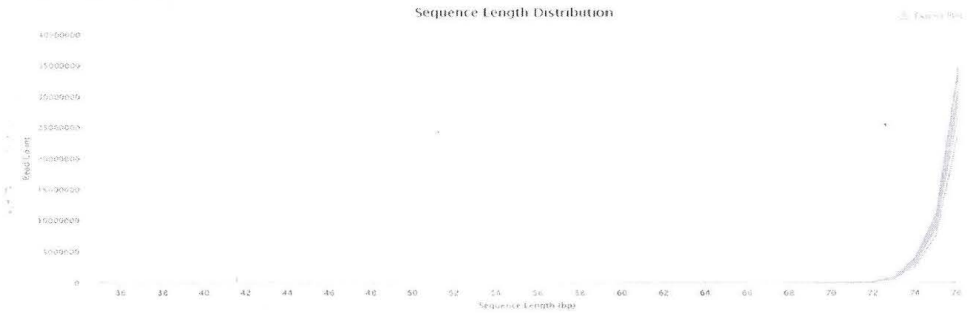
Study Number: G16084

Overall GC percentage



The average GC content of reads. Normal random library typically has a roughly normal distribution of GC content.

Read length distribution

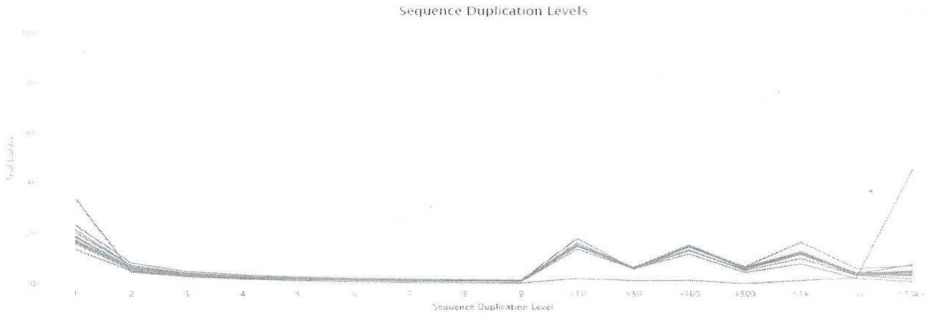


The distribution of fragment sizes (read lengths) found. Variable read length is observed due to adapter trimming process.

Sequence Duplication levels

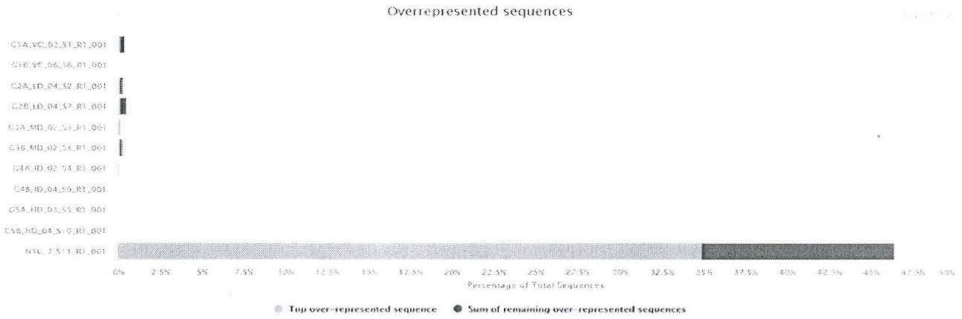
APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084



The relative level of duplication found for every sequence. RNA-Seq is known to have high duplication levels so as to have high coverage/depth of genes.

Over-represented sequences



The total amount of overrepresented sequences found in each library. Here NTC sample is expected to show this pattern due to ERCC Spike-in.

Adapter contamination:

- The cumulative percentage count of the proportion of your library which has seen each of the adapter sequences at each position. Only samples with ≥ 0.1% adapter contamination must be carefully observed for downstream analysis.
- All the 10 samples + NTC sample passed this QC, depicting there is no adapter sequence remaining in the read sequence.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

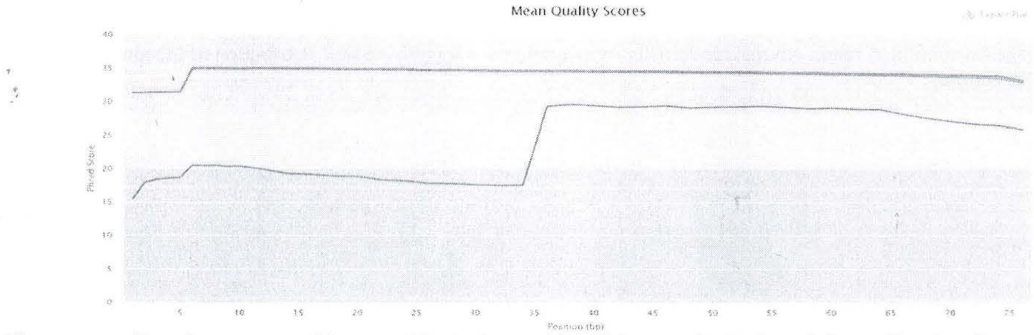
Batch 2 (Run Name: 180309_NB501695_0016_AHKV2VBGX5)

- Illumina NextSeq500 RNA-Seq Single end data
- Final pool concentration : 2 nM
- Final loading concentration : 1.4 pM
- Run details:
  - Flowcell type : 1x75
  - PhiX loading : 5%
  - Cluster density : 217 K/mm2
  - bases >=Q30 = 94.1%
  - PhiX aligned reads = 0.87%

Sample details (10 samples + NTC)

Sample Name	% Duplicates	% GC	Average Read Length	Read Count (in millions)
G1A_VC_05_S1	76.6%	50%	75 bp	53.0
G1B_VC_05_S6	71.4%	49%	75 bp	33.2
G2A_LD_05_S2	78.1%	50%	75 bp	50.3
G2B_LD_03_S7	78.4%	52%	75 bp	49.0
G3A_MD_06_S3	75.8%	49%	75 bp	53.7
G3B_MD_06_S8	75.0%	49%	75 bp	53.5
G4A_ID_06_S4	77.5%	50%	75 bp	55.6
G4B_ID_05_S9	74.7%	49%	75 bp	49.8
G5A_HD_04_S5	74.6%	50%	75 bp	50.9
G5B_HD_01_S10	71.0%	49%	75 bp	46.4
NTC_2_S11	68.6%	62%	57 bp	0.3

Sequence Quality Histograms

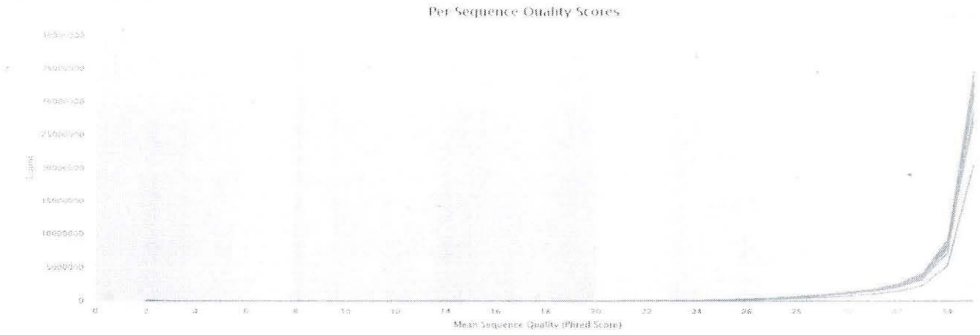


The mean quality value across each base position in the read for all the samples is plotted above. Phred quality score is defined as a property which is logarithmically related to the base calling error probabilities. For example, if Phred assigns a quality score of 20 to a base, the chances that this base is called incorrectly are 1 in 100.

APPENDIX 10 Contd. Toxicogenomics Phase Report

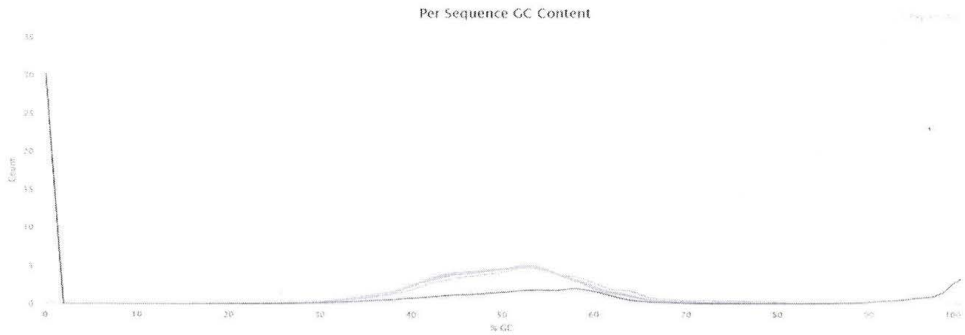
Study Number: G16084

Per sequence quality histograms



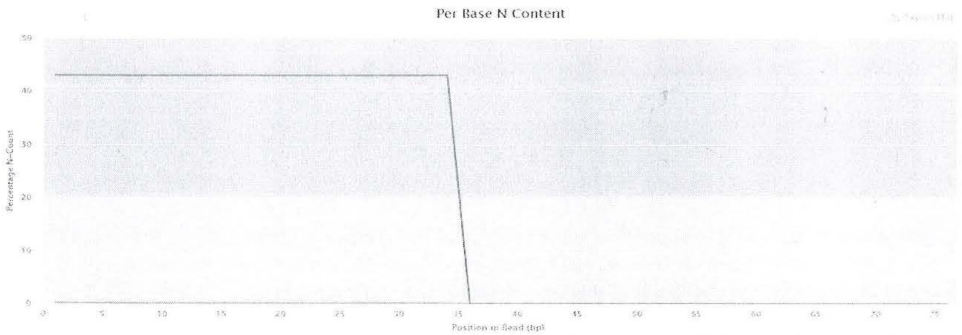
The number of reads with average quality scores. Shows if a subset of reads has poor quality. Here, all the samples have a minimum sequence quality of 32, which is good.

Overall GC percentage



The average GC content of reads. Normal random library typically has a roughly normal distribution of GC content.

Per base N content

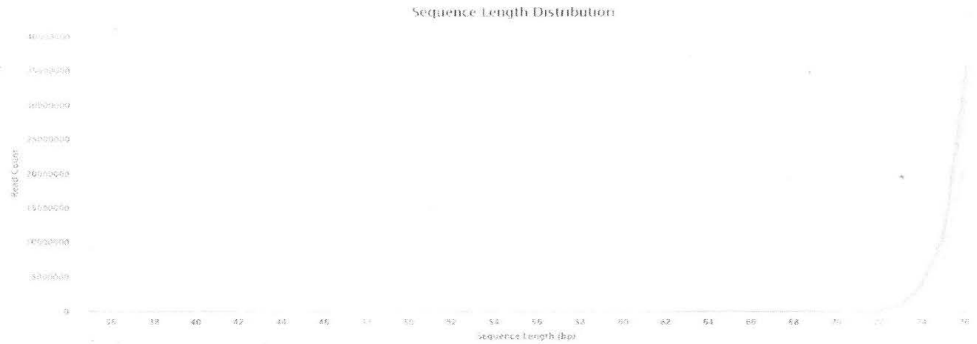


The percentage of base calls at each position for which an N was called. Here, most of the samples do not have N content.

APPENDIX 10 Contd. Toxicogenomics Phase Report

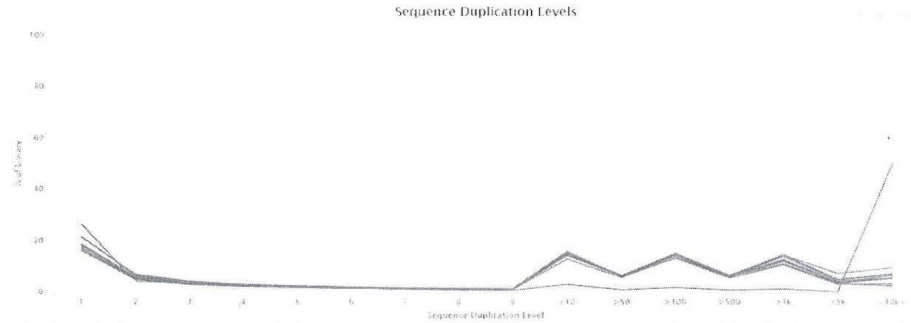
Study Number: G16084

Read length distribution



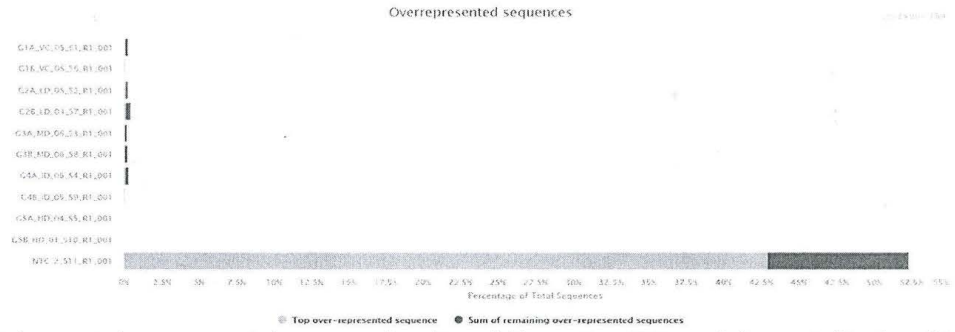
The distribution of fragment sizes (read lengths) found. Variable read length is observed due to adapter trimming process.

Sequence Duplication levels



The relative level of duplication found for every sequence. RNA-Seq is known to have high duplication levels so as to have high coverage/depth of genes.

Over-represented sequences



The total amount of overrepresented sequences found in each library. Here NTC sample is expected to show this pattern due to ERCC Spike-in.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Adapter contamination

- The cumulative percentage count of the proportion of your library which has seen each of the adapter sequences at each position. Only samples with ≥ 0.1% adapter contamination must be carefully observed for downstream analysis.
- All the 10 samples + NTC sample passed this QC, depicting there is no adapter sequence remaining in the read sequence.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

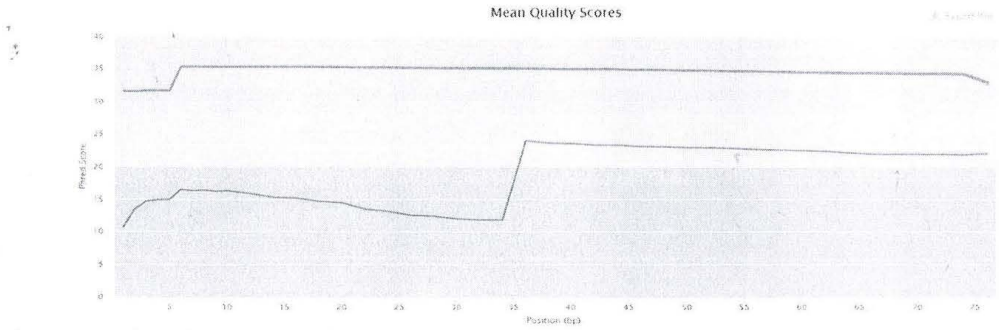
Batch 3 (Run name: 180313_NB501695_0018_AHTH2JBGX3)

- Illumina NextSeq500 RNA-Seq Single end data
- Final pool concentration : 4 nM
- Final loading concentration : 1.4 pM
- Run details:
  - Flowcell type : 1x75
  - PhiX loading : 5%
  - Cluster density : 220 K/mm2
  - bases >=Q30 = 95.2%
  - PhiX aligned reads = 2.99%

Sample details (10 samples + NTC)

Sample Name	% Duplicates	% GC	Average Read Length	Read Count (in millions)
G1A_VC_04_S1	77.5%	50%	75 bp	59.5
G1B_VC_04_S6	76.3%	50%	75 bp	53.9
G2A_LD_01_S2	76.5%	50%	75 bp	54.2
G2B_LD_02_S7	73.9%	50%	75 bp	43.4
G3A_MD_03_S3	74.9%	50%	75 bp	50.7
G3B_MD_03_S8	76.3%	50%	75 bp	46.8
G4A_ID_05_S4	76.2%	49%	75 bp	53.2
G4B_ID_03_S9	75.7%	49%	75 bp	53.4
G5A_HD_05_S5	70.6%	50%	75 bp	41.9
G5B_HD_03_S10	73.2%	50%	75 bp	55.4
NTC_1_S11	76.1%	74%	52 bp	0.2

Sequence quality histograms

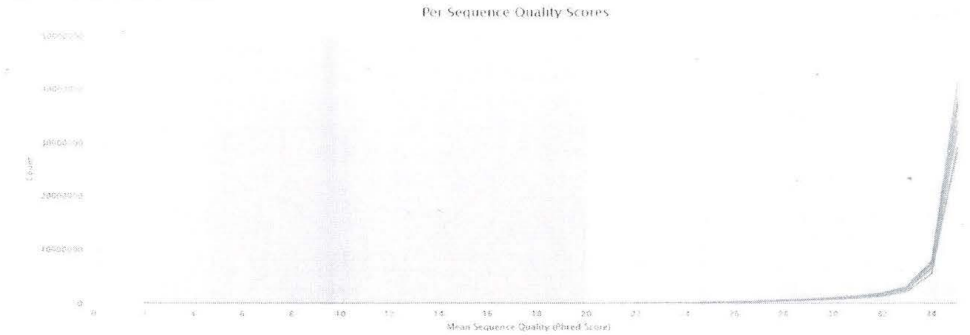


The mean quality value across each base position in the read for all the samples is plotted above. Phred quality score is defined as a property which is logarithmically related to the base calling error probabilities. For example, if Phred assigns a quality score of 20 to a base, the chances that this base is called incorrectly are 1 in 100.

APPENDIX 10 Contd. Toxicogenomics Phase Report

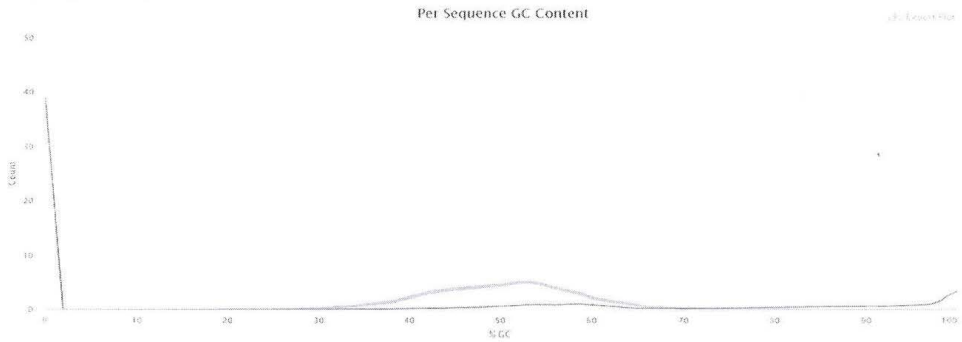
Study Number: G16084

Per sequence quality histograms



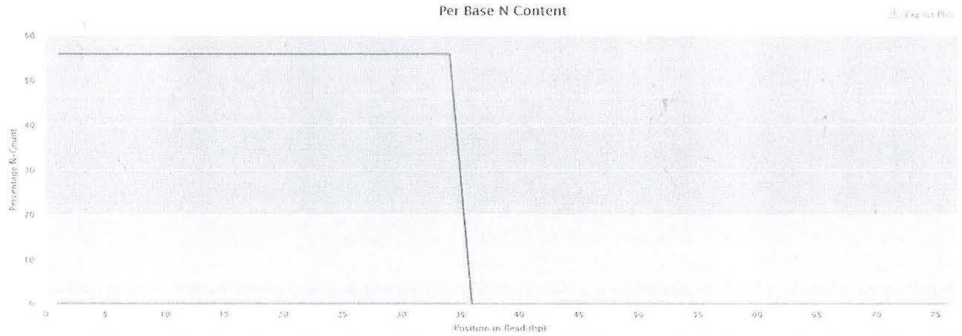
The number of reads with average quality scores. Shows if a subset of reads has poor quality. Here, all the samples have a minimum sequence quality of 32, which is good.

Overall GC percentage



The average GC content of reads. Normal random library typically has a roughly normal distribution of GC content.

Per base N content

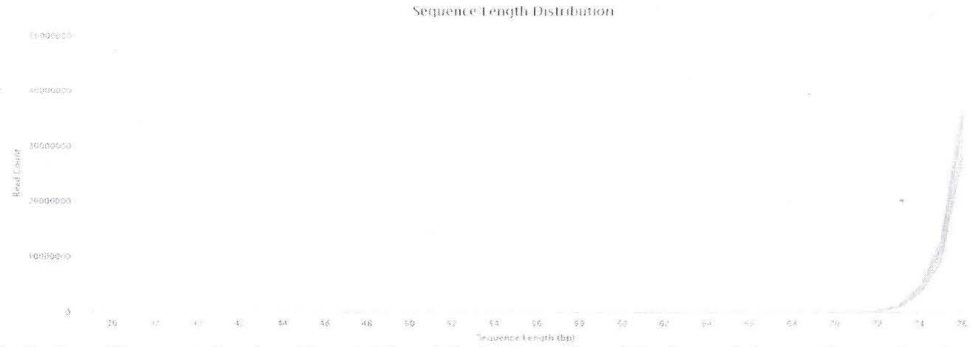


The percentage of base calls at each position for which an N was called. Here, most of the samples do not have N content.

Read length distribution

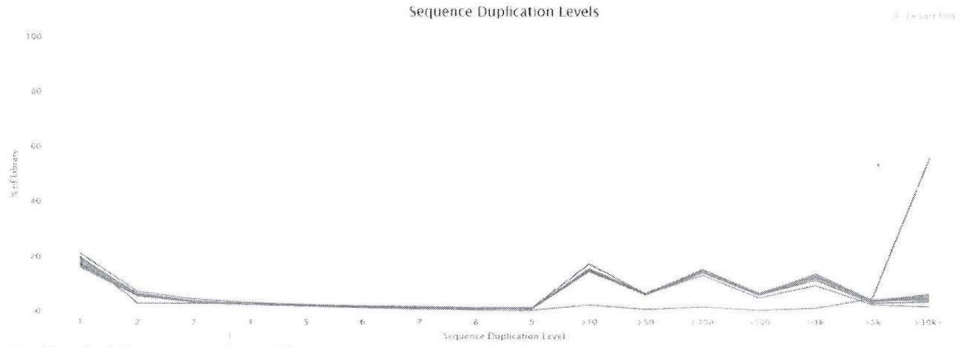
APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084



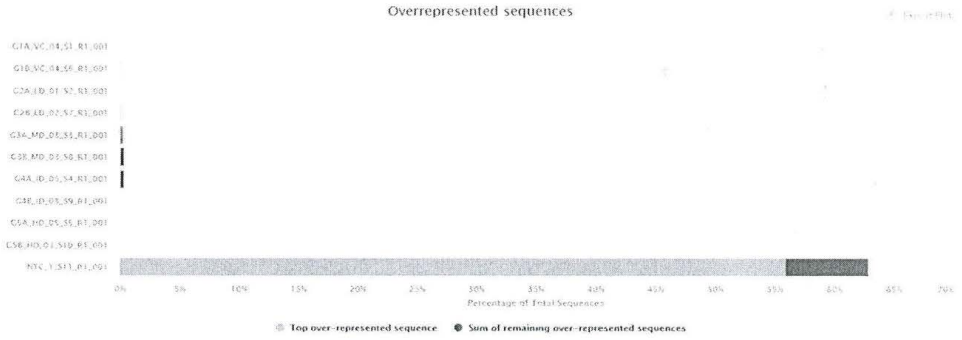
The distribution of fragment sizes (read lengths) found. Variable read length is observed due to adapter trimming process.

Sequence duplication levels



The relative level of duplication found for every sequence. RNA-Seq is known to have high duplication levels so as to have high coverage/depth of genes.

Over-represented sequences



APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084
The total amount of overrepresented sequences found in each library. Here NTC sample is expected to show this pattern due to ERCC Spike-in.

Adapter contamination:

- The cumulative percentage count of the proportion of your library which has seen each of the adapter sequences at each position. Only samples with ≥ 0.1% adapter contamination must be carefully observed for downstream analysis.
- All the 10 samples + NTC sample passed this QC, depicting there is no adapter sequence remaining in the read sequence.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084
Batch 4(Run name: 180315_NB501695_0019_AHTH3CBGX3)

- Illumina NextSeq500 RNA-Seq Single End data
- Final pool concentration : 4 nM
- Final loading concentration : 1.4 pM
- Run details:
  - Flowcell type : 1x75
  - PhiX loading : 5%
  - Cluster density : 183 K/mm2
  - bases >=Q30 = 95.3%
  - PhiX aligned reads = 2.8%

Sample details (10 samples + NTC)

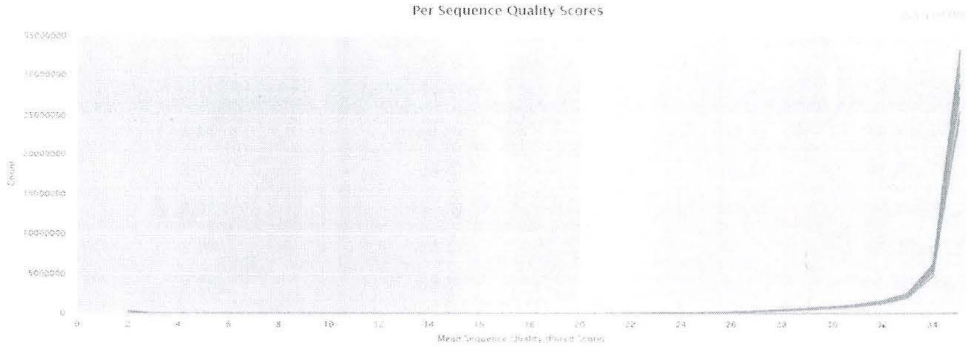
Sample Name	% Duplicates	% GC	Average Read Length	Read Count (in millions)
G1A_VC_03_S1	73.7%	50%	75 bp	44.4
G1B_VC_03_S6	73.9%	50%	75 bp	37.8
G2A_LD_03_S2	77.0%	49%	75 bp	36.3
G2B_LD_01_S7	77.1%	49%	75 bp	41.1
G3A_MD_04_S3	77.9%	49%	75 bp	42.8
G3B_MD_04_S8	77.8%	50%	75 bp	47.6
G4A_ID_01_S4	76.6%	50%	75 bp	46.4
G4B_ID_06_S9	77.3%	49%	75 bp	39.2
G5A_HD_06_S5	68.6%	50%	75 bp	45.8
G5B_HD_02_S10	71.4%	49%	75 bp	42.6
NTC_4_S11	83.1%	56%	47 bp	0.4

Sequence quality histograms:

APPENDIX 10 Contd. Toxicogenomics Phase Report

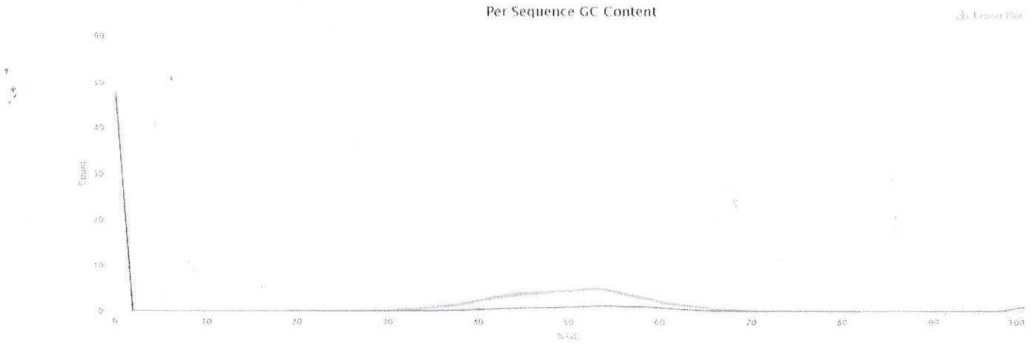
Study Number: G16084
The mean quality value across each base position in the read for all the samples is plotted above. Phred quality score is defined as a property which is logarithmically related to the base calling error probabilities. For example, if Phred assigns a quality score of 20 to a base, the chances that this base is called incorrectly are 1 in 100.

Per sequence quality histograms



The number of reads with average quality scores. Shows if a subset of reads has poor quality. Here, all the samples have a minimum sequence quality of 32, which is good.

Overall GC percentage:

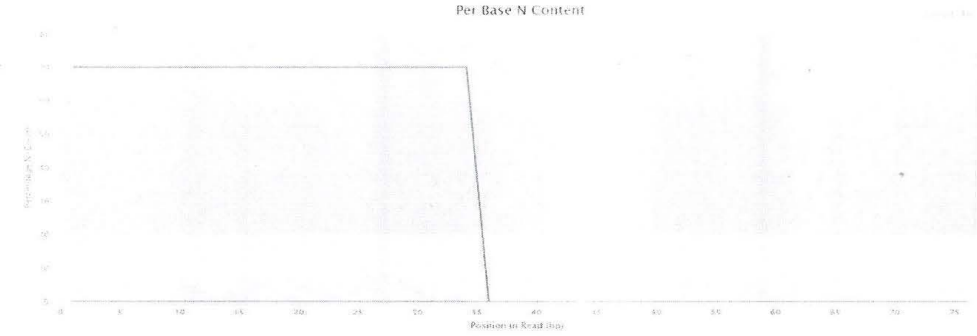


The average GC content of reads. Normal random library typically has a roughly normal distribution of GC content.

APPENDIX 10 Contd. Toxicogenomics Phase Report

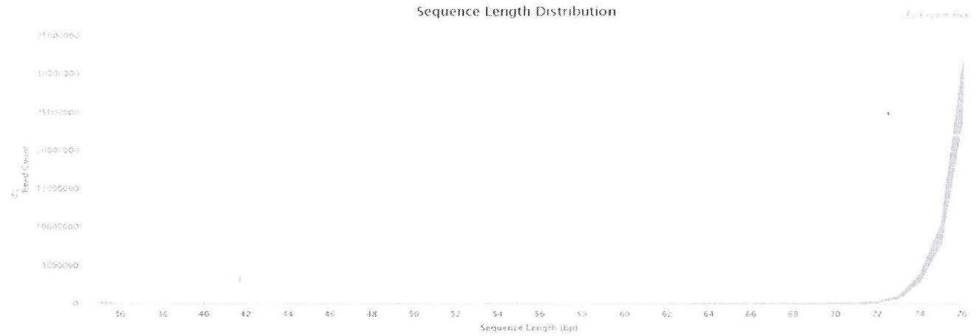
Study Number: G16084

Per base N content:



The percentage of base calls at each position for which an N was called. Here, most of the samples do not have N content.

Read length distribution:

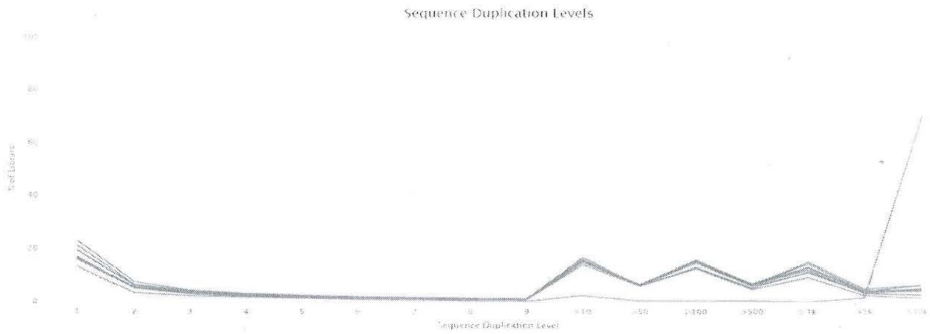


The distribution of fragment sizes (read lengths) found. Variable read length is observed due to adapter trimming process.

APPENDIX 10 Contd. Toxicogenomics Phase Report

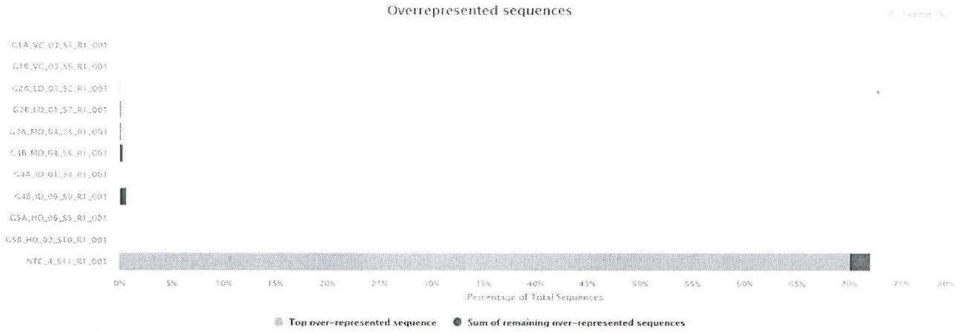
Study Number: G16084

Sequence duplication levels



The relative level of duplication found for every sequence. RNA-Seq is known to have high duplication levels so as to have high coverage/depth of genes.

Over-represented sequences:



The total amount of overrepresented sequences found in each library. Here NTC sample is expected to show this pattern due to ERCC Spike-in.

Adapter contamination:

- The cumulative percentage count of the proportion of your library which has seen each of the adapter sequences at each position. Only samples with ≥ 0.1% adapter

contamination must be carefully observed for downstream analysis.

- All the 10 samples + NTC sample passed this QC, depicting there is no adapter sequence remaining in the read sequence.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084
Batch 5 (Run name: 180316_NB501695_0020_AHTHCJBGX3)

- Illumina NextSeq500 RNA-Seq Single end data
- Final pool concentration : 4 nM
- Final loading concentration : 1.4 pM
- Run details:
  - Flowcell type : 1x75
  - PhiX loading : 5%
  - Cluster density : 212 K/mm2
  - bases >=Q30 = 95%
  - PhiX aligned reads = 2.76%

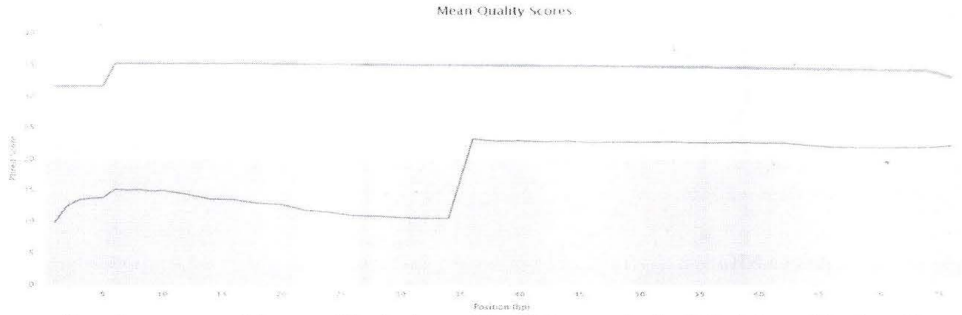
Sample details (10 samples + NTC)

Sample Name	% Duplicates	% GC	Average Read Length	Read Count (in millions)
G1A_VC_01_S1	73.7%	50%	75 bp	47.0
G1B_VC_02_S6	76.8%	49%	75 bp	46.9
G2A_LD_02_S2	75.7%	50%	75 bp	58.1
G2B_LD_05_S7	74.5%	49%	75 bp	46.3
G3A_MD_05_S3	75.7%	50%	75 bp	49.3
G3B_MD_05_S8	72.9%	49%	75 bp	50.7
G4A_ID_04_S4	74.6%	49%	75 bp	47.4
G4B_ID_02_S9	74.9%	49%	75 bp	50.5
G5A_HD_02_S5	73.4%	50%	75 bp	50.2
G5B_HD_05_S10	71.7%	50%	75 bp	45.0
NTC_5_S11	73.6%	74%	51 bp	0.1

APPENDIX 10 Contd. Toxicogenomics Phase Report

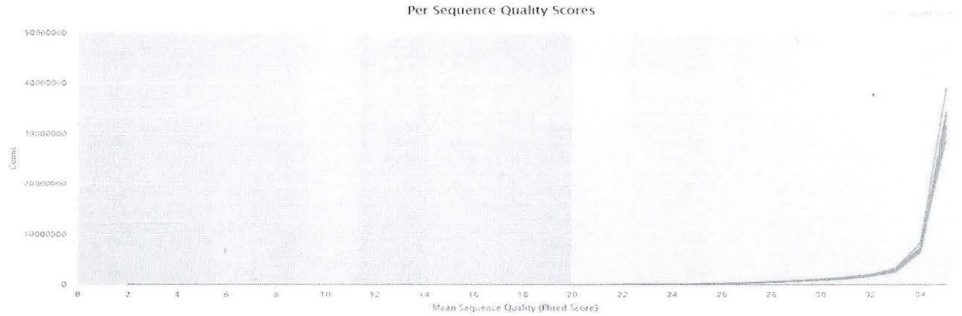
Study Number: G16084

Sequence Quality histograms



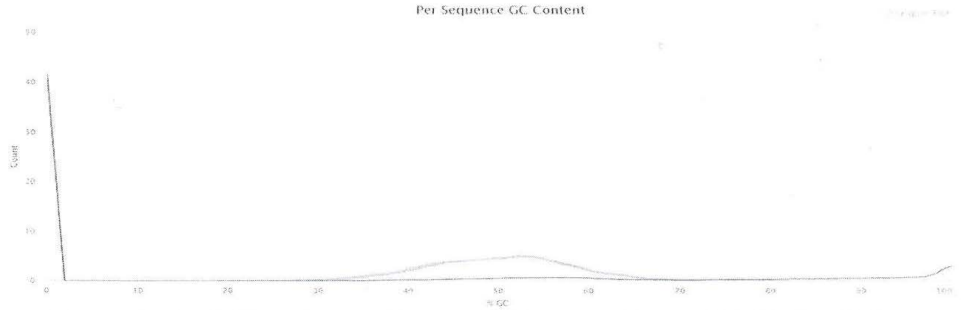
The mean quality value across each base position in the read for all the samples is plotted above. Phred quality score is defined as a property which is logarithmically related to the base calling error probabilities. For example, if Phred assigns a quality score of 20 to a base, the chances that this base is called incorrectly are 1 in 100.

Per sequence quality histogram



The number of reads with average quality scores. Shows if a subset of reads has poor quality. Here, all the samples have a minimum sequence quality of 32, which is good.

Overall GC percentage

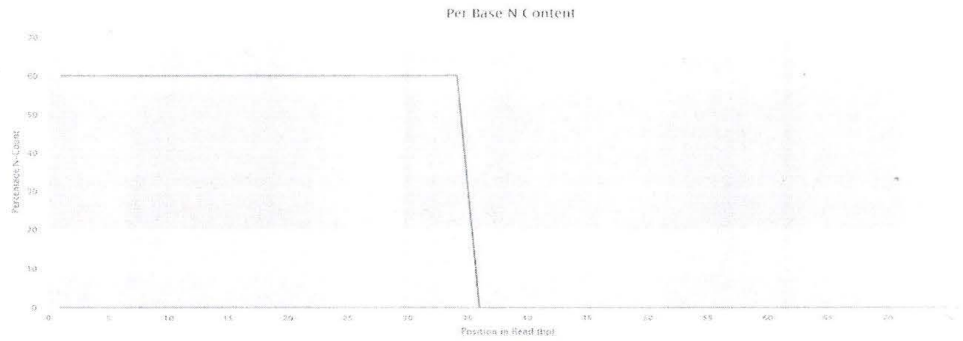


The average GC content of reads. Normal random library typically has a roughly normal distribution of GC content.

APPENDIX 10 Contd. Toxicogenomics Phase Report

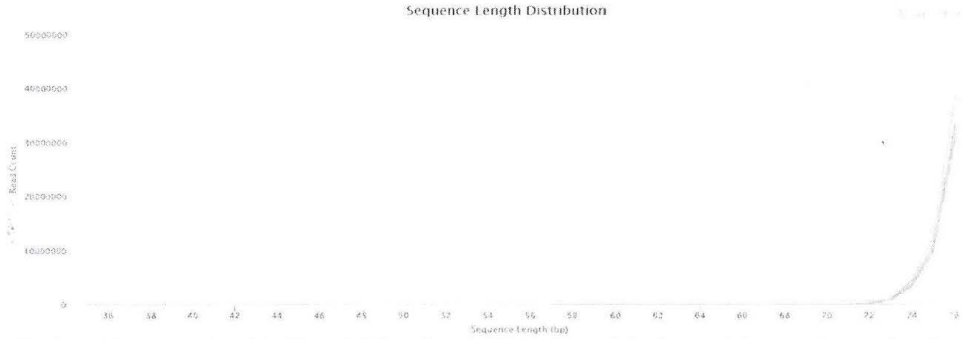
Study Number: G16084

Per base N content



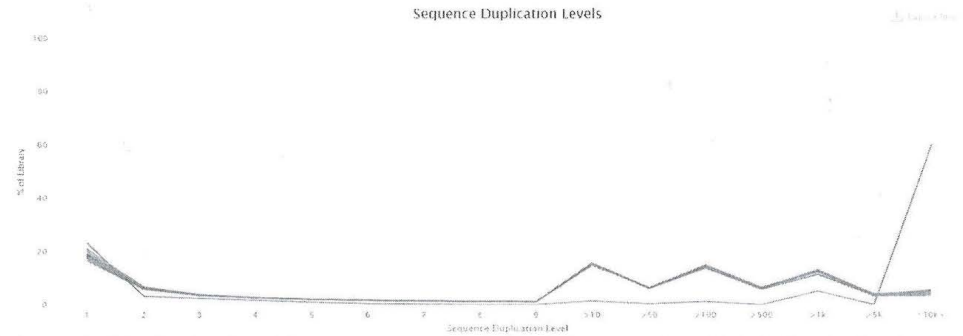
The percentage of base calls at each position for which an N was called. Here, most of the samples do not have N content.

Read length distribution



The distribution of fragment sizes (read lengths) found. Variable read length is observed due to adapter trimming process.

Sequence duplication levels

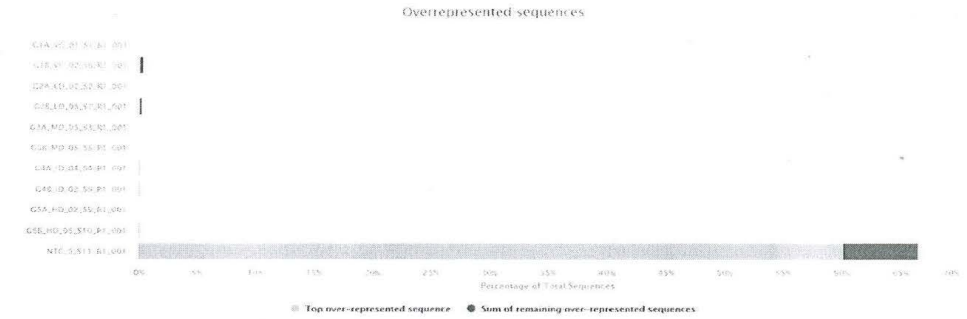


The relative level of duplication found for every sequence. RNA-Seq is known to have high duplication levels so as to have high coverage/depth of genes.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Over-represented sequences



The total amount of overrepresented sequences found in each library. Here NTC sample is expected to show this pattern due to ERCC Spike-in.

Adapter contamination

- The cumulative percentage count of the proportion of your library which has seen each of the adapter sequences at each position. Only samples with ≥ 0.1% adapter

contamination must be carefully observed for downstream analysis.

- All the 10 samples + NTC sample passed this QC, depicting there is no adapter sequence remaining in the read sequence.

Conclusions

- Data quality seems to be good across 50 samples. NTC sample fails almost all the QC parameters as expected.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084
Record of study plan deviation

RECORD OF STUDY PLAN DEVIATION

Study No.: G16084
Study Title: Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats with Toxicogenomics
Study Phase Identification No.: RP.17.0954

As in Study Plan	Deviation	Impact on outcome of study phase
This phase of study to be performed in accordance with the principles of GLP.	Study was not conducted in compliance with the principles of GLP as the Biology laboratory is a discovery laboratory.	There is no adverse impact on the outcome of study as complete study has been performed with scientific integrity by qualified and experienced staff. This was also communicated to the SD and agreed.
Test site QA shall perform the QA functions for these phases.	Experimental part of Study Phase was not inspected by Test site QA during the course of the study.	The test site QA has audited the study raw data for integrity, traceability and security followed by study report. No discrepancies were observed in the data. Therefore, there is no adverse impact on the study outcome.

9 Aug 2018

Seetha K, Ph.D
Principal Investigator
Toxicogenomics Phase

SYNGENE INTERNATIONAL LTD
SEZ-16 UNIT-IX
PLOT NO. 2, 3, 4 & 5, PHASE IV
BIOCON PARK
JIGANI LINK ROAD
BENGALURU-560099
INDIA

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084
7. Data Transfer

De-multiplexed data was transferred to Hard-Drive and shipped to ScitoVation through courier. The md5sums for the files shared are as follows:

Rat number	Filename	md5sum
Ru5681	G1A_VC_01_S1_R1_001.fastq.gz	6a58e60e2a3e90783aabe47081572728
Ru5682	G1A_VC_02_S1_R1_001.fastq.gz	1a565e2eae7077ca1254bb6d5e78b049
Ru5683	G1A_VC_03_S1_R1_001.fastq.gz	31b6c1ee11b4a116ec655e07550cac66
Ru5684	G1A_VC_04_S1_R1_001.fastq.gz	dce4e4fb51758b9ca5a65254c974961f
Ru5685	G1A_VC_05_S1_R1_001.fastq.gz	4ed9c2e54b20820a887675cf41bc7ecb
Ru5712	G1B_VC_02_S6_R1_001.fastq.gz	fe29d5cd69acdaf13bcb0622f2854f16
Ru5713	G1B_VC_03_S6_R1_001.fastq.gz	d6c758308a8c745c14f48ccb3ab40e65
Ru5714	G1B_VC_04_S6_R1_001.fastq.gz	6b3b3aaf6055d89224717d213b3c7adf
Ru5715	G1B_VC_05_S6_R1_001.fastq.gz	1f9e65f98fc4afabc7a6a62860e61cd2
Ru5716	G1B_VC_06_S6_R1_001.fastq.gz	584bea7ac0ef0dd801eb0a70bf058bc2
Ru5687	G2A_LD_01_S2_R1_001.fastq.gz	59095f6f9068711f13885367a6f9b05e
Ru5688	G2A_LD_02_S2_R1_001.fastq.gz	3b95e86be4dd8db9b9610be6ac43c0dc
Ru5689	G2A_LD_03_S2_R1_001.fastq.gz	05be3741f282eadb1bccff4cc807746c
Ru5690	G2A_LD_04_S2_R1_001.fastq.gz	f1b0a34862bd02d7a7b7badbf9d4697c
Ru5691	G2A_LD_05_S2_R1_001.fastq.gz	ef386c98007f69f729ca421428e62fde
Ru5717	G2B_LD_01_S7_R1_001.fastq.gz	c0af62580e657b978d04bcdf18ea9ff2
Ru5718	G2B_LD_02_S7_R1_001.fastq.gz	fcbf5e74e2aabe3e6bdecbe2212348d9
Ru5719	G2B_LD_03_S7_R1_001.fastq.gz	fde9daee8e069bfecdb0d207ea84d14a
Ru5720	G2B_LD_04_S7_R1_001.fastq.gz	2770d19877df415d7ea406f581056dae
Ru5721	G2B_LD_05_S7_R1_001.fastq.gz	07bdbfb1799def6d5aecd74d54486004
Ru5694	G3A_MD_02_S3_R1_001.fastq.gz	ff564bbb6db074070b9217d602c281a3
Ru5695	G3A_MD_03_S3_R1_001.fastq.gz	185b3b3d16eaf4ed67faab5727ae8722
Ru5696	G3A_MD_04_S3_R1_001.fastq.gz	2bb33552efee4e7b77570a362f40d8a7
Ru5697	G3A_MD_05_S3_R1_001.fastq.gz	4cc1dc5a09b4cdce4b1a0a372294ea71
Ru5698	G3A_MD_06_S3_R1_001.fastq.gz	d7a4a0c40926e042da067757b7d7cf37
Ru5724	G3B_MD_02_S8_R1_001.fastq.gz	dd711e691624df0596f72af3cf4fb3ff
Ru5725	G3B_MD_03_S8_R1_001.fastq.gz	04edb555cab4b2ca8f0985a555e3f7f1
Ru5726	G3B_MD_04_S8_R1_001.fastq.gz	49fcbe27af08638d31bfcf92b260a0fb
Ru5727	G3B_MD_05_S8_R1_001.fastq.gz	0ad2360ca4891e6c10642f0f93fb457a
Ru5728	G3B_MD_06_S8_R1_001.fastq.gz	f8bbd3adf72ee0f71cb6b0edf1c59206
Ru5699	G4A_ID_01_S4_R1_001.fastq.gz	c967ac782876d797ef92fe6c0339dd7b
Ru5700	G4A_ID_02_S4_R1_001.fastq.gz	a292a8cbf5d61969f157c43a097d2dad
Ru5702	G4A_ID_04_S4_R1_001.fastq.gz	f1d7653a7c994bb3349e8f7ab79fdc43
Ru5703	G4A_ID_05_S4_R1_001.fastq.gz	bde5deed50a08a5c8ca10858e3ea032e
Ru5704	G4A_ID_06_S4_R1_001.fastq.gz	eb7f80d059a0ef523867913f66db23a2
Ru5730	G4B_ID_02_S9_R1_001.fastq.gz	71292be19cd8984a49045c24eaa883b1
Ru5731	G4B_ID_03_S9_R1_001.fastq.gz	f2e4da8b0a5db986cca9c06a4588554c
Ru5732	G4B_ID_04_S9_R1_001.fastq.gz	9012a8cf7b659e2bab96adaf61f7e8e6
Ru5733	G4B_ID_05_S9_R1_001.fastq.gz	4c6e3594205fb480f443e4dba840efc8
Ru5734	G4B_ID_06_S9_R1_001.fastq.gz	dbe775ff0cf0d39e9b32386406e8c916
Ru5706	G5A_HD_02_S5_R1_001.fastq.gz	94b7906348c9660dbdf83e3777b8305e
Ru5707	G5A_HD_03_S5_R1_001.fastq.gz	45d151691beffd8b4be038eecc6aeeb5
Ru5708	G5A_HD_04_S5_R1_001.fastq.gz	a176b8c650a8040cce490c3ce746476f
Ru5709	G5A_HD_05_S5_R1_001.fastq.gz	015b7846ffc0bb3470f43928b56f8505

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Ru5710	G5A_HD_06_S5_R1_001.fastq.gz	8b94bedf73c0e539c191647aaabf006c
Ru5735	G5B_HD_01_S10_R1_001.fastq.gz	43c89b53d652fe2707e11edac27a4c60
Ru5736	G5B_HD_02_S10_R1_001.fastq.gz	37284ad3ad3a639770d25d7981f97abb
Ru5737	G5B_HD_03_S10_R1_001.fastq.gz	84c651d92f41e85303df79b69146c819
Ru5738	G5B_HD_04_S10_R1_001.fastq.gz	b396ceeebc6a349932652b25951b9515
Ru5739	G5B_HD_05_S10_R1_001.fastq.gz	a01359c9073056b3589d910aeeebe471

APPENDIX 10 Contd. Toxicogenomics Phase Report

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Research Triangle Park, NC 27709
www.scitovation.com

ScitoVation
INNOVATIVE CELL BASED SCIENCE

RNA-seq gene expression results for male Wistar rats exposed to four concentrations of Acetamide for 1 week

prepared for:

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May 21, 2018

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Study Number: G16084

Summary

The following is a brief summary of the findings from whole transcriptomic gene expression analyses in male Wistar rats treated with four doses of acetamide plus a vehicle only control group (0 (vehicle controls), 30, 100, 300 and 1000 mg/kg-d). Five biological replicates were included in each group. Gene expression was measured by single-end read RNA-Seq, mapped to the UCSC Rn6 reference genome (genome.ucsc.edu/). This short report summaries the differential gene expression (DGE) results, ontology enrichment (Reactome - reactome.org/), ChEA enrichment (transcription factor inference from enrichment with ChiP-X public data sets [1]) and Benchmark Dose Pathway enrichment [2].

Methods

In-life study

Details of the in-life study and sequencing experiments have been described elsewhere in this report. RNA-Seq read data was delivered to ScitoVation as FASTQ files, one for each animal exposed. FASTQ format sequence files are simple ASCII text files which include both the read sequence itself (75 base pair single-end reads) as well as the base pair quality scores. These files form the input data used in ScitoVation's mapping of reads to the *Rattus norvegicus* reference genome.

Differential Gene Expression (DGE)

We used a ScitoVation standard pipeline for RNA-seq analyses using open source tools. RNA-seq read quality was assessed using the FastQC*. Based on worst case scenarios observed in the quality control assessment, reads were universally trimmed (Trimmomatic†), to remove the leading 7 base pairs (bp) and the trailing 3 bp (as well as rejecting any such edited reads with a remaining total length less than 50 bp). The high quality of the sequence reads meant that this trimming scheme typically resulted in the rejection of less than 0.5% of the reads for any one sample. Sequence reads by sample ranged from ~44M reads to as much as ~60M reads with an average across all of ~50M reads.

Trimmed reads were mapped to the USCS Rn6 indexed *Rattus norvegicus* reference genome‡ using the short read mapper BowTie2§. Mapped reads were counted for Rn6 CDS features using the Python tool HtSeq**. Of the reads used in mapping (>99% of the available reads per sample after trimming), typically 98% or more mapped uniquely to the reference genome. ScitoVation retains the short read mapping results as BAM files (a compressed binary file format for storing reference mapped gene sequence information), one for each animal sample sequenced, for future reference.

Counted reads for each annotated CDS feature were analyzed for differential expression in R (v.3.4.3) using the BioConductor library DESeq2 (v.1.20.0) [3]. DESeq2 uses a dispersion correction of the count data based on the negative binomial distribution and a maximum likelihood model to impute the prior data distribution for statistical testing. Empirical Bayesian statistics are applied to linear combinations of factors to test differential expression for multiple contrasts simultaneously. In order to avoid bias and unnecessary computation in the dispersion correction, the data is pre-filtered to exclude any annotated genomic feature for which there were no counts in any biological sample. DESeq2 itself is designed to otherwise deal with genes for which there are only counts in a very few or a minority of the samples when

* Babraham Institute, Cambridge UK (www.bioinformatics.babraham.ac.uk/projects/fastqc/)
† Usadel Lab, RWTH Aachen University, Aachen Germany (www.usadellab.org/cms/?page=trimmomatic)
‡ iGenomes, Illumina support (https://support.illumina.com/sequencing/sequencing_software/igenome.html)
§ Johns Hopkins University (bowtie-bio.sourceforge.net/bowtie2/index.shtml)
** Simon Anders, EMBL Heidelberg, Germany (htseq.readthedocs.io/en/release_0.10.0/)

performing tasks such as FDR p-value corrections. The final output of DESeq2 is a table of estimated Log_2 fold change, p-values for the defined contrasts tested, as well as Benjamini-Hochberg corrected false discovery p-values (FDR) [4].

We determined significance of differential expression using a number of criteria, either singly or in combination. In our analyses, we use as a statistical criterion the Benjamini and Hochberg step-up false discovery rate corrected p-value of less than 0.05 (FDR<0.05), a commonly used threshold in whole transcriptomic analysis. Additionally, it is useful to define a minimum magnitude of change in gene expression. Common values for differential expression experiments would be a change in expression (e.g., the ratio of the mean expression of treated samples relative to mean expression of control samples), up or down regulated relative to controls of 1.5 fold or 2 fold (|FC|>1.5 or 2). In our toxicogenomic analyses, we typically use a magnitude of change threshold of 1.5 fold. As treated samples can be either up-regulated (a positive fold change) or down-regulated (a negative fold change) relative to vehicle only controls, this threshold was applied as the absolute value of estimated fold change (|FC|>1.5).[††] Finally, the application of both a statistical threshold and a magnitude of change threshold (FDR<0.05 & |FC|>1.5 fold) was used; these criteria have been shown to produce the most robust gene lists relative to indicated functional enrichment with a known cellular gene ontology [5].

Functional Ontology Enrichment

Gene lists for functional ontology enrichment were selected based on the application of one or both of the significance thresholds used for differential gene expression analysis. We used in-house software—GOFigure Maps [6, 7]—to perform traditional hypergeometric over-representation analysis of selected gene lists relative to the curated lists of ontology elements associated with functional ontology categories in the public Reactome pathway database (www.reactome.org). Reactome is a curated biochemical pathway-based cell biology ontology. Reactome's hierarchy progresses from broad, collective functional categories such as metabolism or cell signaling, to progressively more defined sub-collections of functionally related cellular pathways, and finally to discrete biochemical cellular process pathways. GOFigure Maps determines significance of overlap in a query list of elements with the hierarchical ontology lists of elements using a standard Fisher's one-way exact test with an FDR corrected p-value [8]. This software also produces a visualization of the enrichment pattern in the form of an interactive bubblemap. The bubblemap displays hierarchical structured data in a manner where the relationship of parent to child process is indicated by defining each process category as a node, or bubble, and the relationship of parent and child processes is indicated by a line, or edge, connecting related processes in the ontology hierarchy.

Transcription factor binding predictions

The ChEA database is derived from publicly available ChIP-Seq and ChIP-on-chip microarray data and gene expression data (primarily from RNA-Seq experiments). The database uses iterative analyses to define lists of genes with co-expression associated with transcription factors. Exhaustive iterative gene list comparisons and Fishers exact tests are used to compute p-values of gene list associations and adjusted q-values, z-scores and a combined score (the product in log space of the p-value and z-score) are computed to rank order gene lists by significance of association with a given transcription factor. ChEA thus links known transcriptional coregulated genes with high degrees of gene expression correlation to derive transcription factor associations with functional gene lists.

The Enrichr algorithm searches for overlap of the query list and subsets of the query list with the ChEA database of gene association lists. Enrichr uses conventional hypergeometric overlap analysis with Fisher exact tests of significance to find all significant enrichment between the query list and those gene association lists in the ChEA database. The resulting gene association data sets can be rank ordered by combined score, p-value, q-value, or z-score. These gene association

[††] A linear fold change of 1.5 fold is equal to a Log_2 fold change of 0.585. All computations used Log_2 fold change as normalized expression values are log transformed for statistical analyses.

datasets can then be further explored for the individual gene lists associated with a transcription factor identified from the Enrichr gene set enrichment analysis (see the primary database and tool links at labs.icahn.mssm.edu/maayanlab/).

Benchmark dose analyses

The application of benchmark dose (BMD) to transcriptomic studies was developed at the Hamner Institutes for Health Sciences [9]. Benchmark dose was developed as an alternative to the NOAEL approach to set points of departure in risk assessment studies. BMD takes into account the specifics of a dose response curve to derive a point of departure that is based on a pre-defined degree of biological response to an exposure. The software BMDExpress was designed to adapt EPA's BMDS modeling and statistical algorithms to gene expression data to allow for estimations of BMD from the dose response observed in expression changes in individual genes [10]. Once BMD estimates are available for individual genes, these can be associated with known functional processes using existing gene ontologies and estimates of cellular pathway BMD can be deduced from the BMD of the genes associated with those pathways.

Gene expression data from both microarray and RNA-Seq experiments can be analyzed using the BMDExpress approach developed originally for microarray data. First, normalized log-transformed expression values are computed for each probe in a microarray experiment or each gene (or annotated feature) in a RNA-Seq experiment. These data are then fit to a series of dose response models — typically Hill model, Power model, linear, second-degree and third-degree polynomials. Each probe set or gene is fit independently using the BMDExpress software program which in turn utilizes models developed by the EPA's BMDS group. Each model is computed assuming constant variance for microarrays, but non-constant variance for RNA-Seq as these data would not be expected to display constant variance across the measured range of exposures due to the distribution of count data (which typically is Poisson or negative binomially distributed). The benchmark response (BMR) factor is set to 1.349 multiplied by the SD in the control samples. This value is derived from the assumption of a normal (or log-normal) distribution for control samples, and it is assumed, a priori, that the transcriptional response could occur in either tail, with a 1% change of that occurring in the absence of exposure (0.5% in each tail). A BMR of 1.349 is the amount required to shift the mean transcriptional response of a normal control distribution such that the treated distribution contains 11% in a single tail, i.e., a 10% increase over the assumed background rate of response (Fig.1).

Model selection follows a hierarchical approach. First, Hill models with a k-parameter estimate that is small or less than the lowest exposure concentration (e.g., less than one third of the lowest dose) are problematic as they tend to give biologically irrelevant BMD estimates of extremely low concentrations. Thus, the software is set to flag such models and exclude them from best model consideration. Then, a nested likelihood ratio test is performed on the linear and higher order polynomial models to select the best amongst the polynomial models run. Then, the Akaike information criterion (AIC) for the selected best polynomial model is compared with the AIC for the power and non-flagged Hill models to select the best overall model which is then used to calculate a BMD and benchmark dose lower confidence limit (BMDL). To avoid model extrapolation, probe sets with a BMD value greater than the highest concentration used in the experiment were removed from further analysis, as were poor fitting best overall models with a goodness of fit p-value less than or equal to 0.1.

The final selection of best models passing these quality control thresholds can then be matched to elements in an ontology (GO, MetaCore, Reactome, etc). Any ontology category with at least 10 or more best model elements found among the defined ontology category elements can then be given an estimated pathway or category BMD and BMDL by averaging (or using the median value of) the estimates for the individual elements found. This aggregation of gene BMDs allows comparison of BMD and sensitivity of potentially altered pathways for both toxicity assessment and comparison with other compounds or across species comparisons. BMDExpress was recently updated and is now maintained by and available through a NIEHS repository (github.com/auerbachs/BMDExpress-2)

Study Number: G16084

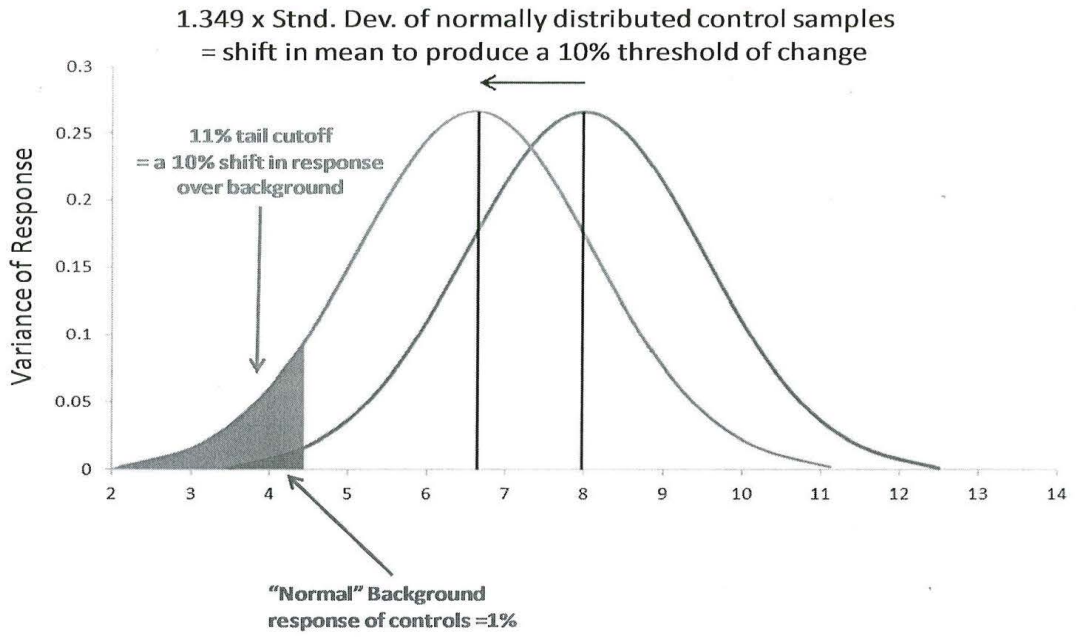

Figure 1. With control samples are normally (or log normally) distributed and show a normal background response of 1%, a BMD of 1.349 times the standard deviation results in a 10% change in the assumed background rate of response (a BMR_{10}).

Results

Differential Gene Expression

Shown in Table 1 are the numbers of differentially expressed genes detected using various thresholds of significance for the acetamide only dosed animals. There were five animals analyzed in each of the concentrations tested (30, 100, 300 and 1000 mg/kg-d) and the vehicle controls. Fold change indicated is the ratio of the mean of each of the five treated animals' normalized expression value relative to the mean of the five vehicle animals' normalized expression.

As indicated, response by fold change alone was evident at all treatment concentrations. However, none of these changes were statistically significant for the two lowest concentrations of 30 and 100 mg/kg-d, and only a single gene was statistically significant for the 300 mg/kg-d treated group. At 1000 mg/kg-d there were numerous genes that were either up- or down-regulated by at least 1.5 fold relative to vehicle, and were statistically significantly altered (FDR<0.05). It was these genes (1000 mg/kg-d, genes significant by both FDR<0.05 & |FC|>1.5 fold) that were used to investigate ontology enrichment (Fig.2) with the Reactome Ontology.

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Table 1. Numbers of differentially expressed genes by statistical significance, magnitude of change and both thresholds applied simultaneously

Differential Gene Expression for Acetamide Only exposures in male Wistar rats for 1 week				
Dose dependent response (all concentrations) FDR<0.05			0	
Dose dependent response (all concentrations) P-value<0.01			71	
Concentration:	30 mg/kg-d	100 mg/kg-d	300 mg/kg-d	1000 mg/kg-d
FDR< 0.05	0	0	1	3909
|FC|> 1.5 fold	1821	1785	1892	4816
FC> +1.5 fold	875	1020	1072	3068
FC< -1.5 fold	946	765	820	1748
FDR< 0.05 & |FC|> 1.5 fold	0	0	1	2685
FDR< 0.05 & FC> +1.5 fold	0	0	1	1717
FDR< 0.05 & FC< -1.5 fold	0	0	0	968

APPENDIX 10 Contd. Toxicogenomics Phase Report

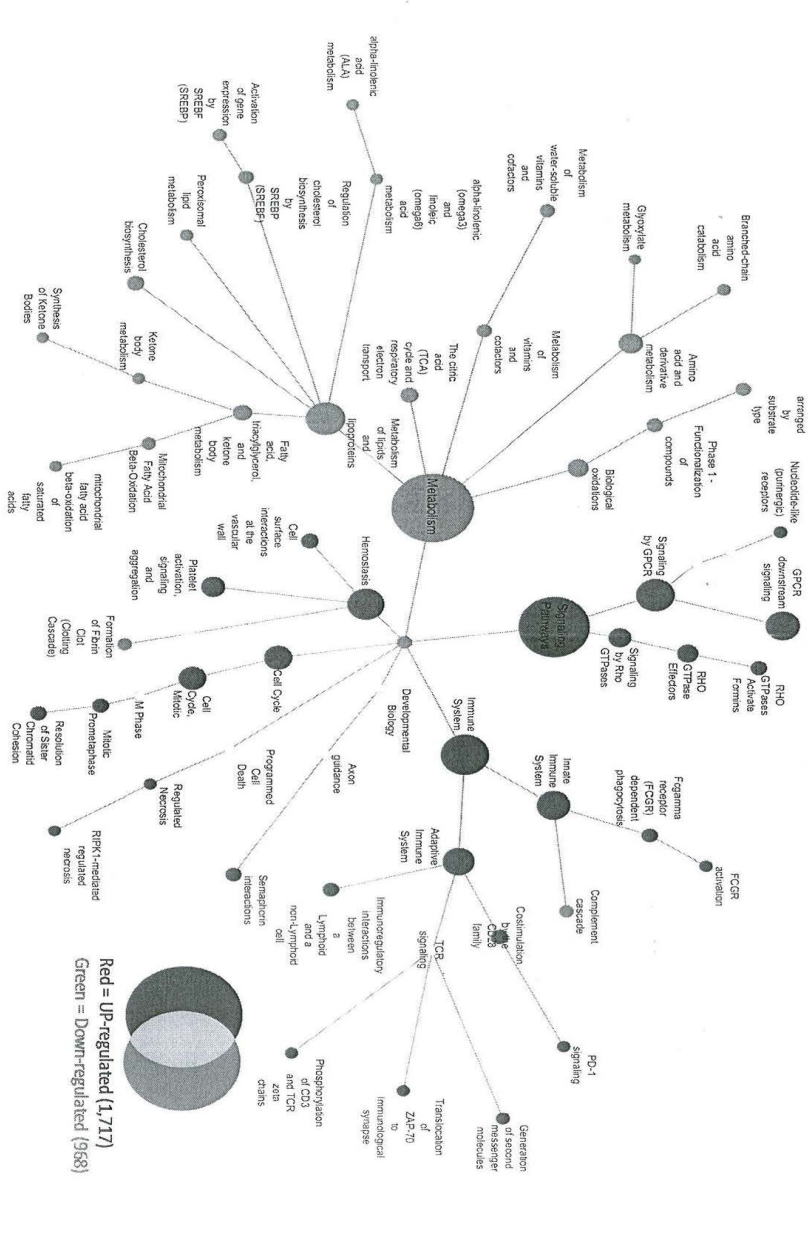

Figure 2. Acetamide only male Wistar rats dosed with 1000mg/kg-d. Genes that were both statistically significantly altered relative to the vehicle control group (FDR<0.05) and were either up- or down-regulated by at least 1.5 fold were included in creating the bubblemap. Nodes indicate the categories in Reactome, connected by edges with indicate relative relationships between categories. Red nodes are those enriched only with up-regulated genes, while green nodes are those enriched only with down-regulated genes. Mustard color indicate a node that was simultaneously enriched with both up- and down-regulated genes.

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Acetamide – 1000 mg/kg-d

FOXM1_23109430_ChIP-Seq_U2OS_Human
E2F4_17652178_ChIP-ChIP_JURKAT_Human
FOXM1_25889361_ChIP-Seq_OE33_AND_U2OS_Human
E2F7_22180533_ChIP-Seq_HELA_Human
IRF8_27001747_Chip-Seq_BMDM_Mouse
MYB_26560356_Chip-Seq_TH2_Human
BPI_19119308_ChIP-ChIP_HS578T_Human
IRF8_22095565_ChIP-ChIP_GC-B_Mouse
IRF8_21731497_ChIP-ChIP_J774_Mouse
NR1H3_23393188_ChIP-Seq_ATHEROSCLEROTIC-FOAM_Human

Up-regulated genes

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	FOXM1_23109430_ChIP-Seq_U2OS_Human	1.3128e-22	8.937e-20	-3.08	155.43
2	E2F4_17652178_ChIP-ChIP_JURKAT_Human	8.631e-10	1.836e-7	-3.21	67.07
3	FOXM1_25889361_ChIP-Seq_OE33_AND_U2OS_Human	1.495e-10	4.768e-8	-2.11	47.72
4	E2F7_22180533_ChIP-Seq_HELA_Human	0.0001346	0.008589	-3.75	33.41
5	IRF8_27001747_Chip-Seq_BMDM_Mouse	1.119e-8	0.0000001784	-1.48	27.12
6	MYB_26560356_Chip-Seq_TH2_Human	9.521e-8	0.00001215	-1.57	25.42
7	BPI_19119308_ChIP-ChIP_HS578T_Human	0.009582	0.2211	-4.90	22.76
8	IRF8_22095565_ChIP-ChIP_GC-B_Mouse	0.0005401	0.02833	-3.07	22.62
9	IRF8_21731497_ChIP-ChIP_J774_Mouse	0.002928	0.1038	-3.21	18.75
10	NR1H3_23393188_ChIP-Seq_ATHEROSCLEROTIC-FOAM_Human	0.0005773	0.02833	-2.30	17.16

RXR_22158963_ChIP-Seq_LIVER_Mouse
PPARA_22158963_ChIP-Seq_LIVER_Mouse
ESR1_17901129_ChIP-ChIP_LIVER_Mouse
LXR_22158963_ChIP-Seq_LIVER_Mouse
PPARG_19300518_ChIP-PET_3T3-L1_Mouse
CEBPA_23403033_ChIP-Seq_LIVER_Mouse
EGR1_23403033_ChIP-Seq_LIVER_Mouse
CEBPB_23403033_ChIP-Seq_LIVER_Mouse
FOXO1_23076095_ChIP-Seq_LIVER_Mouse
NR1I2_20693526_ChIP-Seq_LIVER_Mouse

Down-regulated genes

Index	Name	P-value	Adjusted p-value	Z-score	Combined score
1	RXR_22158963_ChIP-Seq_LIVER_Mouse	2.250e-27	1.417e-24	-1.44	88.60
2	PPARA_22158963_ChIP-Seq_LIVER_Mouse	3.303e-24	1.041e-21	-1.48	80.26
3	ESR1_17901129_ChIP-ChIP_LIVER_Mouse	2.623e-12	4.131e-10	-2.79	74.31
4	LXR_22158963_ChIP-Seq_LIVER_Mouse	1.065e-21	2.236e-19	-1.41	68.31
5	PPARG_19300518_ChIP-PET_3T3-L1_Mouse	0.00001830	0.001647	-3.44	37.50
6	CEBPA_23403033_ChIP-Seq_LIVER_Mouse	8.394e-7	0.00008813	-2.41	33.66
7	EGR1_23403033_ChIP-Seq_LIVER_Mouse	4.824e-7	0.00006091	-2.16	31.47
8	CEBPB_23403033_ChIP-Seq_LIVER_Mouse	0.0006751	0.05316	-2.70	19.72
9	FOXO1_23076095_ChIP-Seq_LIVER_Mouse	0.001253	0.09771	-2.77	18.51
10	NR1I2_20693526_ChIP-Seq_LIVER_Mouse	0.002499	0.1431	-2.70	16.18

Figure 3. Graphical representation of gene set enrichment in the Enrichr database (http://amp.pharm.mssm.edu/Enrichr/) using as query genes the 500 most up- or down regulated genes that were differentially expressed by both statistical and magnitude of change thresholds (FDR<0.05 & FC> +1.5). The bar graphs are ranked by Enrichr combined score (Log(p-value) * z-score) and the table indicates the actual FDR corrected p-values for the enrichment of the top ranked gene sets in each case.

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The Reactome ontology enrichment showed responses that are consistent with previous treatment of hepatocytes and intact rats with acetamide (i.e., TG-GATEs [11]) and included cellular changes in metabolic processes, particularly fatty acid metabolism, as well as immune and immune signaling responses. In the current data, metabolic processes appear enriched for the down-regulated genes and cell cycle, signaling and immune system responses are enriched with up-regulated genes.

In Fig.3 we used a different type of enrichment analysis, submitting gene lists (again, up- and down-regulated) through the Enrichr tool at the Icahn School of Medicine at Mount Sinai (amp.pharm.mssm.edu/Enrichr/). For the query genes, we used the list of genes which were both statistically significant (FDR<0.05) and with a magnitude of change of at least 1.5 fold after exposure to acetamide at 1000 mg/kg-d. We further constrained these lists to include only the 500 most up-regulated and 500 most down-regulated from the larger set of genes fulfilling both criteria. This analysis revealed that PPARa- and RXR-associated genes overrepresented among the down-regulated gene lists, a finding consistent with the observed Reactome enrichment in fatty acid metabolic processes. The up-regulated genes had enrichment for various FOX controlled pathways and regulated elements. FOX proteins are key transcription factors in cell growth and proliferation and are among some of the most conserved transcription factors in mammals.

Finally, Fig.4 presents a Reactome pathway analysis of BenchMark Dose for the 11,373 genes for which all 25 animals in the study had a feature count of at least one. We exclude any gene where even a single replicate failed to detect the gene (a feature count = 0) to avoid issues with mathematical curve fitting to missing data or creating a need to impute missing data for parts of the dataset. BMDExpress 2.2 software was used to fit five models to every one of the 11,373 genes - power model (power coefficient restricted to >1), the Hill model, a linear model and a 2° polynomial and exponential model. Hill models where the shape (or slope) parameter, k, was less than 1/3rd of the maximum concentration were rejected from consideration as these tend to have extreme inflections the shape of the curve leading to biologically non-sensical (e.g., vanishingly small) BMD estimates. Genes where a best fitting model was found that had both a gene-based BMD estimate less than the maximum treatment concentration of 1000 mg/kg-d (to avoid extrapolation of estimates) and which also had a goodness of fit p-value >0.1 were then used in an enrichment analysis using Reactome. Pathways with 10 or more such genes found had a pathway BMD estimated as the median of the BMD of the genes which matched ontology elements in the pathway. Of the pathways indicated in Fig.4 many were also enriched pathways shown in Fig.2 (see Table 2 for examples).

A formal and detailed analyses and discussion of the gene expression implications for cellular mode of action will be forthcoming when the final acetamide exposure data is available later this year.

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Table 2. Reactome pathways indicated amongst the 472 plotted in Fig.4

Reactome Pathway	Genes found	BMD Median	Reactome Pathway	Genes found	BMD Median
Metabolism	540	716.254	Signaling by Rho GTPases	109	674.689
Immune System	434	669.352	GPCR downstream signalling	109	759.329
Innate Immune System	276	695.855	M Phase	96	650.866
Metabolism of lipids	188	692.041	Cell Cycle Checkpoints	95	667.082
Gene expression (Transcription)	180	724.791	Signaling by Receptor Tyrosine Kinases	93	761.577
Adaptive Immune System	177	690.070	Translation	79	617.725
Cell Cycle	167	647.397	Fatty acid metabolism	72	619.087
Cell Cycle, Mitotic	151	618.229	Mitotic Metaphase and Anaphase	64	662.985
Signaling by GPCR	113	758.699	Mitotic Anaphase	63	673.089

APPENDIX 10 Contd. Toxicogenomics Phase Report

Figure 4. Benchmark Dose pathway enrichment results. BMDExpress 2.2 software was used to fit five mathematical dose response models to all genes for which there was a feature count of at least 1 for every sample in the dataset (25 animals and 11,373 genes). Only genes with a best fitting model which had a BMD estimate less than the maximum concentration used (to avoid extrapolation) and had a best model goodness of fit -p-value >0.1 were used in the pathway enrichment. This produced 472 pathways for which a pathway BMD could be estimated. Those pathways are presented along the X-axis in rank order from lowest to highest estimated BMD. The dashed red line is a simple trend line of those rank ordered pathway BMD values.

APPENDIX 10 Contd. Toxicogenomics Phase Report

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APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Report prepared and submitted on behalf of the SeitoVation Authors by

Michael B. Black

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USA

May 21st, 2018

APPENDIX 10 Contd. Toxicogenomics Phase Report

Study Number: G16084

Sample Disposition:
The left-over liver samples will be discarded after signature of the final phase report and upon approval from sponsors.

Archiving:
The original raw data and final phase report will be archived at the archives of Eurofins Advinus Limited for 9 years.

Phase Report Distribution:
Study Director: One signed final phase report in original and an electronic copy in PDF Format.

APPENDIX 11. Certificate of Analysis – Acetamide

Certificate of Analysis

Dec 19, 2017 (JST)

TOKYO CHEMICAL INDUSTRY CO.,LTD.
4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan

Chemical Name: Acetamide		
Product Number: A0007 CAS: 60-35-5	Lot: XHH4A	

Tests	Results	Specifications
Purity(GC) Melting point Solubility in Water	100.0 % 81.4 deg-C transparency	min. 98.0 % 80.0 to 84.0 deg-C almost transparency

TCI Lot numbers are 4-5 characters in length. Characters listed after the first 4-5 characters are control numbers for internal purpose only.
The contents of the specifications are subject to change without advance notice. The specification values displayed here are the most up to date values. There may be cases where the product labels display a different specification, however, the product quality still meets the latest specification.

Customer service:

TCI Chemicals (India) Pvt. Ltd.
Tel: 044-2262 0909 / 044-2262 8878
Fax: 044-2262 8902
E-mail: Sales-IN@TCIchemicals.com

APPENDIX 12. Certificate of Analysis – Glucose

SIGMA-ALDRICH®

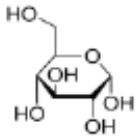
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
D-(+)-Glucose - ≥99.5% (GC)

Product Number:	**G8270**
Batch Number:	**SLBT7078**
Brand:	SIGMA
CAS Number:	50-99-7
MDL Number:	MFCD00063774
Formula:	C6H12O6
Formula Weight:	180.16 g/mol
Quality Release Date:	13 APR 2017
Recommended Retest Date:	MAR 2023



Test	Specification	Result
Appearance (Color)	White	White
Appearance (Form)	Powder	Powder
1H NMR Spectrum	Conforms to Structure	Conforms
Solubility (Color)	Colorless	Colorless
Solubility (Turbidity) 2 g + 15 mL, H2O	Clear	Clear
Purity (GC)	≥ 99.5 %	99.9 %

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

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Version Number: 1

Page 1 of 1

APPENDIX 13. Certificate of Analysis – BrdU

SAFC®

Sigma-Aldrich Biochemie GmbH
Georg-Heyken-Strasse 14, 21147 Hamburg GERMANY
Tel: 49 40 79702 0 Fax: 49 40 79702 100

Certificate of Analysis

Product
5-BROMO-2'-DEOXYURIDINE

Product No.:	B5002-5G
Bulk No.:	HMBF7780
Lot No.:	HMBQ2687V
Storage:	0 TO -20 DEGREE C
Print Date:	20.11.2017
Recommended Retest Date:	05.01.2021
Place of Manufacture:	Hamburg, Germany

Analysis

TestID	Specification	Actual	Unit
Appearance	white to off white powder	white powder	
HPLC Purity	>= 99	100	%area
UV/VIS EMM (lambda max 279-280nm pH 1.0)	8.9 - 9.2	8.9	%
App. of Sol. (50 mg/mL in DMSO)	clear colorless to very faint yellow sol	clear colorless to very faint yellow sol	

This document was produced electronically and is valid without a signature.

Quality Control Sigma-Aldrich Biochemie GmbH

APPENDIX 14. Peer Review Certificate

PATHOLOGY PEER REVIEW CERTIFICATION

Study No.: G16084 Study Code: 7-OGR

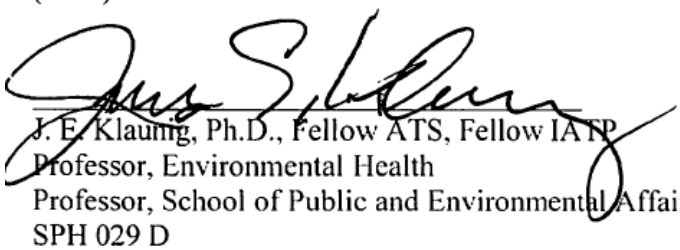
Test Item: ACETAMIDE

The purpose of this independent peer review was a pathology data review and quality assessment of the study pathologist's evaluation of the findings. Attention was directed to the completeness, accuracy, and consistency of the original evaluation.

A peer review of pathology data of the Study No. G16084 Acetamide: 7-day repeat dose oral toxicity study in Wistar rats with Toxicogenomics was conducted at Indiana University, Bloomington, Indiana on 18 June 2018. Hematoxylin and eosin stained liver slides and Ki67 proliferation antigen immuno-stained liver slides from control and high dose groups were reviewed.

The body weight data, clinical pathology data, organ weight data, necropsy findings and histopathology findings with interpretation (Draft Pathology Report) of the study pathologist were available and referred to during the review process.

Differences in diagnoses were discussed and resolved by consensus with the study pathologist. The histopathology results and pathology interpretation are the consensus of the study pathologist and peer review pathologist. Ki67 staining of hepatocytes was optimum and I am in general agreement with quantitation of cell proliferation antigen (Ki67).



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APPENDIX 15. Deviations from Approved Study Plan

Sl. No.	AS IN STUDY PLAN	DEVIATIONS
1.	**Page 4/32 and 5/32** **1 STUDY DETAILS** **Study Schedule** Experiment Completion: Latest by 28 February 2018 Draft Report Date: Latest by 31 March 2018	 **1 STUDY DETAILS** **Study Schedule** Experiment Completion: 22 May 2018 Draft Report Date: 11 June 2018
Justification and impact of deviation: To include the exact dates of experiment completion and draft report date to Sponsor. This deviation will not affect the integrity of the study or interpretation of results.		
2.	**Page 19/32** **11.9 Anatomic Pathology** **11.9.1 Necropsy** To begin with, in the total of 8 groups with 6 rats each, the randomization will be done among the 8 groups and among the 6 rats in each group. For the following round, the 8 groups will be randomized with the randomization among the remaining 5 rats in each group.	 **9.9 Anatomic Pathology** **9.9.1 Necropsy** To begin with, in the total of 10 groups with 6 rats each, the randomization was done among the 10 groups and among the 6 rats in each group. For the following round, the 10 groups were randomized with the randomization among the remaining 5 rats in each group.
Justification and impact of deviation: To correct the typo error in total number of groups. This deviation will not affect the integrity of the study or interpretation of results.		

20 ANNEXURES

ANNEXURE 1. Contaminant Analysis Report For Bedding Material (Corn Cob)

TÜV SÜD South Asia

Choose certainty.
Add value.

Test Report No. BLR/F(C)/17/012621 A1
Dated 2017-05-26

Issued To	:	**ROWAN AGRO NATURE PRIVATE LIMITED** **Ramachandra Niwas, Godbole Mala,** **Near Tilak Kanya Shala,** **Vijayapura, Karnataka,** **India - 586101.**
Attention	:	Dr. Umesh,
Customer Ref. No.	:	Test Request Form
Sample Particulars		
Sample Name	:	Corn Cob Grits
Sample Description	:	Off White Color Solid
Batch No. / Lot No. / Mfg. Date / Best Before Date	:	Lot No. 4, May 2017, Size 06 08
Sample Package / Quantity	:	Ziploc Pouch, 500 gms X 1 No
Sampled by	:	Customer
Sampling Location	:	Not Applicable
Sampling Protocol	:	Not Applicable
Sample Condition	:	Received in Good Condition
Date of Sample Receipt	:	Received on 2017-05-18 4:16 pm
Date of Analysis	:	2017-07-13
Date of Completion	:	2017-07-17

Note : The submitted sample is not Drawn by the Laboratory

Rowan Agro Nature Pvt. Ltd. Vijayapur - 586101

Remark : The report no BLR/F(C)/17/012621 dated 2017-05-26 has been superseded. The test report is amended in terms of Test addition of PCB"s

Authorised By

Veena D
Authorized Signatory

By accepting this document the customer hereby agrees and accepts the 'Terms & Conditions' and the relevant 'Testing & Certification Regulations' of TÜV SÜD South Asia Pvt. Ltd. which are available at Company's website at the link-http://www.tuv-sud.in/in-en/resource-centre/terms-and-conditions
Note: The test report is electronically generated. Hence original signature is not required. For any technical query, please contact Satya Srinivas at Satya.Srinivas@tuv-sud.in and for any complaint please contact Libin Kumar K.V. at Libinkumar.KV@tuv-sud.in .
Note : (1) The results relate only to the items tested, (2) the test report shall not be reproduced except in full without the written approval of the laboratory, (3) For details of the accredited scope, please contact laboratory or accreditation body website

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Regd. Office:
TUV SUD South Asia Pvt. Ltd.
Off Saki Vihar Road, Saki
naka, Andheri (East),
Mumbai-400072. India

ANNEXURE 1 contd. Contaminant Analysis Report for Bedding Material (Corn Cob)

TÜV SÜD South Asia

Test Report No. BLR/F(C)/17/012621 A1
Dated 2017-05-26

S. NO.	PARAMETERS	TEST RESULT	TEST METHOD
	Chemical :		
	Aflatoxin		
1	B1, B2, G1, G2,µg / kg	Not Detected (DL : 1.0)	Lab_P_SOP_217 / HPLC
	Analytical :		
	Pesticide Residues		
2	o,p -DDT,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
3	Alpha HCH,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
4	p,p-DDE,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
5	Gamma HCH,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
6	trans-Chlordane,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
7	Chlorfenvinphos,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
8	Heptachlor,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
9	Parathion-Methyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
10	Chlorpyriphos-ethyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
11	Bromophos-methyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
12	Methidathion,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
13	Endosulfan alpha,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
14	o,p-DDE,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
15	Dieldrin,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
16	Endosulfan beta,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
17	Quintozene,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
18	Pirimiphos methyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
19	Endrin,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
20	cis-Chlordane,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
21	Fenthion,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
22	Chlorpyrifos methyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202

Laboratory:
TÜV SÜD South Asia Pvt. Ltd.
No. A 151, 2nd C Main,
2nd Stage,
Peenya Industrial Estate
Bangalore,

Phone :+91 80 67458096
Fax :+91 80 67458058
E-Mail :satya.srinivas@tuv-sud.in
Url : www.tuv-sud.in
ControlNo:15360250.200517.020

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naka, Andheri (East),
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ANNEXURE 1 contd. Contaminant Analysis Report for Bedding Material (Corn Cob)

TÜV SÜD South Asia

Test Report No. BLR/F(C)/17/012621 A1
Dated 2017-05-26

S. NO.	PARAMETERS	TEST RESULT	TEST METHOD
23	Beta HCH,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
24	Fenitrothion,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
25	Diazinon,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
26	Delta HCH,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
27	Bromophos-ethyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
28	Dimethoate,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
29	Heptachlor Epoxide,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
30	p,p-DDD,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
31	Malathion,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
32	p,p -DDT,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
33	Parathion-ethyl,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
34	Mecarbam,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
35	Ethion,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
36	Hexachlorobenzene / Benzene Hexachloride,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
37	Aldrin,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
38	o,p-DDD,mg / kg	Not Detected (LOQ : 0.01)	Lab_P_SOP_202
	Poly Chlorinated Biphenyls (PCBs)		
39	2, 2, 3, 4, 4, 5, 6 - Heptachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
40	2, 2, 4, 4, 5, 5 - Hexachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
41	2 - Chlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
42	2, 2, 5 - Trichlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
43	2, 2, 3, 3, 4, 4, 5 - Heptachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition

Laboratory:
TÜV SÜD South Asia Pvt. Ltd.
No. A 151, 2nd C Main,
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Phone :+91 80 67458096
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E-Mail :satya.srinivas@tuv-sud.in
Url : www.tuv-sud.in
ControlNo:15360250.200517.020

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naka, Andheri (East),
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ANNEXURE 1 contd. Contaminant Analysis Report for Bedding Material (Corn Cob)

TÜV SÜD South Asia

Test Report No. BLR/F(C)/17/012621 A1
Dated 2017-05-26

S. NO.	PARAMETERS	TEST RESULT	TEST METHOD
44	2, 2, 3, 4, 4, 5, 5 - Heptachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
45	2, 4, 5 - Trichlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
46	2, 2, 3, 4, 5, 5 - Hexachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
47	2, 2, 3, 5 - Tetrachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
48	2, 2, 3, 3, 4, 4, 5, 5, 6 - Nonachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
49	2, 2, 3, 4, 5, 5, 6 - Heptachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
50	2, 3 - Dichlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
51	2, 2, 3, 5, 5, 6 - Hexachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
52	2, 3, 4, 4 - Tetrachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
53	2, 2, 3, 4, 4, 5 - Hexachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
54	2, 2, 3, 4, 5 - Pentachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
55	2, 2, 5, 5 - Tetrachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
56	2, 3, 3, 4, 6 - Pentachlorobiphenyl,mg / kg	Not Detected (LOQ : 0.01)	983.21, Chapter 10, AOAC, 20th Edition
	Metals & Minerals :		
57	Mercury as Hg,mg / kg	Not Detected (DL : 0.02)	Lab_P_SOP_36 / AAS
58	Cadmium as Cd,mg / kg	Below Detection Limit (DL : 0.2)	Lab_P_SOP_36 / ICP - OES

Laboratory:
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E-Mail :satya.srinivas@tuv-sud.in
Url : www.tuv-sud.in
ControlNo:15360260.200517.020

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naka, Andheri (East),
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ANNEXURE 1 contd. Contaminant Analysis Report for Bedding Material (Corn Cob)

South Asia

Test Report No. BLR/F(C)/17/012621 A1
Dated 2017-05-26

S. NO.	PARAMETERS	TEST RESULT	TEST METHOD
59	Arsenic as As,mg / kg	Below Detection Limit (DL : 0.2)	Lab_P_SOP_36 / AAS
60	Lead as Pb,mg / kg	Not Detected (DL : 0.2)	Lab_P_SOP_36 / ICP - OES
61	Selenium as Se,mg / kg	Below Detection Limit (DL : 0.005)	Lab_P_SOP_36 / ICP - OES
62	Copper as Cu,mg / kg	1.5	Lab_P_SOP_36 / ICP - OES

Authorised By
Ramesh V G
(Lab Manager)

Authorised By
Sagar Utture
(Lab Manager)

- END OF TEST REPORT-

Laboratory:
TÜV SÜD South Asia Pvt. Ltd.
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ANNEXURE 2. Analysis Report and Contaminant Analysis Report for Teklad Rats/Mice Diet – Maintenance Pellet

2014C

ENVIGO

Teklad Certified Global 14% Protein Rodent Maintenance Diet

Lot Number	2014C-081517MA
Date of Manufacture	08/15/17
Expiry Date	5/12/2018
Report Date	09/01/17

Laboratory Diet Certification Report

The following data is a consolidation of results obtained from one or more independent testing laboratories. The actual laboratory results are available upon request.

Kurt Schafer
Quality Assurance Specialist
Envigo RMS, Inc. - Teklad Diets

2017.09.05
09:11:12
-05'00'

Analysis	Result (%)
Proximate Analysis	
Protein	14.30
Fat	3.61
Fiber	4.53
Moisture	11.41
Ash	4.67
Calcium	0.70
Phosphorus	0.70

Analysis	Result	Units	Established Maximum Concentration
Heavy Metals			
Arsenic	< 0.10	ppm	1.00
Cadmium	< 0.10	ppm	0.50
Lead	< 0.20	ppm	1.50
Mercury	< 0.05	ppm	0.20
Selenium	0.22	ppm	0.50
Mycotoxin			
Aflatoxin B1, B2, G1, G2	< 5.00	ppb	5.00
Chlorinated Hydrocarbons			
Aldrin	< 0.01	ppm	0.03
Lindane	< 0.01	ppm	0.05
Chlordane	< 0.01	ppm	0.05
DDT & related substances	< 0.03	ppm	0.15
Dieldrin	< 0.02	ppm	0.03
Endrin	< 0.02	ppm	0.03
Heptachlor	< 0.01	ppm	0.03
Heptachlor Epoxide	< 0.01	ppm	0.03
Toxaphene	< 0.10	ppm	0.15
PCB's	< 0.10	ppm	0.15
a-BHC	< 0.01	ppm	0.05
b-BHC	< 0.01	ppm	0.05
d-BHC	< 0.01	ppm	0.05
Hexachlorobenzene	< 0.01	ppm	0.03
Mirex	< 0.01	ppm	0.02
Methoxychlor	< 0.05	ppm	0.50
Organophosphates			
Thimet	< 0.15	ppm	0.50
Diazinon	< 0.14	ppm	0.50
Disulfaton	< 0.15	ppm	0.50
Methyl Parathion	< 0.14	ppm	0.50
Malathion	< 0.14	ppm	0.50
Parathion	< 0.12	ppm	0.50
Thiodan	< 0.02	ppm	0.50
Ethion	< 0.14	ppm	0.50
Trithion	< 0.15	ppm	0.50

Teklad Global Diets is a trademark of Envigo. © Envigo 2015

Envigo Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

ANNEXURE 3. Analysis Report – Water Sample

EUROFINS ADVINUS LIMITED
21 & 22, PEENYA INDUSTRIAL AREA, II PHASE
BENGALURU- 560 058

ANALYSIS REPORT - WATER SAMPLE

From: Analytical R&D Department
Eurofins Advinus, Bengaluru-560 058

To: Department of Safety Assessment.
Eurofins Advinus, Bengaluru-560 058

Our Ref. No: WC/TWS/339 Date: 16/04/2018

Sample Details: Source of Collection: Outlet of the Aquaguard (At use point)

Date of Collection: 17/01/2018

ANALYSIS RESULTS

Sl. No.	Parameter	Content
1.	Colour	Colourless
2.	Odour	Odourless
3.	Turbidity	Clear
4.	pH	7.01
5.	Electrical Conductivity, dSm^{-1}	NA
6.	Total solids, (ppm)	NR
7.	Suspended solids, (ppm)	NR
8.	Dissolved solids, (ppm)	130
9.	Dissolved oxygen (ppm)	NA
10.	Biochemical Oxygen Demand 5 days at 20°C, (ppm)	NA
11.	Chemical Oxygen Demand (ppm)	NA
12.	Total hardness as $CaCO_3$	45
13.	Calcium as Ca^{2+}	15
14.	Magnesium as Mg^{2+}	NA
15.	Chlorides as Cl^-	64
16.	Sulphates as SO_4^{2-}	13.33
17.	Carbonates as CO_3^{2-}	NA
18.	Bicarbonates as HCO_3^-	NA
19.	Sodium as Na	NA
20.	Potassium as K	NA

Sl. No.	Parameter	Content (ppm)
21	Iron as Fe	NA
22	Manganese as Mn	NA
23	Copper as Cu	BQL (0.00990)
24	Zinc as Zn	NA
25	Arsenic as As	BQL (0.00990)
26	Cobalt as Co	NA
27	Cadmium as Cd	BQL (0.00198)
28	Lead as Pb	BQL (0.00990)
29	Mercury as Hg	BQL (0.000247)
30	Selenium as Se	BQL (0.00198)
31	Magnesium as Mg	NA
32	Fluorides as F	NA

NA: Not Analysed
NR: Not Reported
BQL: Below Quantification Limit

16/4/2018
Analytical R&D Department

ANNEXURE 4. Contaminant Analysis Report for Water Sample

Auriga Research

Auriga Research Private Limited
Govt. Approved Test House
No. 136, 6th Cross, 2nd Stage, Yeshwanthpur Industrial Suburb, Bangalore - 560 022.
Ph. : +91-80-28396878
Email : admin@aurigaresearch.com, Website : www.aurigareasearch.com

Certificate of Analysis **Report No.** : BG201705220050

Sample	: WATER SAMPLE				
Mfgd. By	: NS		**Received On**	:	22/05/2017
Supplied By	: NS		**Mfg lic. No.**	:	NS
Submitted By	: ADVINUS THERAPEUTICS LTD.		**Ref. No.**	:	NS
Address	: 21&22,PHASE II,PEENYA INDUSTRIAL AREA, BANGALORE, KARNATAKA- 560058				

Batch No	Mfg. Date	Expiry Date	Batch Size	Sample Quantity
NS	NS	NS	NS	1 Ltr

Date of start of analysis 31/05/2017 **Date of completion of analysis** 06/06/2017

Reference to protocol :- **IS:10500 : 2012 (Amendment No. 1 June 2015)**

<Test>	<Results>	<Acceptable Limit>	<Permissible Limit>	<Method>
1 Description	Clear colourless liquid		-	-
TABLE 3. PARAMETERS CONCERNING TOXIC SUBSTANCES (mg/L)				
2 Polychlorinated biphenyle (PCB)	Below Detection Limit	0.0005 Max.	-	APHA
TABLE 5 : PESTICIDE RESIDUES (µg/l)				
PESTICIDE RESIDUES:				
3 Alpha - HCH	Below Detection Limit	0.01 Max.	-	AOAC 990 . 06
4 Lindane	Below Detection Limit	2.0 Max.	-	AOAC 990 . 06
5 Monocrotophos	Below Detection Limit	1.0 Max.	-	AOAC 990 . 06
6 Butachlor	Below Detection Limit	125.0 Max.	-	AOAC 990 . 06
7 Malathion	Below Detection Limit	190.0 Max.	-	AOAC 990 . 06
8 Alachlor	Below Detection Limit	20.0 Max.	-	AOAC 990 . 06
9 Ethion	Below Detection Limit	3.0 Max.	-	AOAC 990 . 06
10 Chlorpyrifos	Below Detection Limit	30.0 Max.	-	AOAC 990 . 06
11 Phorate (sum of phorate its oxygen alalogue and their sulfones expressed as phorate).	Below Detection Limit	2.0 Max.	-	AOAC 990 . 06
12 Atrazine	Below Detection Limit	2.0 Max.	-	AOAC 990 . 06
13 Methyl Parathion	Below Detection Limit	0.3 Max.	-	AOAC 990 . 06
14 Aldrin & Dieldrin	Below Detection Limit	0.03 Max.	-	AOAC 990 . 06
15 Beta HCH	Below Detection Limit	0.04 Max.	-	AOAC 990 . 06
16 Delta HCH	Below Detection Limit	0.04 Max.	-	AOAC 990 . 06
17 DDT (O, P & P, P Isomers of DDT, DDE & DDD)	Below Detection Limit	1.0 Max.	-	AOAC 990 . 06
18 Endosulfan (Alpha, Beta & Sulphate)	Below Detection Limit	0.4 Max.	-	AOAC 990 . 06

Page 1 of 2 Date :- Tuesday,Jun 06, 2017

Authorized Signatory
T. GANESAN
Sr. Technical Manager

Note : The results listed refer only to the tested sample and applicable parameter, Endorsement of products is neither inferred nor implied.
This report is not to be reproduced wholly or in part and cannot be used as evidence in the court of Law and should not be used in advertising media without our special permission in writing
The samples will be destroyed in appropriate time from the date of issue of test certificate unless otherwise specified (in case of non-perishable items only).

ANNEXURE 4 contd. Contaminant Analysis Report for Water Sample

Auriga Research

Auriga Research Private Limited
Govt. Approved Test House
No. 136, 6th Cross, 2nd Stage, Yeshwanthpur Industrial Suburb, Bangalore - 560 022.
Ph. : +91-80-28396878
Email : admin@aurigaresearch.com, Website : www.aurigareasearch.com

Certificate of Analysis **Report No.** : BG201705220050

Sample	:	WATER SAMPLE			
Mfgd. By	:	NS	**Received On**	:	22/05/2017
Supplied By	:	NS	**Mfg lic. No.**	:	NS
Submitted By	:	ADVINUS THERAPEUTICS LTD.	**Ref. No.**	:	NS
Address	:	21&22,PHASE II,PEENYA INDUSTRIAL AREA, BANGALORE, KARNATAKA- 560058			

Batch No	Mfg. Date	Expiry Date	Batch Size	Sample Quantity
NS	NS	NS	NS	1 Ltr

<Test>	<Results>	<Acceptable Limit>	<Permissible Limit>	<Method>
19 2, 4 Dichlorophenoxy acetic acid	Below Detection Limit	30.0 Max.	-	USEPA 515.1
20 Isoproturon	Below Detection Limit	9.0 Max.	-	USEPA 532

Tests parameters marked with *were subcontracted to our concerned laboratory

Report : PARTY ASKED OR THE ABOVE TESTS ONLY

Page 2 of 2 Date :- Tuesday,Jun 06, 2017

T.GANESAN
Authorised Signatory
Sr. Technical Manager

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This report is not to be reproduced wholly or in part and cannot be used as evidence in the court of Law and should not be used in advertising media without our special permission in writing
The samples will be destroyed in appropriate time from the date of issue of test certificate unless otherwise specified (in case of non-perishable items only).

ANNEXURE 5. GLP Certificate

The summary of GLP accreditations received by the test facility from multiple Monitoring Authorities and the latest GLP certificate issued by the Indian National GLP Compliance Monitoring Authority are given below:

(i) Summary of Accreditations Received by the Test Facility from Multiple GLP Monitoring Authorities

GLP Monitoring Authority	Accreditation received in the year
Germany	1992, 1997, 2001, 2005 and 2010.
Netherlands	1999, 2003, 2007 and 2009.
India	2005, 2008, 2012, 2014 and 2017.

Note:

1. The test facility was inspected / monitored as per the OECD GLP system by the respective Monitoring Authorities stated above, and maintained the accreditations continuously.

2. India accepted the OECD Council invitation and received the Full Adherence status for Mutual Acceptance of Data (MAD) in March 2011, and also became a member of the OECD GLP Working Group. As per the OECD provisions, the Monitoring Authorities in OECD member countries as well as those in Full Adherent countries would inspect test facilities in the respective countries for the purpose of monitoring and accreditation. Accordingly, since 2011, the test facility was inspected only by the Indian National GLP Compliance Monitoring Authority (NGCMA) for monitoring and accreditation.

ANNEXURE 5 contd. GLP Certificate

(ii) GLP Certificate – India

National Good Laboratory Practice Compliance Monitoring Authority

Certificate of GLP Compliance

सत्यमेव जयते

GOVERNMENT OF INDIA
Department of Science and Technology
Technology Bhawan, New Mehrauli Road, New Delhi-110016
www.dst.gov.in/ngcma

ANNEXURE 5 contd. GLP Certificate

सत्यमेव जयते

भारत सरकार
विज्ञान और प्रौद्योगिकी विभाग
राष्ट्रीय उत्तम प्रयोगशाला पद्धति (जी एल पी) अनुपालन निगरानी प्राधिकरण (एन जी सी एम ए)

जी एल पी अनुपालन प्रमाण-पत्र

निरीक्षण और तत्पश्चात अनुवर्ती कार्रवाईयों के आधार पर

यूरोफिन्स एडवीनस लिमिटेड

21एवं22 फेस II, पिन्या इंडस्ट्रियल एरिया
बेंगलुरू – 560058

को जीएलपी के आर्थिक सहयोग एवं विकास संगठन (ओईसीडी) सिद्धांतो के अनुसार निम्नलिखित जांच/अध्ययन करने में सक्षम होने के लिए प्रमाणित किया जाता है:

- **पाँच बेच विश्लेषण सहित भौतिक-रासायनिक जांच**
- **विषैलापन अध्ययन**
- **उत्परिवर्तजनियता अध्ययन**
- **जलीय एवं पार्थिव जीवों के संबंध में पर्यावरणीय विषैलापन अध्ययन**
- **जल, मृदा और वायु में व्यवहार अध्ययन: जैवसंचयन**
- **अवशिष्ट अध्ययन**
- **विश्लेषणात्मक एवं नैदानिक रसायन जांच**
- **अन्य**

विशेषज्ञता के विशिष्ट क्षेत्र, रसायनों के प्रकार और जांच प्रणालियों की सूची अनुलग्नक में दी गई है।

वैधता की अवधि : 07 नवम्बर, 2017 – 06 नवम्बर, 2020

यह प्रमाण पत्र इस शर्त के अध्यधीन है कि जांच सुविधा एनजीसीएमए के प्रलेख संख्या जीएलपी–101 के निबंधन एवं शर्तों तथा जीएलपी के ओईसीडी सिद्धांतों का अनुपालन करती है।

प्रमाण पत्र सं. : जी एल पी/सी–111/2017
जारी करने की तारीख : 26–12–2017

(डॉ. नीरज शर्मा)
प्रमुख, राष्ट्रीय जी एल पी
अनुपालन निगरानी प्राधिकरण

ANNEXURE 5 contd. GLP Certificate

सत्यमेव जयते

GOVERNMENT OF INDIA
Department of Science and Technology
National Good Laboratory Practice (GLP) Compliance Monitoring Authority (NGCMA)

Certificate of GLP Compliance

Based on the Inspection and the subsequent follow-up actions

Eurofins Advinus Limited

21&22 Phase II, Peenya Industrial Area
Bengaluru – 560058

is certified capable of conducting the below-mentioned tests/studies in compliance with Organization for Economic Co-operation & Development (OECD) Principles of GLP:

- **Physical-chemical Testing including Five batch analysis**
- **Toxicity Studies**
- **Mutagenicity Studies**
- **Environmental Toxicity Studies on Aquatic & Terrestrial Organisms**
- **Studies on Behaviour in Water, Soil, and Air; Bioaccumulation**
- **Residue Studies**
- **Analytical and Clinical Chemistry Testing**
- **Others**

The specific areas of expertise, types of chemicals and test systems are listed in annexure overleaf.

Validity: November 07, 2017 – November 06, 2020

This certificate is subject to the condition that the test facility complies with the Terms & Conditions of the NGCMA's Document No. GLP-101 and OECD Principles of GLP.

Certificate No. : GLP/C-111/2017
Issue Date : 26-12-2017

(Dr. Neeraj Sharma)
Head, NGCMA

ANNEXURE 5 contd. GLP Certificate

National GLP Compliance Monitoring Authority (NGCMA)

Annexure to Certificate of GLP Compliance No. GLP/C-111/2017

Areas of Expertise:

Physical-chemical Testing including Five-batch Analysis

Toxicity Studies

- Acute Toxicity
- Skin irritation/ corrosion
- Eye irritation
- Skin Sensitization
- Dermal Toxicity
- Inhalation Toxicity
- Sub-acute/ Sub-chronic Toxicity
- Chronic Toxicity
- Reproductive Toxicity
- Carcinogenicity studies

Mutagenicity Studies

- Chromosomal Aberration Test (*In-vitro/ In-vivo*)
- Micronucleus Assay (*In-vitro/ In-vivo*)
- Bacterial Reverse Mutation Assay (AMES Test)
- Gene Mutation Test (*In-vitro*)

Environmental Toxicity Studies on Aquatic & Terrestrial Organisms

Studies on Behaviour in Water, Soil, and Air; Bioaccumulation

- Biodegradability Studies
- Environmental Fate Studies

Residue Studies

Analytical and Clinical Chemistry Testing

Others

- Bioanalytical and Toxicokinetics
- Drug Metabolism and Pharmacokinetics & Tissue Distribution
- Safety Pharmacology
- PK and Immunogenicity analysis of Biologics

Types of Chemicals:

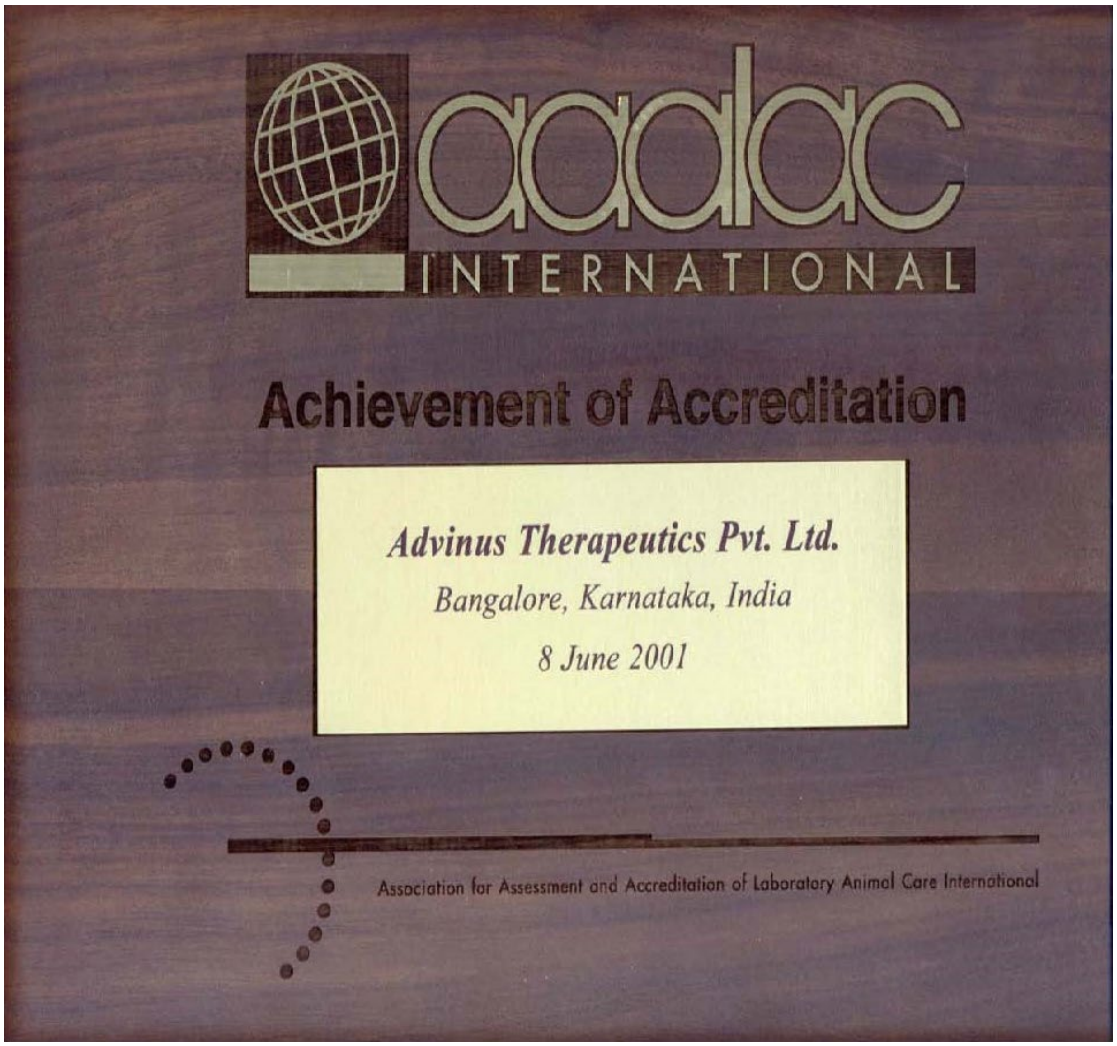
Industrial Chemicals, Pharmaceuticals, Veterinary Drugs, Pesticides, Cosmetic Products, Food Additives, Feed Additives, GMOs, Biomaterials, Ophthalmic Devices, Packaging Materials, Nutraceuticals and Medical Devices.

Test Systems:

Rat, Mice, Rabbit, Guinea Pig, Dog, Goat, Chicken, Quail, Pigeon, Hamster, Fresh Water Fish, Daphnia, Algae, Honeybee, Earthworm, *Salmonella typhimurium*, *Escherichia coli*, Mouse Lymphoma Cell line, Chinese Hamster Ovary (CHO) Cell Line, RhE and Human Hepatocellular Carcinoma Cell Line.

(Dr. Neeraj Sharma)
Head, NGCMA

ANNEXURE 6. AAALAC Certificate



ANNEXURE 7. Study Plan

STUDY PLAN

ACETAMIDE: 7-DAY REPEAT DOSE ORAL TOXICITY STUDY IN WISTAR RATS WITH TOXICOGENOMICS

STUDY No.: G16084

SPONSOR

MICHIGAN STATE UNIVERSITY
COLLEGE OF ENGINEERING, MICHIGAN STATE UNIVERSITY
EAST LANSING
MICHIGAN 48824

TEST FACILITY

DEPARTMENT OF SAFETY ASSESSMENT
EUROFINS ADVINUS LIMITED
POST BOX No. 5813, PLOT Nos. 21 & 22
PEENYA II PHASE, BENGALURU 560 058
INDIA

TEST SITE
(TOXICOGENOMICS)

SYNGENE INTERNATIONAL LTD
SEZ-16 UNIT-IX
PLOT NO. 2, 3, 4 & 5, PHASE IV
BIOCON PARK
JIGANI LINK ROAD
BENGALURU-560099
INDIA

ANNEXURE 7 contd. Study Plan

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1. STUDY DETAILS

Monitoring Scientist	:	Dr. Bobby Bringi Research Professor and AFEX Project Director 3815 Technology Blvd. Lansing, MI 48910 Telephone: +1-517-336-4602 Email: bringi@msu.edu

Key Study Personnel

Study Director	:	D. P. Santhosh kumar, M.Sc., Ph.D., ERT
Clinical Veterinarian	:	G. Sacravarty, M.V.Sc., DABT
Study Pathologist	:	K.C.Jayachandra, M.V.Sc., DABT
Formulation in-charge and Bioanalyst	:	Alagappan L., M.Pharm.
Test Site Management	:	Dr. Jayashree Aiyar Vice President, Discovery Biology Syngene International Ltd. SEZ-16 UNIT-IX, Plot No.2,3,4 & 5, Phase IV, Biocon Park, Jigani Link Road Bangalore-560099 India Mobile: +91 9008488022 Email: Jayashree.Aiyar@syngeneintl.com
Principal Investigator (Toxicogenomics)	:	Seetha K, Ph.D Lead Investigator, Biology Syngene International Ltd. SEZ-16 UNIT-IX, Plot No.2,3,4 & 5, Phase IV, Biocon Park, Jigani Link Road Bengaluru-560 099, India Telephone: +91 804014 8607 Mobile: +91 99005 80779 Email: seetha.krishnan@syngeneintl.com
Test Site name and Address (Toxicogenomics)	:	Syngene International Ltd. SEZ-16 UNIT-IX, Plot No.2,3,4 & 5, Phase IV, Biocon Park, Jigani Link Road Bangalore-560 099

ANNEXURE 7 contd. Study Plan

India
Telephone: +91 08 2808 8616

Test Site QAU	:	Shivakumar Madenahalli Manager Syngene International Ltd. SEZ-16 UNIT-IX, Plot No.2,3,4 & 5, Phase IV, Biocon Park, Jigani Link Road Bangalore-560099 India Mobile: +919880303771 Email: Shivakumar.Madenahalli@syngeneintl.com
Lead QAU	:	Rajeeva G S, M. Pharm. RQAP-GLP Quality Assurance Unit, GLP Section, Eurofins Advinus Limited Post Box No. 5813, Plot Nos. 21 & 22 Peenya II Phase, Bengaluru – 560 058, India Telephone: 080-28394959 Ext.391 Email: rajeeva.s@advinus.com
Test Facility	:	Department of Safety Assessment Eurofins Advinus Limited Post Box No. 5813, Plot Nos. 21 & 22 Peenya II Phase, Bengaluru 560 058 India

Study Schedule

Study Initiation Date	:	19 January 2018
Experiment Start Date	:	19 January 2018
Acclimatization	:	19 January 2018 to 29 January 2018
Treatment	:	30 January 2018 to 05 February 2018
Sacrifice	:	06 February 2018
Experiment Completion	:	Latest by 28 February 2018
Draft Report Date	:	Latest by 31 March 2018
Study Completion Date	:	Latest by 30 April 2018

ANNEXURE 7 contd. Study Plan

2. QUALITY ASSURANCE

Test Facility QAU: The Quality Assurance Unit (QAU) of Eurofins Advinus Limited, Bengaluru 560 058, India, will inspect the study, review the raw data, the draft and final reports. Findings of all inspections dates will be reported to the Management and to the Study Director. Inspection and reporting dates will be reflected in the Quality Assurance Statement in the report. The Quality Assurance Unit has reviewed the Study Plan and will receive a copy thereof.

Test Site QAU: The QAU of Syngene International Ltd. will have responsibilities of the test site QAU and will inspect the phase work, draft and final phase reports generated at test site and audit the phase report generated at the test site. Also to provide QA statement signed by QA personnel for inclusion in the Final Report.

3. ANIMAL WELFARE

Use of live animals is inevitable to accomplish the purpose of the study. This study is designed to use minimum number of animals to meet the scientific objectives, the goals of the sponsor and considering applicable regulatory requirements.

This study will be performed in an AAALAC approved laboratory. All procedures will be in compliance with the guidelines provided by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) India. This study plan has been approved by the Institutional Ethics Committee (IAEC) of Eurofins Advinus Limited (Approval No. 024 September 2017).

4. STUDY COMPLIANCE

The study will be performed in compliance with the following:

- OECD Principles of Good Laboratory Practice [C (97)186/Final].
- US FDA Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58).
- The mutually agreed Study Plan and the Standard Operating Procedures of Eurofins Advinus Limited.
- Toxicogenomics analysis will be performed at Syngene International Ltd., SEZ-16 UNIT-IX, Plot No.2,3,4 & 5, Phase IV, Biocon Park, Jigani Link Road, Bengaluru-560 099, India. This test site is non-GLP laboratory.

ANNEXURE 7 contd. Study Plan

5. STUDY GUIDELINES

This study will be conducted in accordance with the following guideline:

- OECD Guideline No. 407 for Testing of Chemicals, "Repeated Dose 28-Day Oral Toxicity Study in Rodents'' adopted by the Council on 3rd October, 2008 with exception to the duration of treatment period. In this study, only males will be used and age of rats will be as per the study plan.

6. JUSTIFICATION FOR THE SELECTION OF TEST SITE

The test site has already developed the analytical methods for toxicogenomics analysis. The test site QA shall perform the QA functions for these phases.

7. AMENDMENT AND DEVIATION PROCEDURES

This Study Plan may be amended or subjected to alterations.

The amendment to the approved Study Plan will be put in writing and realized only after written consent from the Study Sponsor. If immediate action is necessary, verbal agreement with the Sponsor will be confirmed as soon as possible by Study Plan Amendment.

Any unintended change(s) to the Study Plan will be documented in the raw data and mentioned in the report as deviation(s).

8. SAFETY PRECAUTIONS

Gloves, cap, face mask and goggles (if required) will be used in addition to protective body garments and shoes to ensure adequate personal health and safety and to avoid inhalation and skin contact with the test item. In case of eye contact, the eye will be washed thoroughly with water and medical treatment will be sought. In case of skin contact, it will be washed with soap and lukewarm water and subsequent medical aid would be sought if necessary.

9. OBJECTIVES

The objectives of the study are:

- To assess the toxicological and toxicogenomic profile of the test item Acetamide when administered by oral gavage to Wistar rats for a minimum period of 7 consecutive days.
- To provide information on major toxic effects, target organs and an estimate of the No Observed Effect Level (NOEL) / No Observed Adverse Effect Level (NOAEL).

ANNEXURE 7 contd. Study Plan

10. MATERIALS AND METHODS

10.1 Materials

10.1.1 Test Item Information

(Reference: TIDS and / or COA)

Test Item	:	Acetamide
Lot No.	:	XHH4A
Batch Manufactured by (Name and Address)	:	Tokyo Chemical Industry Co.,Ltd. 4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan
Batch Supplied by (Name and Address)	:	Tokyo Chemical Industry Co.,Ltd. 4-10-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-0023 Japan
Manufactured Date	:	7 December 2017
Retest Date	:	7 December 2018
Purity as per Certificate of Analysis	:	100.0 %
Physical Appearance	:	Crystal- Powder
Recommended Storage Condition	:	Ambient (+15 to +25ºC)

Notes

1. Date of receipt of test item at test facility: 19 December 2017
2. Test item code by test facility: M033-01

10.1.2 Identity of the Test Item

The identity of the test item has been provided by the Study Sponsor by a Certificate of Analysis. The test item will not be authenticated at the test facility. The correct identity and purity rests with the Sponsor.

10.1.3 Test System

Species	:	Rats (males)
Strain	:	HsdHan™: WIST Rats

ANNEXURE 7 contd. Study Plan

Source	:	Envigo Laboratories
Justification for the selection of species	:	Rat is one of the standard rodent species recommended by various regulatory authorities for toxicity assessment.
Justification for route of administration	:	The oral route is chosen because it is a potential route of human exposure.
No. of groups	:	10 Toxicity groups a. Acetamide groups: G1A - Vehicle control G2A - Low dose G3A - Mid dose G4A - Intermediate G5A - High dose b. Acetamide + Brdu groups: G1B - Vehicle control G2B - Low dose G3B - Mid dose G4B - Intermediate G5B - High dose
No. of rats/group	:	6 males Total number of rats: 60 males
Age at the start of treatment	:	9 - 11 weeks (exact age will be provided in the report)
Body weight range	:	At the commencement of treatment, the weight variation of rats used will not exceed ± 20 % of the mean body weight in each sex and group.
Identification	:	Temporary Identification: The temporary identification of animals during acclimatization period will be done with crystal violet body marking and cage cards Permanent Identification: After grouping all animals will be identified using rat accession number, cage cards, body marking with turmeric solution

ANNEXURE 7 contd. Study Plan

Acclimatization	:	After detailed clinical examination, for good health and the suitability for the study, the rats will be acclimatized for at least 7 days before start of the treatment.

10.2 Methods

10.2.1 Husbandry

10.2.1.1 Environmental Conditions

Rats will be housed in an environment controlled room at 22 ± 3°C and relative humidity of 30 to 70 percent. The photoperiod will be 12 hours light and 12 hours dark. Adequate fresh air supply of 12 - 15 air changes/hour will be maintained in the experimental room.

The maximum and minimum temperature and relative humidity in the experimental rooms will be recorded once daily. The relative humidity in the experimental room will be calculated daily from dry and wet bulb temperature recordings.

10.2.1.2 Housing

Two rats per sex per cage will be housed in sterilized standard polysulfone cages with stainless steel top grill having facilities for pelletted food and drinking water in polycarbonate bottle with stainless steel sipper tube. Cage size is approximately 425 mm (length) x 266 mm (breadth) x 185 mm (height). Additionally, polycarbonate rat huts will be placed inside the cage as environmental enrichment objects. These enrichment objects will be replaced at least once a week. During the experimental period, animals will be housed in experimental room of barrier area.

10.2.1.3 Bedding

Steam sterilized corn cob will be used as bedding and changed along with the cage at least once a week.

10.2.1.4 Diet

Teklad Certified (2014C/2018C) Global 14%/18% Protein Rodent Maintenance Diet – pellet (Certified) manufactured by Envigo, PO Box 44220, Madison, WI 53744-4220, will be provided *ad libitum* to rats.

ANNEXURE 7 contd. Study Plan

10.2.1.5 Water

Deep bore-well water passed through activated charcoal filter and exposed to UV rays in Aquaguard on-line water filter-cum-purifier manufactured by Eureka Forbes Ltd., Mumbai 400 001, India, will be provided *ad libitum* to rats in polycarbonate bottles with stainless steel sipper tubes.

The feed and water provided to the rats will be tested for contaminants. Analysis and contaminant analysis reports of feed, water and bedding material (close to the experimental period) will be included in the study report.

Based on the latest analytical certificate/s available, there are no known contaminants in the feed, water and bedding that are expected to interfere with the results of this study.

About 200 grams of the diet and 200 mL of drinking water will be stored under -50°C which will be utilized for future investigation if required or else discarded/dispatched to Sponsor as per Sponsor's recommendation. If analysis results are planned from Sponsor, then such analysis will not be included in the current report.

10.2.2 Dose Levels and Dose Justification

The dose levels of 30, 100, 300 and 1000 mg/kg/day were selected based on the available literature and in consultation with the Sponsor.

The selection of low dose (30 mg/kg/day) is based upon acetamide levels anticipated in foods if certain ammoniation feed-production technologies are widely adopted. Selected of high dose (1000 mg/kg/day) is based upon acetamide levels known to cause rodent cancers in historical studies. The mid (100 mg/kg/day) and intermediate (300 mg/kg/day) doses are selected to bridge the low and high doses, given that one of the experimental objectives is to explore a threshold level for acetamide carcinogenesis.

10.2.3 Grouping

Rats will be randomly distributed to different groups by the body weight stratification method using Provantis™ software (8.7.3, Instem LSS, Staffordshire ST15OSD, United Kingdom). In case of any emergency (i.e. failure of network, power, system, etc.) grouping will be documented in standard SOP formats. Rats with extreme body weights will be excluded from the study. Grouping will be done during acclimatization period/prior to initiation of the treatment.

ANNEXURE 7 contd. Study Plan

10.2.4 Experimental Design, Group Allocation and Number of Rats

The selected rats will be assigned to vehicle control and different treatment groups as shown below:

Toxicity Groups

a. Acetamide groups:

Group No.	Group	Colour of cage card	Dose (mg/kg /day)	Concent-ration (mg/mL)	Dose volume (mL/kg)	No. of Rats	Sex	Rat Numbers	
								From	To
G1A	Vehicle control	White	0	0	10	6	M	Ru5681	Ru5686
G2A	Low dose	Yellow	30	3	10	6	M	Ru5687	Ru5692
G3A	Mid dose	Green	100	10	10	6	M	Ru5693	Ru5698
G4A	Intermediate dose	Blue	300	30	10	6	M	Ru5699	Ru5704
G5A	High dose	Pink	1000	100	10	6	M	Ru5705	Ru5710

b. Acetamide + Brdu groups:

Group No.	Group	Colour of cage card	Dose (mg/kg/ day)	Concent-ration (mg/mL)	Dose volume (mL/kg)	No. of Rats	Sex	Rat Numbers	
								From	To
G1B	Vehicle control	White	0	0	10	6	M	Ru5711	Ru5716
G2B	Low dose	Yellow	30	3	10	6	M	Ru5717	Ru5722
G3B	Mid dose	Green	100	10	10	6	M	Ru5723	Ru5728
G4B	Intermediate dose	Blue	300	30	10	6	M	Ru5729	Ru5734
G5B	High dose	Pink	1000	100	10	6	M	Ru5735	Ru5740

M = Male

10.2.5 Justification for Selection of Vehicle

As per the information provided by the Sponsor, the test item Acetamide is soluble and forms solution in Milli Q water. Hence, Milli Q water is selected as vehicle for this study.

10.2.6 Stability of the Test Item in the Vehicle

The stability and homogeneity of the test items in dose formulations will be established separately under Eurofins Advinus Study No.: G16081 in the vehicle and the results will be presented in this study report.

10.2.7 Dose Formulation Preparation

The dose formulations will be prepared prior to administration of the test item and/or on need basis. The prepared dose formulations will be used within the prescribed stability period.

The required quantities of test item product for each dose will be weighed and taken in a beaker. Then small volume of vehicle will be added and mixed to

ANNEXURE 7 contd. Study Plan

form a solution, the same will be transferred to the measuring cylinder/volumetric flask. The beaker will be rinsed and the contents will be transferred to the measuring cylinder/volumetric flask. Required volume will be made up with vehicle to get the desired concentration.

Example for formulation preparation is as in the following table:

Groups	G1A/G1B	G2A/G2B	G3A/G3B	G4A/G4B	G5A/G5B
Dose (mg/kg/day)	0	30	100	300	1000
Dose volume (mL/kg)	10	10	10	10	10
Test item weight (mg)	-	150	500	1500	5000
Final volume made up with vehicle (mL)	50	50	50	50	50
Concentration (mg/mL)	0	3	10	30	100

The weight of the test item and volume of preparation may vary depending on the requirement.

10.2.8 Dose Formulation Analysis

Dose formulations of Acetamide will be analyzed for active ingredient concentration (a.i.) from the first preparation.

The prepared formulations will be sampled in duplicate sets wherein one set will be used for analysis and another as back up set which will be stored at ambient / refrigerated condition. The composite sample will be drawn from the prepared formulations at all the groups and will be sent to Department of DMPK & CP, for analysis. The analyses will be done as per the analytical method validated under Advinus Study No.: G16081. The analytical method for Analysis of Test Item Concentration in the dose formulation samples will be provided in amendment to the study plan. The dose formulation analysis will be performed within the stability period.

Dose formulations will be considered acceptable if the overall mean result is within ± 15% of the nominal concentration and the relative standard deviation (%RSD, calculated using all the replicate values) of assay of all layers is less than or equal to 15%.

If there is any variation in the % overall mean, the analysis will be carried out from the backup samples or subsequent days of preparation during the treatment period and will be recorded in the raw data and the details will be provided in the report. The unused back up samples will be disposed after the results of dose formulation analysis are reviewed by the Study Director.

Dose formulation analysis report will be included as an Appendix to the main report.

ANNEXURE 7 contd. Study Plan

10.2.9 BrdU Preparation in Drinking Water

Materials: BrdU (5-Bromo-2'-deoxyuridine) from Sigma, Cat# B5002-5G and Glucose from Sigma, Cat# G8270-1KG

Procedure: BrdU (0.8 mg/ml + 1% glucose) in drinking water will be prepared freshly in a fume hood and protected from light. The bottles containing drinking water will be covered with aluminum foil. Rats assigned to Brdu groups (G1B to G5B) will be given BrdU containing drinking water continuously from Day 1 of treatment till sacrifice in order to stain the accumulated nuclear antigen (BrdU) over a period of 7 days.

The BrdU-containing drinking water will be changed twice/week. Excess remaining water will be diluted with 10% bleach, disposed into sink, flushed and the bottle will be rinsed thoroughly.

10.2.10 Treatment

Acetamide dose formulations or vehicle will be administered by oral gavage to rats in specific treatment or vehicle control groups once daily at approximately the same time each day (varying by ± 3 hours) for a minimum period of 7 consecutive days.

The dose volume administered to all the rats will be as specified in the 'Experimental design' table. The dose volume will be calculated for individual animal on the first day of the treatment and will be adjusted according to the recent body weights recorded at different intervals of the study.

The unused dose formulations will be stored and sent for disposal.

In addition, BrdU formulations will be provided through drinking water to G1B to G5B groups rats from Day 1 till sacrifice.

11. OBSERVATIONS

11.1 Clinical Signs and Mortality

Each rat will be observed at least twice daily for mortality and morbidity. Routine observations for checking general clinical signs will be performed at least twice a day (pre-dose and post-dose) during treatment days. However, more frequent observations will be performed depending upon observed clinical signs.

Based on the assessment, if there are no clinical signs of concern, then the observation for morbidity and mortality will be carried out once during weekends and public holidays. Dead rats (if any) will be necropsied or preserved for necropsy. Moribund rats will be sent for necropsy.

ANNEXURE 7 contd. Study Plan

11.2 Detailed Clinical Examination

Detailed clinical examination will be done prior to start of treatment and on Day 7. On days of detailed clinical examination, these observations will replace the daily clinical sign (post-dose) observations except for Day 1.

During detailed clinical examination, all rats will be observed for changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern), changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling and bizarre behaviour (e.g. self-mutilation, walking backwards).

11.3 Ophthalmological Examination

Ophthalmological examination of all animals will be performed with an ophthalmoscope prior to start of treatment and at the end of the treatment period. Prior to examination, mydriasis will be induced using a 1 % solution of Tropicamide.

11.4 Body Weights

Individual body weights (g) will be recorded prior to test item administration on Day 1 and on a daily basis during the treatment period. Fasting body weight will be recorded prior to sacrifice for all surviving rats.

11.5 Food Consumption

The food consumption will be measured on days 1-2, 2-5 and 5-7. The cage wise average food intake (g/rat/day) will be calculated and presented in the report. The food spillage, if any, will be weighed and recorded at each food leftover recording session/during cage change. Amount of spillage, if any, will be considered for calculation of food consumption.

11.6 Water Consumption

The water consumption will be measured twice a week during the treatment Period.

The cage wise water consumption will be estimated using the water consumed at each measuring interval per cage and dividing by the number of animals per cage and the number of days in the intervening period to determine the water intake/rat/day.

ANNEXURE 7 contd. Study Plan

11.7 Bioanalysis

The objective of bioanalysis study is to determine the plasma exposures of Acetamide following once daily oral gavage administration of Acetamide dose formulation to Wistar rats for 7 days. The blood samples will be collected once on Day 8 from all rats for bioanalysis.

About 0.5 mL of blood will be collected from retro orbital plexus into tubes containing K_2EDTA as an anticoagulant. Plasma will be separated by centrifuging at 5000 rpm, 2 to 8 ºC for 5 minutes within 30 minutes of blood collection. Plasma samples will be stored at ≤-70 ºC until analysis.

The concentration of Acetamide, in rat plasma will be determined using GC-MS method in the Bioanalytical section, Drug Metabolism, Pharmacokinetics and Clinical Pharmacology department, Eurofins Advinus Limited, Bengaluru as per Advinus Study No. G16082. The plasma concentration will be observed and reported.

Tissue Sample

At necropsy, right caudal liver lobe will be collected from all surviving rats after completion of 7-days treatment. The liver tissue collected for drug concentration analysis will be flash frozen with liquid Nitrogen and will be stored at -70^0 C. These samples will be sent to Department of DMPK & CP, Eurofins Advinus Limited for bioanalysis. These samples will be transferred along with labelled tubes/containers, and stored at ~ -70 ºC until bioanalysis for Acetamide.

The liver tissue concentration of Acetamide will be determined using a validated GC-MS method as per Advinus Study No. G16083.

Bioanalytical report will be included as an appendix to the main report.

11.8 Clinical Pathology Investigations

11.8.1 Blood Collection

Blood will be collected at the end of the treatment period (prior to necropsy on Day 8) from all surviving rats. All surviving rats will be fasted overnight (water allowed) and approximately 3 mL of blood will be collected from each rat (as indicated in the Table below) under isoflurane anesthesia, with the help of a fine capillary tube, by retro-orbital plexus puncture.

Parameter	Approximate volume (mL)	Anticoagulant	
		Name	Concentration per mL of blood
Hematology	0.7	K_2 EDTA	1.6 mg
Clinical chemistry	1.8	Lithium heparin	10 Unit
Coagulation	0.5	Trisodium citrate	3.2 mg

ANNEXURE 7 contd. Study Plan

After analysis and data review by the analyst, the residual hematology samples will be stored at 2-8 ºC and will be discarded after data review. The plasma samples used for clinical chemistry analysis will be stored frozen. The fate of these samples will be decided at the time of final report preparation.

11.8.2 Haematology

The following haematological parameters will be determined using ADVIA 2120i haematology system (Siemens Healthcare Diagnostics Inc., NY, USA).

Sl. No.	Parameter	Abbreviations	Units[2]
1	Red Blood Corpuscles	RBC	10^{12}/L
2	Haemoglobin	Hb	g/L
3	Haematocrit	Hct	L/L
4	Mean Corpuscular Volume	MCV	fL
5	Mean Corpuscular Haemoglobin	MCH	pg
6	Mean Corpuscular Haemoglobin Concentration	MCHC	g/L
7	Reticulocytes count	Retic	10^{12}/L
8	White Blood Corpuscles	WBC	10^{9}/L
9	Differential leukocyte count[1]	DLC	10^{9}/L
10	Platelets	Plat	10^{9}/L
11	Red Blood Cell Morphology		
	a. Red Cell Distribution Width	RDW	%
	b. Haemoglobin Distribution Width	HDW	g/L
	c. Hyperchromic cells	Hyper	%
	d. Hypochromic cells	Hypo	%
	e. Macrocytes	Macro	%
	f. Microcytes	Micro	%
	g. RBC Fragments	RBC Frag	10^{12}/L
	h. RBC Ghosts	RBC Gh	10^{12}/L

[1]: Differential Leukocyte parameters and their respective abbreviations are: Neutrophils (Neut), Lymphocytes (Lymp), Monocytes (Mono), Eosinophils (Eosi) and Basophils (Baso).

[2]: Expansion of units are as follows: L:Litre, g: Gram, pg: Picogram, fL:Femtolitre,

11.8.3 Coagulation

Blood samples collected for coagulation analysis will be centrifuged at 2500 times gravity (x*g*) for 10 minutes for separation of plasma and analysed for the following parameters in plasma sample using STart-4 coagulation analyzer (Diagnostica stago, 92600 Asnieres, France):

Sl. No.	Parameter	Abbreviations	Units
1	Prothrombin Time	PT	Sec
2	Activated Partial Thromboplastin Time	APTT	Sec

ANNEXURE 7 contd. Study Plan

11.8.4 Clinical Chemistry

Plasma will be separated in a refrigerated centrifuge at 4000-6000 rpm, 4±1° C for 5-10 minutes and analysed using Dimension RxL MaX Clinical Chemistry System (Dade Behring Inc. Newark, DE 19714, USA) or Roche/Hitachi 902 (Hitachi High-Technologies Corporation, Tokyo, Japan) Automatic Analyser for the following parameters:

Serial Number	Parameters	Abbreviations	Units [b]
1	Alkaline Phosphatase	ALP	U/L
2	Alanine aminotransferase	ALT	U/L
3	Albumin	Alb	g/L
4	Albumin/Globulin ratio (calculated)	A/G	-
5	Aspartate aminotransferase	AST	U/L
6	Blood Urea Nitrogen	BUN	mmol/L
7	Calcium	Ca	mmol/L
8	Chloride	Cl	mEq/L
9	Creatinine	Creat	µmol/L
10	Direct Bilirubin [a]	D.Bil	µmol/L
11	Gamma Glutamyl Transpeptidase	GGT	U/L
12	Globulin (calculated)	Glob	g/L
13	Glucose	Glu	mmol/L
14	Indirect Bilirubin [a] (calculated)	Ind. Bil	µmol/L
15	Inorganic phosphorous	Pi	mmol/L
16	Lactate dehydrogenase	LDH	U/L
17	Potassium	K	mEq/L
18	Sodium	Na	mEq/L
19	Total Cholesterol	T.Chol	mmol/L
20	Total Protein	T.Pro	g/L
21	Total Bilirubin	T.Bil	µmol/L
22	Triglycerides	Trig	mmol/L

Notes:

[a]: Direct and indirect bilirubin will be determined if total bilirubin values are >10 µmol/L

[b]: Expansion of units are as follows: U:Unit, L:Litre, g: Gram, mmol: Millimole, mEq: Milli equivalent, µmol: Micromole

11.8.5 Urinalysis

Urine will be collected in urine collection tubes from all surviving main group rats on Day 7.

Each rat will be placed in a specially fabricated cage (water allowed) for about 3-4 hours and the collected urine will be sent for analysis. If particular rat does not yield urine, “No sample collected” will be indicated against that rat numbers in the raw data.

ANNEXURE 7 contd. Study Plan

Urine will be analyzed for the following parameters:

Sl. No.	Parameters
1	Specific gravity[1]
2	Nitrite[2]
3	pH[2]
4	Proteins[2]
5	Glucose[2]
6	Ketone bodies[2]
7	Urobilinogen[2]
8	Bilirubin[2]
9	Appearance (colour and clarity)[3]
10	Volume (approximate)[3]

[1]: Will be measured using Refractometry method (Refractometer-PAL-10S. Atago Co., Ltd., Japan).
[2]: Will be analyzed using Multistix 10 SG strips, read with Clinitek status analyzer (Bayer Healthcare LLC, UK)
[3]: Will be recorded manually.

Urine will also be subjected to microscopic examination for sediments such as crystals, epithelial cells, erythrocytes, leukocytes and casts.
An aliquot of urine samples from all rats subjected for collection will be stored under -50°C which will be utilized for future investigation if required or else discarded/dispatched to Sponsor as per Sponsor's recommendation. If analysis results are planned from Sponsor, then such analysis will not be included in the current report.

11.9 Anatomic Pathology

11.9.1 Necropsy

All rats in the study including the moribund and dead will be subjected to detailed necropsy (examination of external surfaces of the body, all orifices, cranial, thoracic and abdominal cavities and their contents) and findings will be recorded. All surviving rats will be subjected to necropsy after overnight fasting (water allowed) at the end of treatment period. Terminal body weights will be recorded for all surviving animals immediately prior to sacrifice. Rats to be sacrificed (moribund or terminal necropsy) will be euthanized by exsanguination under isoflurane anesthesia. The terminal necropsy will be done as per the random numbers generated. Between the different groups and the rats in each group will be randomized for each round of sacrifice, one animal in each group will be necropsied. To begin with, in the total of 8 groups with 6 rats each, the randomization will be done among the 8 groups and among the 6 rats in each group. For the following round, the 8 groups will be randomized with the randomization among the remaining 5 rats in each group. This procedure will be followed till the last round of rats subjected for terminal necropsy.

ANNEXURE 7 contd. Study Plan

11.9.2 Tissue Collection, Weighing and Preservation

On completion of the gross pathology examination, the tissues and organs noted below will be collected from all rats. The tissues will be weighed from all terminally sacrificed rats. The organ weight ratios (organ to terminal fasting body weight and organ to brain weight) will be determined and presented in the report. Paired organs will be weighed together and combined weight will be presented. Tissues will be preserved in 10% Neutral Buffered Formalin (NBF) unless noted otherwise.

Sl. No.	Tissue	Organ weight	Collection and preservation
1	Adrenals	X	X
2	All gross lesions		X
3	Aorta		X
4	Bone marrow smear		X
5	Brain including cerebrum, cerebellum and medulla/pons	X	X
6	Cecum		X
7	Colon		X
8	Duodenum		X
9	Epididymides	X	X
10	Eyes with optic nerves[1]		X
11	Femur bone with distal joint[6]		X
12	Heart	X	X
13	Ileum (with Peyer's patches)		X
14	Jejunum		X
15	Kidneys	X	X
16	Liver	X	X
17	Larynx		X
18	Lungs[2]	X	X
19	Mandibular lymph nodes		X
20	Mesenteric lymph nodes		X
21	Nose		X
22	Oesophagus		X
23	Pancreas		X
24	Pituitary[3]	X	X
25	Prostate[5]	X	X
26	Rectum		X
27	Salivary glands		X
28	Sciatic nerves		X
29	Seminal vesicles and coagulating glands [5]	X	X
30	Skeletal muscle		X
31	Skin		X
32	Spinal cord at 3 levels (cervical, mid thoracic and lumber)		X
33	Spleen	X	X
34	Sternum[6]		X
35	Stomach		X
36	Tongue		X

ANNEXURE 7 contd. Study Plan

Sl. No.	Tissue	Organ weight	Collection and preservation
37	Testes[4]	X	X
38	Thymus	X	X
39	Thyroid with parathyroids[3]	X	X
40	Trachea		X
41	Urinary bladder		X
42	Ureters		X

[1]: To be collected in Davidson's fluid
[2]: To be inflated with 10 % neutral buffered formalin and then immersed in formalin
[3]: Will be weighed after formalin fixation
[4]: To be collected in modified Davidson's fluid
[5]: Prostate + Seminal vesicles with coagulating glands will be weighed as a whole; subsequently prostate will be separated and weighed. The derived weight will be presented for the seminal vesicles and coagulating glands.
[6]: Will be decalcified prior to sectioning
X: Organs to be weighed, collected and preserved

Note 1: A section of right caudal liver lobe will be preserved for determining the tissue concentration of acetamide.

Note 2: A section of liver specimen from left lobe will be snap frozen and shipped to Syngene for toxicogenomics assay adopting the following procedure:

- To prevent microbial contamination (and subsequent RNAse contamination) disposable gloves will be used and good sterile technique will be practiced when handling samples and reagents.
- Before starting isolation, work surface area will be wiped out with 10% sodium hypochloride followed by 70% ethanol and then by RNAse ZAP RNAse ZAP™ to ensure an RNAse free environment.
- All surgical instruments which will be used for dissection will be autoclaved. After tissue collection from each animal, surgical instruments will be cleaned with RNase Zap.
- Aluminum foil will be wiped with RNAse ZAP before placing in balance for weighing.
- 20 to 30 mg of liver will be placed into individually labelled sterile / RNAse / DNAse free cryo vials, and will be snap frozen in liquid nitrogen container as soon as possible.
- RNaseZAP agent will be used to wipe surfaces between samples, as well as cryo vials after labeling.
- Work surface area will be wiped with RNAse ZAP™ after each collection.

ANNEXURE 7 contd. Study Plan

- These samples will be stored -70° C deep freezer and stored until shipment. The samples will be shipped in frozen condition in dry ice to Syngene for toxicogenomics assay.

Address for shipment:

Seetha K, Ph.D
Lead Investigator, Biology
Syngene International Ltd.
SEZ-16 UNIT-IX,
Plot No.2,3,4 & 5, Phase IV,
Biocon Park,
Jigani Link Road
Bengaluru-560099
Telephone: +91 804014 8607
Mobile: +91 99005 80779
Email: seetha.krishnan@syngeneintl.com

The above mentioned samples will be collected in duplicate and one set of samples will be stored at Eurofins Advinus Limited. The fate of these samples will be decided at the time of report finalization in consultation with Sponsor.

Note 3: A small quantity of liver sample will also be collected and stored in frozen condition for any future investigation. The fate of these samples will be decided at the time of report finalization in consultation with Sponsor.

11.9.3 Histopathology

Histopathological examination will be carried out only for Liver of all rats A section of left lobe of liver will be used.

Tissues will be processed for routine paraffin embedding and 4-5 micron sections will be stained with Mayer's Haematoxylin and Eosin stain. Unused tissues will be archived.

Additional procedures to identify and/or clarify histologic features of lesions may be performed at the discretion of the pathologist and/or Sponsor/Study director and will be presented in the report (if done).

The microscopic examination can be extended to the remaining tissues based on the results available from the remaining laboratory investigations. The details will be included as an amendment to study plan and at additional cost. Photographs of microscopic findings will be taken at the discretion of pathologist.

ANNEXURE 7 contd. Study Plan

11.9.4 Immunohistochemistry

From all rats, paraffin embedded liver sections will be subjected for Ki67 immunohistochemistry staining. From all rats administered with BrdU, the immunohistochemistry staining for BrdU will be performed. The formalin preserved tissues will be processed for paraffin embedding during 48-72 hour post fixation. The procedure for BrdU and Ki67 immunohistochemistry staining is given below:

Detection of Brdu in Formalin-Fixed Paraffin-Embedded Rat Tissue

Reagent and Antibody Information

- 1x Wash Buffer (PBS)
- 3% Hydrogen Peroxide
- 1% BSA Diluent
- 1x citrate buffer
- DAB chromogen and substrate. Dako Cytomation
- Hematoxylin solution
- Blocking Solution: Dakocytomation protein block (Ready-to-Use)
- Avidin / Biotin Blocking Kit. Vector Labs.
- Primary Antibody: Mouse Monoclonal BRDU Antibody (Clone BU20a) Dako Cytomation.
- Secondary Antibody: Biotinylated Horse Anti-Mouse IgG (H+L). Vector Labs
- Label complex: R.T.U. Vectastain Elite ABC reagent Vector Labs

Staining Procedure

Positive Control Tissue: Intestine section for each Brdu incorporated rat
Stain localization: Nuclear

1. Deparaffinize and hydrate slides through the following solutions:

Solution	Repetitions	Time
Xylene	2 times	5 minutes
100% Ethanol	2 times	3 minutes
95% Ethanol	2 times	3 minutes
1X Wash Buffer	2 times	5 minutes

2. Heat-Induced Epitope Retrieval Using the Decloaker :
   Add 500 ml of distilled water to the pan inside the decloaker. Place a full rack of slides into container with 200 ml of 1X citrate buffer. (Insert blank slides into any empty slots in the rack to ensure even heating of slides). Place the container stably inside the pan and decloak the slides for 15 minutes at 110°C. Remove pan top and cool for 10 minutes.
3. Rinse the slides in 2 changes of distilled water for 3 minutes each time.
4. Quench endogenous peroxidase by placing the slides in 3% hydrogen peroxide for 15 minutes.
5. Rinse the slides in 2 changes of 1X wash buffer for 5 minutes each.

ANNEXURE 7 contd. Study Plan

6. Block with the Dako protein-blocking reagent for 10 minutes at room temperature.
7. Do not rinse slides. Continue to Avidin-Biotin block.
8. Apply Avidin block for 15 minutes at room temperature. Quick rinse in 1X wash buffer. Apply biotin block for 15 minutes at room temperature. Do not rinse sections with buffer before adding primary antibody, only wipe excess block.
9. Apply the primary antibody at a 1:200 dilution. Incubate for 1 hour at room temperature.
10. Rinse the slides in 2 changes of 1X wash buffer for 5 minutes each.
11. Apply the horse anti-mouse secondary antibody at a 1:500 dilution. Incubate for 30 minutes at room temperature.
12. Rinse slides in 2 changes of 1X wash buffer for 5 minutes each
13. Apply the Vectastain R.T.U. Elite Label and incubate for 30 minutes at room temperature.
14. Rinse slides in 2 changes of 1X wash buffer for 5 minutes each.
15. Apply the DAB chromogen. Incubate in the dark for 6 minutes at room temperature. (Add 1 drop of DAB per ml of substrate)
16. Rinse the slides in tap water 3 minutes.
17. Counterstain with hematoxylin for 20 seconds.
18. Rinse the slides in tap water until water is clear.
19. Gently agitate slides in 1X wash buffer until the tissues turn blue.
20. Dehydrate through the following solutions:

Solutions	Repetitions	Time
95% Ethanol	1 time	3 minutes
100% Ethanol	3 times	3 minutes
Xylene	2 times	5 minutes

21. Apply 1-2 drops of Vecta Mount to each slide; cover the slide with cover glass. Examine under microscope for brown staining of Nucleus

Detection of Ki 67 proliferation antigen in Formalin-Fixed Paraffin-Embedded Rat Tissue

Reagent and Antibody Information

1. 1X Wash Buffer (PBS)
2. 1X Citrate buffer
3. 3% Hydrogen Peroxide
4 Blocking Solution: Dakocytomation protein block (Ready-to-Use)
5 Anti-Ki67 [SP6] antibody (Abcam – catalog # ab16667)
6 Vectastain Elite ABC Kit Rabbit IgG (Vector Lab – catalog # PK-6101)
   a) Blocking goat serum
   b) Biotinylated secondary antibody
   c) Reagent A (Avidin DH)
   d) Reagent B (Biotinylated Horseradish Peroxidase H)
7 DAB Chromogen and substrate buffer (Dako)
8 Hematoxylin solution

ANNEXURE 7 contd. Study Plan

Staining Procedure

Positive Control Tissue: Intestine
Stain Localization: Nuclear (proliferating cells, distinctly stains nucleoli)

1. Deparaffinize and hydrate slides through the following solutions:

Solution	Repetitions	Time
Xylene	2 times	5 minutes
100% Ethanol	2 times	3 minutes
95% Ethanol	2 times	3 minutes
1X Wash Buffer	2 times	5 minutes

2. Heat-Induced Epitope Retrieval Using The Decloaker:
   Add 500 ml of distilled water to the pan inside the decloaker. Place a full rack of slides into container with 200 ml of 1X citrate buffer. (Insert blank slides into any empty slots in the rack to ensure even heating of slides). Place the container stably inside the pan and decloak the slides for 15 minutes at 110°C. Remove pan top and cool for 10 minutes.

3. Rinse the slides in 2 changes of distilled water for 3 minutes each time.
4. Rinse the slides in 2 changes of 1X wash buffer for 5 minutes each.
5. Quench endogenous peroxidase by placing the slides in 3% hydrogen peroxide for 15 minutes.
6. Rinse the slides in 2 changes of 1X wash buffer for 5 minutes each
7. Block with the Dako protein-blocking reagent for 10 minutes at room temperature.
8. Do not rinse slides.
9. Briefly rinse the slides with 1X PBS, without let dry, put 4-6 drops of biotin solution and incubate at room temperature for 15 minutes.
10. Apply Avidin block for 15 minutes at room temperature. Quick rinse in 1X wash buffer. Apply biotin block for 15 minutes at room temperature.
11. Do not rinse sections with buffer before adding primary antibody, only wipe excess block.
12. Apply the primary antibody at a 1:200 dilution. Incubate for 2 hour at room temperature.
13. Rinse the slides in 2 changes of 1X wash buffer for 5 minutes each.
14. Apply the biotinylated secondary antibody at a 1:1000 dilution. Incubate for 1 hour at room temperature.
15. Prepare ABC reagent right after step 14 since it has to develop for at least 30 minutes before use. Add 1 drop of reagent A into 5ml of 1X PBS and then add 1drop of B.
16. Rinse the slides in 3 changes of 1X wash buffer for 5 minutes each
17. Incubate with ABC reagent for 40 minutes at room temperature.
18. Rinse the slides in 3 changes of 1X wash buffer for 5 minutes each
19. Apply the DAB chromogen. Incubate in the dark for 6 minutes at room temperature. (Add 1 drop of DAB per ml of substrate)

ANNEXURE 7 contd. Study Plan

20. Rinse the slides in tap water 3 minutes.
21. Counterstain with hematoxylin for 20 seconds.
22. Rinse the slides in tap water until water is clear.
23. Gently agitate slides in 1X wash buffer until the tissues turn blue.
24. Dehydrate through the following solutions:

Solutions	Repetitions	Time
95% Ethanol	1 time	3 minutes
100% Ethanol	3 times	3 minutes
Xylene	2 times	5 minutes

25. Apply 1-2 drops of Vecta Mount to each slide; cover the slide with cover glass. Examine under microscope for brown staining of Nucleus

Analysis of BrdU/Ki67 Stained Slides

Procedure for scoring slides:

1. After the slide has been prepared by the appropriate staining procedure, place it under an appropriate microscope.
2. At first, view the slide under low power, 10X. Then view the slide under 40X so that the cells can be easily distinguished from one another.
3. Count the total number of hepatocytes that are contained within one high power (40X) field.
4. Randomly moving the slide stage around the tissue, make 10 different counts of this nature and record.
5. Next, randomly selecting the area of view of the tissue, count the total number of hepatocytes that are positively stained within one high power (40X) field. Make at least 10 counts/tissue slice and >2000 nuclei per slide following this procedure.
6. Calculate the labeling index of the slide by dividing the total number of positively stained hepatocytes by the total number of hepatocytes counted (see below).

$$\text{BrdU/Ki67 Index \%} = \frac{\text{Total number of Brdu/Ki67 stained cells}}{\text{Total number of hepatocytes counted}} \times 100.$$

12. STATISTICAL ANALYSES

Data captured using Provantis™: Parameters such as body weight, body weight gains, food consumption, organ weights and their ratios, laboratory Investigations - Haematology (Coagulation tests PT and APTT data will be entered retrospectively in Provantis™) and Clinical Chemistry data will be analyzed using Provantis built-in statistical tests.

The integrated decision tree of Provantis™:

ANNEXURE 7 contd. Study Plan

(i) Test variance homogeneity by Levene's method will be tested. When variances are heterogeneous, suitable transformation will be performed automatically by the software.

(ii) Further one-way analysis of variance (ANOVA) will be performed. When ANOVA is significant, Dunnett's control versus treatment group mean comparisons will be performed.

All analyses and comparisons will be evaluated at the 5% ($p< 0.05$) level. Statistically significant differences ($p<0.05$), indicated by the aforementioned tests will be designated by the following throughout the report:

+/-: Significantly higher (+) /lower (-) than the vehicle control group

Data may also be subjected to other statistical tests and the same will be mentioned in the report (if done).

Pathology data may be statistically analyzed at the discretion of the Study Director or Study Pathologist with Sponsor's approval and the same will be mentioned in the report, if done.

13. DATA COMPILATION

All individual animal data will be presented in Appendices and/or summarized and presented in Tables along with results of statistical analyses. All findings will be presented in the report as per the standard reporting format.

The Provantis™ system for on-line data capturing in toxicology studies will be used for recording the daily clinical sign observations, detailed clinical examination, treatment, body weights, food input and output records, terminal body weights, organ weights, laboratory investigations – Haematology (except coagulation parameters) and Clinical Chemistry, gross pathology and histopathology data.

Printouts from STart-4 coagulation analyzer will be the raw data for Coagulation tests. The Clinitek status analyzer printouts will be the raw data for the tests analysed using Multistix 10 SG strips in urinalysis, while other urinalysis data will be recorded in respective standard SOP formats.

In case of any emergency (i.e. failure of network, power, system, balance, etc.) this will be recorded in standard SOP formats. Further entry of these data in Provantis™ will be carried out retrospectively. However, for Clinical Pathology, the instrument generated printouts will be preserved as raw data. Further entry of clinical pathology data in Provantis™ will be carried out retrospectively. Other data/ procedures of the study will be recorded and documented manually in the respective standard SOP formats.

ANNEXURE 7 contd. Study Plan

14. REPORTING

The final report will include, but not be limited to:

- A descriptive title
- The name and address of sponsor, test facility along with details of study schedule
- List of Scientists/professionals
- The test item and its code, concentration, purity, composition and other appropriate characteristics and vehicle with identification by name
- A description of the test system, including species, strain, source, number, sex, age at start of the treatment, body weight range, housing conditions, method of identification and food and water and bedding material.
- A description of the doses, dose regimen, route of administration, dose formulation preparations and duration of the treatment period
- Tables of group mean values with standard deviation of body weight, food intake, haematology, clinical chemistry values and urine analysis data (only mean for volume, specific gravity and pH)
- Pathology contributory report
- Dose formulation analysis contributory report
- Bioanalysis contributory report
- Toxicogenomics phase report
- Tables of group wise clinical signs, pre-terminal deaths, body weight at sacrifice and organ weights, gross pathology and histopathological findings.
- A summary and assessment of clinical signs
- A description of the results, discussion and conclusion
- A description of study plan deviations, if any
- Contaminant analysis of bedding material
- Analysis and contaminant analysis of food and water
- A description of all circumstances that may have affected the quality or integrity of the study
- Quality Assurance Statement, signed by Quality Assurance Unit
- GLP compliance statement signed by the Study Director
- The storage locations of all raw data, specimens, reports, the test item reference sample and the archiving period
- GLP certificates
- AAALAC certificate (USA)

ANNEXURE 7 contd. Study Plan

- If response from sponsor for finalization of draft report is not received within six months after sending draft report in spite of repeated reminders, management could approve termination of study, or finalization of report and archiving. In such an event, any subsequent requests from sponsor for modification, correction or addition to the final report will be subject to report amendment at additional cost.

15. ARCHIVING

Eurofins Advinus Limited will archive at the test facility the following for 9 years after completion of the study: Study Plan, Study plan amendments (if any), raw data, draft and final reports. The raw data captured through Provantis™ software will be archived in data base server and also raw data available in Provantis™ will be transferred to EDA (Electronic Data Archives) and archived at the archives of test facility. A sample of test item was sent from the test item stores to the archives before the first dispensing of test item to studies. This sample shall be stored for a period of 9 years from the date of this final report. All tissue specimens, blocks and slides will be archived for 9 years.

16. STUDY PLAN DISTRIBUTION

The final study plan will be distributed as follows:

Test Facility: One signed study plan (Copy No. 1/1).
Sponsor: Electronic copy in PDF (eCTD) format

17. REPORT DISTRIBUTION

Test Facility: One signed final report in original (Copy No. 1/2).
Sponsor: One signed final report in original (Copy No. 2/2) and a searchable electronic copy in the PDF Format (eCTD compliant).

18. REFERENCES

- OECD Principles of Good Laboratory Practice [C (97)186/Final].
- US FDA Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58).
- International Council for Harmonization (ICH), Harmonized Tripartite Guideline M3 (R2); Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, adopted in June 2009.
- OECD Guideline No. 407 for Testing of Chemicals, "Repeated Dose 28-Day Oral Toxicity Study in Rodents'' adopted by the Council on 3rd October, 2008 with exception to the duration of treatment period.

ANNEXURE 7 contd. Study Plan

19. AGREEMENT

This study plan for Study No.: G16084 has been mutually agreed and the final study plan was approved by the Monitoring scientist through e-mail on 13 January, 2018 for the Study Director's signature.

for **TEST FACILITY**

1. _____
D. P. Santhosh kumar, M.Sc., Ph.D., ERT
STUDY DIRECTOR
Date: 19 January 2018

2. _____
Rajeeva G.S., M. Pharm., RQAP-GLP
QUALITY ASSURANCE UNIT
Date: 19 January 2018

3. _____
Vikram K. Ramanathan, Ph.D.
Management
Date: 19 Jan 2018

ANNEXURE 7 contd. Study Plan

20. PRINCIPAL INVESTIGATOR AND TEST SITE MANAGEMENT ACKNOWLEDGEMENT

Study No.: G16084

Study Title: Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats with Toxicogenomics

Delegated Phase (s): Toxicogenomics

Study Phase Identification No.: RP.17.0954

Responsibilities of the Principal Investigator

- Review the study plan, for the delegated phase information and provided comments to the Study director.
- Provide consent to the acceptance of the study phase by signing this document.
- Overall responsibility for the conduct of the study phase in compliance with the principles of GLP.
- Communicate with the Study director, test site QA and Test Site Management (TSM) on need basis.
- Any GLP deviations from the SOP and/or study phase plan, as well as any critical findings noted by the Test Site QA, that could affect the quality or the integrity of the study will be promptly notified to the Study Director.
- Issue a phase report with a GLP compliance statement signed by respective Principal Investigator and QA statement signed by QA personnel. QA statement must include phases inspected, the date of inspections, the date of reporting to PI and TSM, and the date of reporting to SD, Lead QA, and Test Facility Management (TFM) from test site QA.
- Deviations and Amendments: During conduct of study, if any deviation occurs from study plan validated method / SOPs, Principal investigator shall document the same and communicate to study director, sponsor and test site management and same will be mentioned in the phase report. Also communicate the same to Test site and Test facility QA.
- If immediate action is necessary, verbal agreement from the Study director will be sought and an amendment will be issued by the study director at test facility.
- Reporting: Phase report will be prepared as per the standard format of test site or as per SOPs. The audit of study phase report shall be done by Test site QA. After resolution of QA observations, draft study phase report shall be sent to study director for review. Once the draft study phase report is reviewed and comment/ observation resolved, all the generated data shall be archived and at the same time

ANNEXURE 7 contd. Study Plan

finalized signed copy of the phase report shall be dispatched to Test facility. The phase report will be included as an appendix to the final study report.

- Quality Assurance Audits: The actual conduct of the study and the raw data generated during the course of the study phase will be audited by the Quality Assurance department for conformance to the study plan, principles of GLP and all the governing SOPs. After corrective actions have been taken and reviewed, audit certificate will be issued by the QA department, which will be included in the phase report.
- Phase Report Distribution:

  The study phase will be distributed as follows:

  Test Facility: One signed final phase report in original and an electronic copy in the PDF Format.

  The role of principal investigator ends when study phase report is signed off. After that point onward, if any rework needs to be performed, for that study phase, amendment/ errata/ version needs to be generated for the study phase report and again principle investigator (either same or different, as per the situation) shall be assigned by TSM. The final phase report shall be signed and dated by the principal investigator and TSM to indicate acceptance of the responsibility for the validity of the data and to indicate the extent of which the study complies with the Principles of GLP.

- Archive original raw data and report at the test site archives till the retention period mentioned in the study plan.

We hereby acknowledge that we will conduct the delegated phase of the Study according to the Study Plan and the principles of Good Laboratory Practice.

Seetha K, Ph.D
Principal Investigator
Toxicogenomics Phase
Date: 29 Jan 2018

Dr. Jayashree Aiyar
Test Site Management
Date:

ANNEXURE 8. Amendments

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STUDY PLAN AMENDMENT

Amendment No. 1 **Date: January 29, 2018**

Study No. : G16084

Study title : Acetamide: 7-Day Repeat Dose Oral Toxicity Study In Wistar Rats With Toxicogenomics.

Sponsor : Michigan State University
College of Engineering, Michigan State University
East Lansing
Michigan 48824

AS IN STUDY PLAN	AMENDED AS
Page 11/32 **10.2.3 Grouping** Rats will be randomly distributed to different groups by the body weight stratification method using Provantis™ software (8.7.3, Instem LSS, Staffordshire ST15OSD, United Kingdom).	**10.2.3 Grouping** Rats will be randomly distributed to differen groups by the body weight stratification metho using Provantis™ software (10.1.0.1, Instem LSS, Staffordshire ST15OSD, United Kingdom).
Justification and impact of the amendment: To include change in the version of Provantis™ software. The above amendment does not have any impact on the outcome of the study.	

ANNEXURE 8 contd. Amendments

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AS IN STUDY PLAN
Page 13/32 **10.2.8 Dose Formulation Analysis** The analyses will be done as per the analytical method validated under Advinus Study No.: G16081. The analytical method for Analysis of Test Item Concentration in the dose formulation samples will be provided in amendment to the study plan.
AMENDED AS
10.2.8 Dose Formulation Analysis The analyses will be done as per the analytical method validated under Advinus Study No.: G16081. The analytical method for Analysis of Test Item Concentration in the dose formulation samples is provided below: **ANALYTICAL METHOD FOR ANALYSIS OF TEST ITEM CONCENTRATION IN THE DOSE FORMULATIONS** **1.1 Introduction** The method described is applicable to the determination of test item in the dose formulations, with the vehicle. **1.2 Analytical Method** **1.2.1 Principle** Test item in the dose formulations will be determined using Gas Chromatography (GC) with mass spectrometry (MS) detection. Test item and the internal standard will be derivatized to yield Xanthyl derivatives and subjected for GC-MS analysis. **1.2.2 Reagents** • Acetamide, Test-item (Used as standard) • Propionamide (Internal standard) • Milli-Q water • Methanol • Ethyl acetate • Sodium chloride • 0.5M HCl: Prepare by adding 20.625 mL of HCl stock solution (12.1M or ~37%) to 500 mL methanol in a glass bottle. • 0.7M Potassium hydroxide: Prepare by dissolving 39.277g of potassium hydroxide in 1000 mL of water. • Xanthydrol 0.25%: Weigh about 125 mg of Xanthydrol and dissolve in 50 mL of methanol. Protect this solution from light and store in a Refrigerator (2 to 8 °C). Use this solution up to 7 days from the date of preparation. • Solvents, chemicals and reagents of high purity

ANNEXURE 8 contd. Amendments

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1.2.3 Apparatus

- Analytical balance having readability ± 0.01 mg or better.
- Ultra-sonicator
- Gas Chromatography (GC) equipped with mass spectrometer (MS)
- Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm Film Thickness

1.2.4 Glassware

- Volumetric flasks of various volume
- Measuring cylinders of various volume
- Micropipettes of various volume
- HPLC glass vials (2 mL capacity) with caps
- Beakers of various volume

Note:

1. The exact make and grade of the chemicals used during the validation will be recorded in raw data and report.
2. Any other glassware as required may be used, recorded in the raw data and presented in the report.

1.2.5 Preparation of Working Standard Solutions

At least five working standard solutions containing Acetamide in the concentration range, not narrower than 1.0 to 25 µg/mL will be prepared. Any volume, as required, may be prepared. An example of the preparation is given below:

Stock Solution of Acetamide: About 10.0 mg of Acetamide reference standard will be weighed and transferred into a 10 mL volumetric flask. Milli-Q water will be added to the flask and the contents will be sonicated to dissolve (if required). Then the volume will be made up to the mark using Milli-Q water and the content will be mixed thoroughly. This results in a stock solution of about 1000 µg/mL.

Working Standard Solution: Aliquots of 0.025, 0.063, 0.125, 0.250, 0.500 and 0.625 mL of the stock solution of Acetamide will be added in respective order in to 25 mL volumetric flask. The solutions will be made up to mark with Milli-Q water. These will result in the working standard solutions at concentrations of about 1.0, 2.52, 5, 10, 20 and 25 µg/mL of Acetamide.

1.2.6 Preparation of Sample Solutions

Sample of dose formulations will be taken and diluted with diluent (sonicate, if required) in such a way to get the final concentration of about 10 µg/mL.

1.2.7 Preparation of Internal standard Working Solutions

Prepare internal standard (Propionamide) working solution (150 µg/mL) using methanol as diluent. An example of the preparation is given below:

ANNEXURE 8 contd. Amendments

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Internal Standard Stock Solution of Propionamide: About 10.0 mg of Propionamide reference standard will be weighed and transferred into a 10 mL volumetric flask. Methanol will be added to the flask and the contents will be sonicated to dissolve (if required). Then the volume will be made up to the mark using methanol and the content will be mixed thoroughly. This results in a stock solution of about 1000 µg/mL.

Internal Standard Working Solution: Aliquot of 1.50 mL of the stock solution of Propionamide will be transferred in to 10 mL volumetric flask. The volume will be made up to mark with methanol. This will result in the working solution at concentrations of about 150 µg/mL of Propionamide.

1.2.8 Derivatization Procedure

- Transfer accurately 2.5 mL of standard/samples in to a 15 mL polypropylene centrifuge tube.
- Add 50 µL of internal standard (150 µg/mL propionamide in methanol) solution to each sample and vortex to mix.
- Add 2.5 mL of 0.5M HCl in methanol to each tube and sample was vortex to mix.
- Then add 200 µl of xanthydrol (0.25%) solution, vortex to mix and incubate in darkness at 40 ^{0}C for about 90±15 minutes.
- After incubation, add about 3±0.2 g of sodium chloride to each tube.
- Neutralize all the samples by adding 2.0 mL of 0.7 M KOH.
- Add 3.0 mL of ethyl acetate to each tube. Vortex, sonicate and centrifuge all the samples at 4,000 rpm for 5 min.
- Transfer 1.7 mL of supernatant from each samples to a new RIA vial.
- Place all the samples on a nitrogen evaporator until all of the ethyl acetate is been removed.
- Add 250 µL of ethyl acetate to each RIA vial. Vortex all the samples and centrifuge at 4000 rpm for 5 min.
- Transfer 150 µL of the supernatant in to a insert placed in the GC vial for analysis by GC-MS.

1.2.9 Chromatographic Conditions

Column: Agilent HP-5MS, 0.25mm I.D., 30m length, 0.25µm film thickness.
Carrier Gas: Helium
Flow Rate: 1.2 mL/min
Column Oven Temperature:
40 °C (Hold for 2.0 min.) then 20.0 °C/minute to reach 300 °C, (hold for 10 min) -Total of 25 min.
Injector Temperature: 250°C
Injection volume: 1 µL

ANNEXURE 8 contd. Amendments

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Mass Spectrometry: SIM Mode
Solvent Delay Time : 10.0 min
Ion source temperature : 200°C
Interface temperature : 230 °C
Selected Ion Monitoring (SIM) for masses 239.1 (Xanthyl-acetamide) and 253.1 (Xanthyl-Propionamide)

Sampling Rate: 5
Mode: Splitless
Run Time: 25 minutes

- Any change in the above parametrs will be documented and reported.
- The system suitability will be checked through the use of working standard solutions, as detailed in Section 10.2.11.
- Analyte peak in the sample will be identified by comparing the retention time of analyte peak in the standard with that of analyte peak in the sample.
- Peak area of Acetamide and propionaide for each injection will be recorded
- Analytical batch will comprise of Calibration curve standards with at least 5 points, by interspersing the standard injections in between the sample injections, through the use of working standard solutions. At least one standard injection before the first sample injection and at least one standard injection after the last sample injection will also be made.
- Calibration curve will be prepared for Acetamide by plotting peak area ratio versus concentration (µg/mL, after correcting for purity) for each standard.
- Best-line fit equation (Y = a + bX) will be calculated using the method of least square. In this equation, 'Y' is peak area, 'X' is concentration (µg/mL), 'a' is -intercept and 'b' is the slope of the line.

1.2.10 Calculation

$$\text{Concentration in the sample solution } (\mu g/mL) = \frac{\text{Sample peak area} - a}{b}$$

$$\text{Calculated test item concentration } (mg/mL) = \frac{\text{Concentration in the sample solution } (\mu g/mL)}{1000} \times \frac{\text{Dilution } (mL)}{\text{Sample Volume } (mL)}$$

1.2.11 System suitability test

System suitability of the instrument will be calculated by checking the system precision. Chronology of system suitability injections will be as below:

- One diluent injection.
- At least three injections of the highest working standard solution.
- One diluent injection.

ANNEXURE 8 contd. Amendments

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System precision will be checked by calculating relative standard deviation (% RSD from the area ratio values and retention time of three replicate injections of highes working standard solution and the same should not be more than 15 and 15% respectively. Diluent, injected at the end of system suitability test, should not sho injection carryover (if any carryover is present, it should not be more than 10% of th mean standard response). If the system passes the above criteria, it will be considered suitable for carrying out th analysis.
Justification and impact of the amendment: To include analytical method for analysis of tes item concentration in the dose formulations. The above amendment does not have any impact o the outcome of the study.

This study plan amendment no.1 for Study no.: G16084 has been mutually agreed and was approved by the Monitoring Scientist through e-mail on 27 January, 2018 for the Study Director signature.

for **TEST FACILITY**

1. D. P. Santhosh kumar, M.Sc., Ph.D., ERT
STUDY DIRECTOR
Date: 29 January 2018

2. Rajeeva G.S., M. Pharm., RQAP-GLP
QUALITY ASSURANCE UNIT
Date: 29 January 2018

3. Vikram K. Ramanathan, Ph.D.
Management
Date: 29 Jan 2018

ANNEXURE 8 contd. Amendments

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STUDY PLAN AMENDMENT

Amendment No. 2 **Date: March 29, 2018**

Study No. : G16084

Study title : Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats With Toxicogenomics.

Sponsor : Michigan State University
College of Engineering, Michigan State University
East Lansing
Michigan 48824

AS IN STUDY PLAN	AMENDED AS
Page 22/32 **11.9.3 Histopathology .**	**11.9.3 Histopathology** Additionally, histopathological examination will be carried out for femur bone with distal joint, sternum and spleen for rats in control and high dose groups. If treatment-related changes are present in the above tissues, then these tissues will be examined in lower dose groups.
Justification and impact of the amendment: As there were changes observed in haematological parameters (increase in RBC counts, haemoglobin concentration, haematocrit values and decrease in reticulocytes), histopathological examination will be carried out for femur bone with distal joint, sternum and spleen. The above amendment does not have any impact on the outcome of the study.	

ANNEXURE 8 contd. Amendments

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This study plan amendment no.1 for Study no.: G16084 has been mutually agreed and was approved by the Monitoring Scientist through e-mail on 26 March 2018 for the Study Director signature.

for **TEST FACILITY**

1. pp. S
D. P. Santhosh kumar, M.Sc., Ph.D., ERT
STUDY DIRECTOR
Date: 29 March 2018

2. Muktha Bhagavan
Muktha Bhagavan, M.Sc., RQAP-GLP
QAU-GLP
Date: 29th March 2018

3.
Krishnappa H., M. V. Sc., Ph. D., DABT
MANAGEMENT
Date 29 March 2018

ANNEXURE 8 contd. Amendments

eurofins | Advinus

STUDY PLAN AMENDMENT

Amendment No. 3 **Date: May 03, 2018**

Study No. : G16084

Study title : Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats With Toxicogenomics.

Sponsor : Michigan State University
College of Engineering, Michigan State University
East Lansing
Michigan 48824

AS IN STUDY PLAN	AMENDED AS
Page 22/32 **11.9.3 Histopathology**	**11.9.3 Histopathology** In addition, the procedure for calculation of mitotic index is furnished below. **Calculation of Mitotic Index of Hepatocytes:** This will be done only for G1A-G5A groups (BrdU minus groups). **Procedure for scoring slides:** 1. Place Haematoxylin and Eosin stained liver slides under an appropriate microscope. 2. At first, view the slide under low power, 10X. Then view the slide under 40X so that the cells can be easily distinguished from one another. 3. Count the total number of hepatocytes in mitosis, that are contained within one high power (40X) field. 4. Randomly moving the slide stage around the tissue, make 10 different counts of this nature and record. 5. Make at least 10 counts/tissue slice and >2000 hepatocyte per slide.

ANNEXURE 8 contd. Amendments

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AS IN STUDY PLAN	AMENDED AS
	6. Calculate the mitotic index of the slide by dividing the total number of hepatocytes in mitosis by the total number of hepatocytes counted (see below). Mitotic Index % = Total number of hepatocyte in mitosis× 100. / Total number of hepatocytes counted In addition, All the Haematoxylin and Eosin stained liver slides of Acetamide + Brdu groups will be shipped to the Peer Review Pathologist for review.
11.9.4 Immunohistochemistry	All the Ki67 immuno-stained Liver slides of Acetamide + Brdu groups will be shipped to the Peer Review Pathologist for review. **Peer review:** Haematoxylin and Eosin stained and Ki-67 immuno-stained liver slides will be shipped for the Peer review to:Dr. J. E. Klaunig, Ph.D., Fellow ATS, Fellow IATP (Peer Review Pathologist) on the following address: Dr. J. E. Klaunig, Ph.D., Fellow ATS, Fellow IATP Professor, Environmental Health Professor, School of Public and Environmental Affairs Innovation Center Office Room 256 Office Phone 812 856 3957 Laboratory Rooms 230-236 Lab Phone 812-856-4936, 2719E 10th St Bloomington Indiana 47408 Klauniglab.com jklauni@indiana.edu The findings from the Peer review will be discussed by the study pathologist and reviewer. The consensus diagnoses/findings will be included in the report. The review process will be indicated in the peer review statement, signed by both pathologists and will be included in the main report. This document will be issued after receiving the signed pathology report from study pathologist. All the slides will be shipped back to Eurofins Advinus for archiving.
Justification and impact of the amendment: The mitotic index of hepatocytes will be calculated as per Sponsor request as it adds further value to histopathology data. As indicated by Sponsor, the slides will be shipped to Sponsor designated Pathologist for Peer review. The above amendment does not have any impact on the outcome of the study.	

ANNEXURE 8 contd. Amendments

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This study plan amendment No. 3 for Study no.: G16084 has been mutually agreed an was approved by the Monitoring Scientist through e-mail on 02 May 2018 for the Stud Director signature.

for **TEST FACILITY**

1. D. P. Santhosh kumar, M.Sc., Ph.D., ERT
Study Director
Date: 03 May 2018

2. 03, May, 2018
Satish Murthy V., M.Sc. RQAP-GLP.
Head, QAU-GLP.

3. Vikram K. Ramanathan, Ph.D.
Management
Date: 03 May 2018

ANNEXURE 8 contd. Amendments

eurofins | Advinus

STUDY PLAN AMENDMENT

Amendment No. 4 **Date: 18 September, 2018**

Study No. : G16084

Study title : Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats With Toxicogenomics.

Sponsor : Michigan State University
College of Engineering, Michigan State University
East Lansing
Michigan 48824

AS IN STUDY PLAN	AMENDED AS
Page 4/32 and 5/32 **1. STUDY DETAILS** **Study schedule** Study Completion date): Latest by 30 April, 2018	**1. STUDY DETAILS** **Study schedule** Study Completion date): 31 October 2018 or date of Study Director signature on final report
Justification and impact of the amendment: This study could not be completed as per the schedule given in the study plan, and a deviation has been documented. The proposed study completion date is now furnished in this amendment. This amendment has no impact on outcome of the study.	
Page 6/32 **4. STUDY COMPLIANCE**	**Page 6/32** **4. STUDY COMPLIANCE** • The acetamide concentration in diet and water will be performed in a Non-GLP environment. • The left over plasma samples will be analysed for presence of N-hydroxyacetamide (Acetohydroxamic Acid, a putative metabolite of acetamide) in a Non-GLP environment. • The acetamide concentration in urine samples will be performed in a Non-GLP environment. All the above analysis will be done at Department of DMPK & CP, Eurofins Advinus Limited.
Justification and impact of the amendment: To indicate the Non-GLP status for the analysis of diet, water, plasma and urine samples. This amendment has no impact on outcome of the study.	

ANNEXURE 8 contd. Amendments

eurofins | Advinus

AS IN STUDY PLAN	AMENDED AS
Page 10/32 and 11/32 **10.2 Methods** **10.2.1.4 Diet** **10.2.1.5 Water** About 200 grams of the diet and 200 mL of drinking water will be stored under -50°C which will be utilized for future investigation if required or else discarded/dispatched to Sponsor as per Sponsor's recommendation. If analysis results are planned from Sponsor, then such analysis will not be included in the current report.	**Page 10/32 and 11/32** **10.2 Methods** **10.2.1.4 Diet** **10.2.1.5 Water** About 200 grams of the diet and 200 mL of drinking water was stored under -50°C. The acetamide concentration will be determined in drinking water and diet. The analysis will be done on a Non-GLP basis using a fit-for-purpose GC-MS method and results will be included as Appendix to the report.
Justification and impact of the amendment: To confirm that rats could not have been exposed to acetamide either through either drinking water or feed diet used for this study. This amendment has no impact on outcome of the study.	
Page 15/32 **11.7 Bioanalysis**	**Page 15/32** **11.7 Bioanalysis** In addition, the left over plasma samples (after bioanalysis) from Acetamide groups (G1A, G2A, G3A, G4A and G5A) will be analysed for presence of N-hydroxyacetamide (Acetohydroxamic Acid, a putative metabolite of acetamide) on a Non-GLP basis using a fit-for-purpose colorimetric method and the results will be included as Appendix to the report.
Justification and impact of the amendment: To look for the presence of putative metabolite in plasma samples. This amendment has no impact on outcome of the study.	
Page 18/32 **11.8.5 Urinalysis** An aliquot of urine samples from all rats subjected for collection will be stored under -50°C which will be utilized for future investigation if required or else discarded/dispatched to Sponsor as per Sponsor's recommendation. If analysis results are planned from Sponsor, then such analysis will not be included in the current report.	**Page 18/32** **11.8.5 Urinalysis** In addition, the urine samples (after urinalysis) from Acetamide groups (G1A, G2A, G3A, G4A and G5A) will be analysed for presence of Acetamide on a Non-GLP basis using a fit-for-purpose GC-MS method and the results will be included as Appendix to the report.

ANNEXURE 8 contd. Amendments

eurofins | Advinus

AS IN STUDY PLAN	AMENDED AS
Justification and impact of the amendment: To determine how much Acetamide was present in urine samples to provide an insight into clearance rates. This amendment has no impact on outcome of the study.	
Page 20/32 and 21/32 **Toxicogenomics Assay**	 **Toxicogenomics Assay** In addition, the RNA-sequencing data generated at the Syngene test site by Seetha K, Ph.D (Lead Investigator, Biology, Syngene International Ltd.) will be shared with; **Michael B. Black, Ph.D.** Scientist IV ScitoVation 6 Davis Drive Research Triangle Park, NC 27709 Phone: (919)-558-1338 Email: mblack@scitovation.com Website: www.scitovation.com ScitoVation will interpret the data based upon the RNA sequencing data generated by Syngene. The interpretation data from ScitoVation will be included in the Toxicogenomics Phase Report prepared by Seetha K, Ph.D., Syngene International Ltd.
Justification and impact of the amendment: To ensure that the analysis and interpretation of RNA sequencing data will be captured in the study report. This amendment has no impact on outcome of the study.	

ANNEXURE 8 contd. Amendments

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AS IN STUDY PLAN	AMENDED AS
Page 40/46 **APPENDIX 1 Principal Investigator and Test Site Management Acknowledgement** **Responsibilities of the Principal Investigator** • Overall responsibility for the conduct of the study phase in compliance with the principles of GLP. • Any GLP deviations from the SOP and/or study phase plan, as well as any critical findings noted by the Test Site QA, that could affect the quality or the integrity of the study will be promptly notified to the Study Director. • Issue a phase report with a GLP compliance statement signed by respective Principal Investigator and QA statement signed by QA personnel. QA statement must include phases inspected, the date of inspections, the date of reporting to PI and TSM, and the date of reporting to SD, Lead QA, and Test Facility Management (TFM) from test site QA. • Archive original raw data and report at the archives of Eurofins Advinus till the retention period mentioned in the study plan.	**APPENDIX 1 Principal Investigator and Test Site Management Acknowledgement** **Responsibilities of the Principal Investigator** • Overall responsibility for the conduct of the study phase in compliance with the study plan and test site procedures. • Any deviation/s from the SOP and/or study plan, as well as any critical findings noted by the Test Site QA, that could affect the quality or the integrity of the study will be promptly notified to the Study Director. • Issue a phase report with a compliance statement signed by respective Principal Investigator and QA statement signed by QA personnel. QA statement must include phases inspected, the date of inspections, the date of reporting to PI and TSM, and the date of reporting to SD, Lead QA, and Test Facility Management (TFM) from test site QA. • Send original raw data and report to Study Director for archiving at Eurofins Advinus.
Justification and impact of the amendment: To revise the responsibilities of Principal Investigator by considering Non-GLP status of test site. The above amendment does not have any impact on the outcome of the study.	

ANNEXURE 8 contd. Amendments

eurofins | Advinus

This study plan amendment No. 4 for Study no.: G16084 has been mutually agreed and was approved by the Monitoring Scientist through e-mail on 04 September 2018 for the Study Director signature.

for **TEST FACILITY**

1. D P Santhosh kumar
D. P. Santhosh kumar, M.Sc., Ph.D., ERT
Study Director
Date: 18 September 2018

2. Rajeeva
Rajeeva G S, M. Pharm., RQAP-GLP
QAU-GLP
Date: 18 September 2018

3. Rajiv Malik 18/9/18
Rajiv Malik
MANAGEMENT
Date:

ANNEXURE 8 contd. Amendments

eurofins | Advinus

STUDY PLAN AMENDMENT

Amendment No. 5 **Date: 22 November, 2019**

Study No. : G16084

Study title : Acetamide: 7-Day Repeat Dose Oral Toxicity Study in Wistar Rats with Toxicogenomics

Sponsor : Michigan State University
College of Engineering, Michigan State University
East Lansing
Michigan 48824

AS IN STUDY PLAN	AMENDED AS
Page 15/32 and 16/32 **11.7 Bioanalysis**	**11.7 Bioanalysis** The left over liver samples after analysis will be discarded at the time of final report preparation.
Justification and impact of the amendment: To indicate the fate of left over liver samples as requested by Sponsor. The above amendment does not have any impact on the outcome of the study.	
Page 16/32 **11.8.1 Blood Collection** The plasma samples used for clinical chemistry analysis will be stored frozen. The fate of these samples will be decided at the time of final report preparation.	**11.8.1 Blood Collection** The plasma samples used for clinical chemistry analysis were stored frozen. These samples will be discarded at the time of final report preparation.
Justification and impact of the amendment: To indicate the fate of left over plasma samples as requested by Sponsor. The above amendment does not have any impact on the outcome of the study.	
Page 19/32 to 21/32 **11.9.2 Tissue Collection, Weighing and Preservation** The above mentioned samples will be collected in duplicate and one set of samples will be stored at Eurofins Advinus Limited. The fate of these samples will be decided at the time of report finalization in consultation with Sponsor. **Note 3:** A small quantity of liver sample will also be collected and stored in frozen condition for any future investigation. The fate of these samples will be decided at the time of report finalization in consultation with Sponsor.	**11.9.2 Tissue Collection, Weighing and Preservation** The above mentioned samples were collected in duplicate and one set of samples were stored at Eurofins Advinus Limited. The stored second set of samples will be discarded prior to finalization of the report as per the request of the Sponsor. **Note 3:** A small quantity of liver sample was also collected and stored. The stored liver sample will be discarded prior to finalization of the report as per the request of the Sponsor.
Justification and impact of the amendment: To indicate the fate of left over liver samples as requested by Sponsor. The above amendment does not have any impact on the outcome of the study.	

ANNEXURE 8 contd. Amendments

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AS IN STUDY PLAN	AMENDED AS
Page 29/32 **17. REPORT DISTRIBUTION** Test Facility: One signed final report in original (Copy No. 1/2). Sponsor: One signed final report in original (Copy No. 2/2) and a searchable electronic copy in the PDF Format (eCTD compliant)	 **17. REPORT DISTRIBUTION** Test Facility: One signed final report in original (Copy No. 1/1). Sponsor: A searchable electronic copy in the PDF Format (eCTD compliant) of Copy No.1/1
Justification and impact of the amendment: To prepare one copy of the final report and submission of electronic copy in the PDF Format to Sponsor. The above amendment does not have any impact on the outcome of the study.	

AS IN STUDY PLAN AMENDMENT No.4	AMENDED AS
Page 2/5 **Page 10/32 and 11/32** **10.2 Methods** **10.2.1.4 Diet** **10.2.1.5 Water**	 **10.2 Methods** **10.2.1.4 Diet** **10.2.1.5 Water** The left over diet and water samples after analysis will be discarded at the time of final report preparation.
Justification and impact of the amendment: To indicate the fate of left over diet and water samples as requested by Sponsor. The above amendment does not have any impact on the outcome of the study.	
Page 2/5 **Page 15/32** **11.7 Bioanalysis**	 **11.7 Bioanalysis** The left over plasma samples after analysis will be discarded at the time of final report preparation.
Justification and impact of the amendment: To indicate the fate of left over samples as suggested by Sponsor. The above amendment does not have any impact on the outcome of the study.	
Page 2/5 and 3/5 **Page 18/32** **11.8.5 Urinalysis**	 **11.8.5 Urinalysis** The left over urine samples after analysis will be discarded at the time of final report preparation.
Justification and impact of the amendment: To indicate the fate of left over urine samples as indicated by Sponsor. The above amendment does not have any impact on the outcome of the study.	

ANNEXURE 8 contd. Amendments

eurofins | Advinus

This study plan amendment No. 5 for Study no.: G16084 has been mutually agreed and was approved by the Monitoring Scientist through e-mail on 17 November, 2019 for the Study Director signature.

for **TEST FACILITY**

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Study Director
Date: 22 November 2019

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