Protocol for Measuring Neutralizing Antibodies Against HIV-1, SIV and SHIV Using a Luciferase Reporter Gene Assay in TZM-BL Cells

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INTRODUCTION:

This assay measures neutralization of HIV, SIV and SHIV as a function of reductions in Tat-regulated Luc reporter gene expression after a single round of infection in TZM-bl cells. This assay was first developed by Dr. George Shaw and colleagues at the University of Alabama, Birmingham (1) and was later optimized and validated at Duke University (2). TZM-bl cells (also called JC53BL-13) may be obtained from the NIH AIDS Research and Reference Reagent Program (Cat. No. 8129). This is a CXCR4-positive HeLa cell clone that was engineered to express CD4 and CCR5 (3). The cells were further engineered to contain integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under control of an HIV long-terminal repeat sequence (4). TZM-bl cells are permissive to infection by a wide variety of HIV, SIV and SHIV strains, including primary HIV isolates and molecularly cloned Env-pseudotyped viruses. Assay stocks of Env-pseudotyped viruses are produced in 293T/17 cells by co-transfection with an Env expression plasmid and a second plasmid expressing the entire HIV-1 genome except Env. Only the latter *env*-minus plasmid is transcribed into viral genomic RNA that is packaged by the pseudovirions for delivery of the *tat* gene to TZM-bl cells. Thus, co-transfection generates pseudovirus particles that are infectious but are unable to produce infectious progeny virions for subsequent rounds of infection. Reporter gene expression is induced in trans by viral Tat protein soon after single cycle infection. DEAE dextran is added to the medium to enhance infection and has been found to have no obvious effects on NAb activity. Luciferase activity is quantified as relative luminescence units (RLU) and is directly proportional to the number of infectious virus particles present in the initial inoculum over a wide range of values (Fig. 1A). The assay is performed in 96-well plates for high throughput capacity. Use of a clonal cell population provides enhanced precision and uniformity. We have validated this neutralization assay for single-cycle infection with either uncloned viruses grown in human lymphocytes or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T/17 cells.

Samples may be assayed to determine the magnitude, kinetics, breadth and duration of the neutralizing antibody response by using a three tiered algorithm as described (5). Briefly, serum and plasma samples are first tested for an ability to neutralize the homologous vaccine strain(s) and one or more additional strains of virus that are highly sensitive to neutralization (Tier 1). Examples of Tier 1 viruses are MN and SF162 that are easily neutralized by HIV-1-positive serum samples and by sera from Env-immunized animals and humans. These Tier 1 assays should detect any neutralizing antibodies that are present in test samples. Positive neutralization in Tier 1 assays would justify further testing to assess the extent of cross-neutralizing activity. Thus, serum samples would be tested in Tier 2 assays using primary isolate Env clones that are matched in genetic subtype to the vaccine strain(s). These Tier 2 assays may include standard panel of clade B and C HIV-1 reference strains described recently (6, 7). Positive neutralization in Tier 2 assays would be encouraging and would justify a final round of testing in Tier 3 assays using primary isolate Env clones from other genetic subtypes of HIV-1. This three-tiered algorithm minimizes the number of assays to be performed in cases of weak immunogens and allows large datasets to be acquired for enhanced statistical power in cases where stronger immunogenicity is observed.

Additional information, including detailed protocols, macros, educational materials and related links may be found at http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm.

References:

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HEAT-INACTIVATION OF SAMPLES

Clinical specimens may be serum or plasma, although **serum is preferred**. Anticoagulants in plasma are problematic in the assay, especially when heparin is used (some forms of heparin have potent and strain-specific antiviral activity). All anticoagulants (heparin, EDTA, ACD) are toxic to cells at plasma dilutions lower than 1:60. **Samples should be heat-inactivated at 56ºC for 1 hour prior to assay**. It is important to destroy complement activity by heat-inactivation. Complement in the serum of humans and nonhuman primates may enhance virus infection and mask neutralizing activity in cells that express complement receptors. In this regard, the expression of complement receptors on TZM-bl cells has not been thoroughly investigated. In other cases, complement activation may lead to lysis and inactivation of the virus. This latter phenomenon is a major concern for serum and plasma from small animals such as mice, guinea pigs and rabbits.

Samples should be mixed thoroughly by gentle agitation after thawing. Multiple freeze-thaw cycles should be avoided. Centrifuge the samples briefly after mixing to assure that no sample remains adhered to the inside cap or sides of the tube. Place tubes in a pre-calibrated 56ºC water bath, allowing the water level to reach the top of the sample volume but not touching the rim of the cap of the tube (for sterility purposes). Incubate for 1 hour. Remove and gently mix the sample to collect condensation that accumulated on the sides and top of the tube. Centrifuge briefly to pellet any insoluble materials. Store at 4ºC in cases where assays are to be performed within 2 weeks. Store at -80ºC in cases where assays will be delayed for >2 weeks.

CELL CULTURE

TZM-bl is an adherent cell line that we maintain in T-75 culture flasks. Complete growth medium (GM) consists of D-MEM supplemented with 10% fetal bovine serum (FBS, heat-inactivated), 25 mM HEPES and 50 μg/ml gentamicin. Cell monolayers are disrupted and removed by treatment with trypsin/EDTA:

Trypsin-EDTA Treatment for Disruption of TZM-bl Cell Monolayers:

TZM-bl is an adherent cell line that is maintained in T-75 culture flasks. Cell monolayers are disrupted and removed by treatment with trypsin/EDTA at confluency when splitting cells for routine maintenance and when preparing cells for assay.

1. Decant the culture medium and remove residual serum by rinsing monolayers with 6 ml of sterile PBS.

2. Slowly add 2.5 ml of a 0.25% Trypsin-EDTA solution to cover the cell monolayer. Incubate at room temp for 30-45 seconds. Decant the trypsin solution and incubate at 37ºC for 4 minutes. Do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.

3. Add 10 ml of GM and suspend the cells by gentle pipet action. Count cells.

4. Seed new T-75 culture flasks with approximately 10^6 cells in 15 ml of GM. Cultures are incubated at 37ºC in a 5% CO_2/95% air environment. Cells should be split approximately every 3 days.

VIRUS STOCKS

Stocks of uncloned viruses may be produced in either PBMC or T cell lines. Pseudoviruses may be produced by transfection in an appropriate cell type, such as 293T/17 cells. All virus stocks should be made cell free by low speed centrifugation and filtration (0.45-micron) and stored at -80°C in GM containing 20% FBS.

TCID50 DETERMINATION

It is necessary to determine the TCID50 of each virus stock in a single-cycle infection assay (2-day incubation) in TZM-bl cells prior to performing neutralization assays. We use a cut-off value of 2.5-times background RLU when quantifying positive infection in TCID50 assays.

We have observed that too much virus in the neutralization assay can result in strong virus-induced cytopathic effects that interfere with accurate measurements. Most virus stocks must be diluted at least 10-fold to avoid cell-killing. A standard inoculum of 200 TCID50 was chosen for the neutralization assay to minimize virus-induced cytopathic effects while maintaining an ability to measure a 2-log reduction in virus infectivity. It should be noted that different strains vary significantly in their cytopathicity. It may be necessary to use a lower TCID50 in the neutralization assay for highly cytopathic strains of virus. As shown in Fig. 1B, greater sensitivity is achieved in the neutralization assay at lower input TCID50 doses but overall this difference is less than 3-fold between 50 and 1,000 input TCID50 in most cases. Virus-induced cytopathic effects may be monitored by visual inspection of syncytium formation under light microscopy. Cytopathic effects may also be observed as reductions in luminescence at high virus doses in the TCID50 assay (Fig. 1A).

NEUTRALIZING ANTIBODY ASSAY PROTOCOL

NOTE 1: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. Using the format of a 96-well flat-bottom culture plate as illustrated Template A (Fig. 2), place 150 μl of GM in all wells of column 1 (cell control). Place 100 μl in all wells of columns 2-12 (column 2 will be the virus control). Place an additional 40 μl in all wells of columns 3-12, row H (to receive test samples).

2. Add 11 μl of test sample to each well in columns 3 & 4, row H. Add 11 μl of a second test sample to each well in columns 5 & 6, row H. Add 11 μl of a third test sample to each well in columns 7 & 8, row H. Add 11 μl of a fourth test sample to each well in columns 9 & 10, row H. Add 11 μl of a fifth test sample to each well in columns 11 & 12, row H. Mix the samples in row H and transfer 50 μl to row G. Repeat the transfer and dilution of samples through row A (these are serial 3-fold dilutions). After final transfer and mixing is complete, discard 50 μl from the wells in columns 3-12, row A into a waste container of disinfectant.

This format is designed to measure neutralizing antibody titers in the range of 1:20 to 1:43,740. Appropriate adjustments may be made to test a different range of dilutions (see "Standard Dilution Charts"). This format is designed to assay 5 samples in duplicate wells at each serum dilution per plate. Adjustments may be made to test a larger number of samples per plate. For example, 10 samples may be assayed at 4 dilutions in duplicate per plate by simply dividing the plate in half (Fig.

2, Template B). Alternatively, samples may be screened at a single dilution (Fig. 2, Template C). This latter option is advantageous when neutralizing activity is to be determined against a large number of strains. Due to possible nonspecific activity at low sample dilutions, it is recommended that corresponding pre-immune samples be included when performing screening assays.

A positive control with a known neutralization titer against the target virus should be included on at least one plate in series each time assays are performed. Also, at least one negative control sample is strongly advised. Ideally, negative controls consist of corresponding pre-immune or pre-infection samples from either test animals or study subjects. In the case of clinical vaccine trials, a sufficient number of post-inoculation samples from placebo recipients may serve to provide adequate information on negative control values.

3. Thaw the required number of vials of virus by placing in an ambient temperature water bath. When completely thawed, dilute the virus in GM to achieve a concentration of 4,000 $TCID_{50}$/ml.

Cell-free stocks of virus should be prepared in advance and cryopreserved in working aliquots of approximately 1 ml.

4. Dispense 50 μl of cell-free virus (200 $TCID_{50}$) to all wells in columns 2-12, rows A through H.

5. Cover plates and incubate for 1 hour.

6. Prepare a suspension of TZM-bl cells (trypsinize aproximately 10-15 minutes prior to use) at a density of 1 x 10^5 cells/ml in GM containing DEAE dextran (37.5 μg/ml). Dispense 100 μl of cell suspension (10,000 cells per well) to each well in columns 1-12, rows A though H. The final concentration of DEAE dextran is 15 μg/ml.

NOTE 2: The concentrations of DEAE dextran shown above are approximations. The actual optimal concentration should be determined for each new batch of dextran by performing a titration assay: The dextran should be serially diluted in a 96-well plate containing GM, virus and TZM-bl cells added, and incubated for 48 hours. Following the incubation, luminescence should be measured and the appropriate concentration selected that yields the highest RLU but has no detrimental effect on the cells as determined by microscopic examination.

7. Cover plates and incubate for 48 hours.

Assays with replication-competent viruses must be incubated for 48 hours only to minimize virus replication.

8. Remove 150 μl of culture medium from each well, leaving approximately 100 μl. Dispense 100 μl of Britelite Reagent to each well. Incubate at room temperature for 2 minutes to allow complete cell lysis. Mix by pipet action (at least two strokes) and transfer 150 μl to a corresponding 96-well black plate. Read the plate immediately in a luminometer.

9. Percent neutralization is determined by calculating the difference in average RLU between test wells (cells + serum sample + virus) and cell control wells (cells only, column 1), dividing this result by the difference in average RLU between virus control (cell + virus, column 2) and cell control wells (column 1), subtracting from 1 and multiplying by 100. Neutralizing antibody titers are expressed as the reciprocal of the serum dilution required to reduce RLU by 50%.

ANALYZING AND PRINTING RESULTS

The "Luminescence" macro calculates the percent neutralization provided by each serum dilution. Our luminometer is interfaced with a dedicated computer in the BSL-3 laboratory and linked electronically, via a network file server, to a data analysis computer in our general office area. The accompanying software program automatically saves the raw data generated by the luminometer onto the network file server, after each plate is read, using a unique file identification number (ID) for each plate. The file ID corresponds to each operator's user folder, the date, and the assay read number. For example, the file ID for the 1000th assay plate read on February 15, 2006 by Operator A would be: "A20060215;1000." Analyze and print the data using the Microsoft Excel macro, also located on the network file server. **The data print-out must include: i) experiment number, ii) protocol number, iii) cells used in the assay, iv) length of incubation in days, v) name, lot number and dilution of the virus stock used, vi) ID, visit number and bleed date of each sample and vii) signature of technician who performed the assay**.

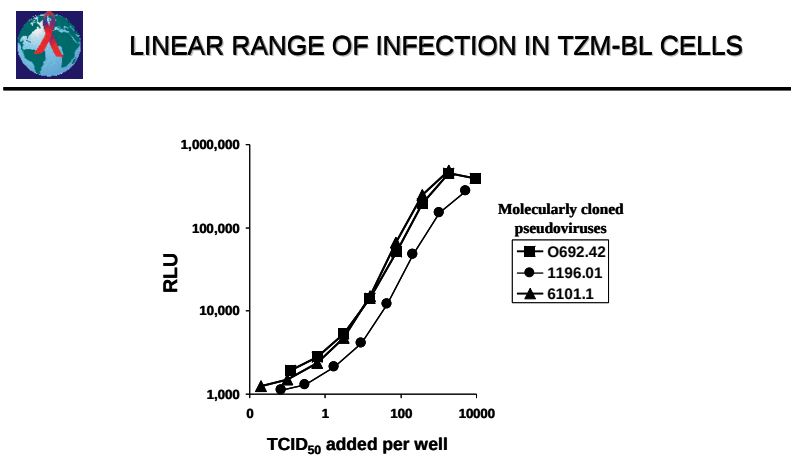
REAGENT PREPARATION

Britelite Reagent:

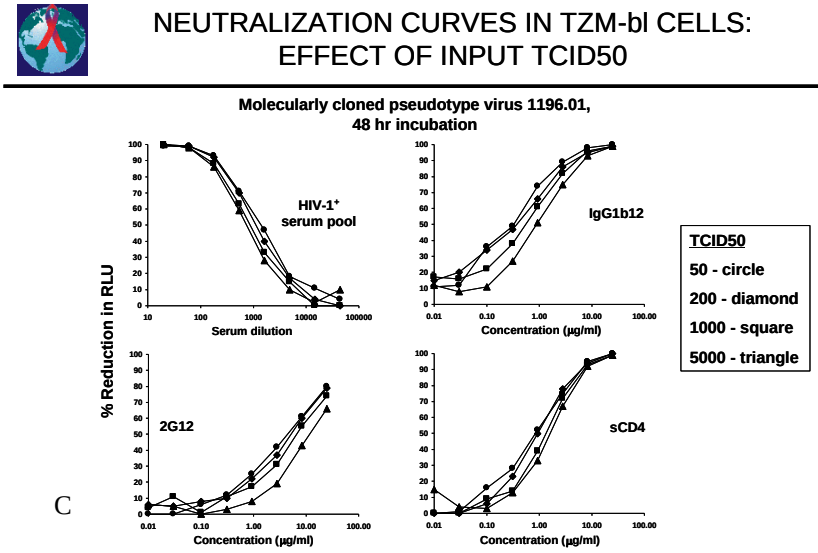
Reconstitute one vial of lyophilized Britelite Substrate Solution with 250 ml of Britelite Substrate Buffer Solution. After the substrate is dissolved completely, store in 10.5 ml aliquots at -70^{o}C for up to 1 month. Thaw aliquots in an ambient temperature water bath in the dark immediately before use. Mix gently prior to use and use within 60 minutes of thawing. Excess reagent may be stored at -70°C and used once more.

Figure 1. Linearity of infection and neutralization in TZM-bl cells.

A



B



C

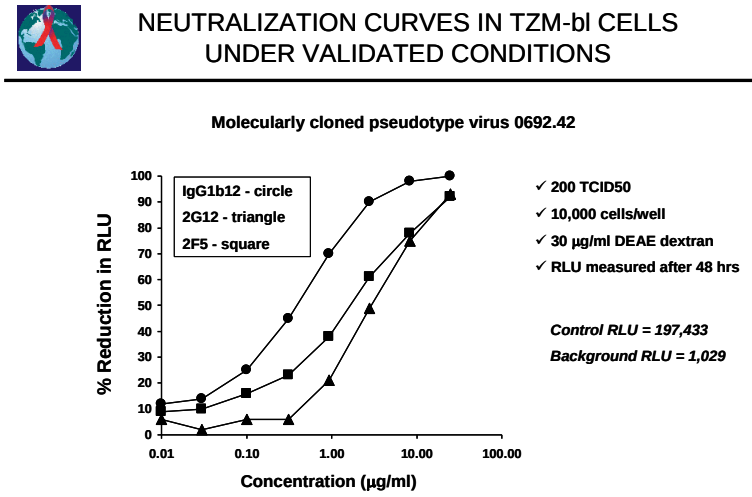


Figure 2. Various templates for neutralization assays

Template A

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8	Dil 8
B	CC	VC	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7	Dil 7
C	CC	VC	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6
D	CC	VC	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Sample 1		*Sample 2*		*Sample 3*		*Sample 4*		*Sample 5*	

Template B

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
B	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
C	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
D	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
E	CC	VC	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4
F	CC	VC	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3
G	CC	VC	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2
H	CC	VC	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1	Dil 1
			Samples 1 & 2		*Samples 3 & 4*		*Samples 5 & 6*		*Samples 7 & 8*		*Samples 9 & 10*	

Template C

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	VC	S#4 Post	S#4 Post	S#4 Post	S#8 Post	S#8 Post	S#8 Post	S#12 Post	S#12 Post	S#12 Post	BLK
B	CC	VC	S#4 Pre	S#4 Pre	S#4 Pre	S#8 Pre	S#8 Pre	S#8 Pre	S#12 Pre	S#12 Pre	S#12 Pre	BLK
C	CC	VC	S#3 Post	S#3 Post	S#3 Post	S#7 Post	S#7 Post	S#7 Post	S#11 Post	S#11 Post	S#11 Post	BLK
D	CC	VC	S#3 Pre	S#3 Pre	S#3 Pre	S#7 Pre	S#7 Pre	S#7 Pre	S#11 Pre	S#11 Pre	S#11 Pre	BLK
E	CC	VC	S#2 Post	S#2 Post	S#2 Post	S#6 Post	S#6 Post	S#6 Post	S#10 Post	S#10 Post	S#10 Post	BLK
F	CC	VC	S#2 Pre	S#2 Pre	S#2 Pre	S#6 Pre	S#6 Pre	S#6 Pre	S#10 Pre	S#10 Pre	S#10 Pre	BLK
G	CC	VC	S#1 Post	S#1 Post	S#1 Post	S#5 Post	S#5 Post	S#5 Post	S#9 Post	S#9 Post	S#9 Post	BLK
H	CC	VC	S#1 Pre	S#1 Pre	S#1 Pre	S#5 Pre	S#5 Pre	S#5 Pre	S#9 Pre	S#9 Pre	S#9 Pre	BLK

Figure 2. Three optional templates for measuring either the titer of neutralizing antibodies or the potency of neutralization at a single dilution. Format A: Template for measuring the titer of neutralizating antibodies using 5 samples per plate. Format B: Template for measuring the titer of neutralizating antibodies using 10 samples per plate. Format C: Template for screening samples at a single dilution. CC, cell control wells (cells only); VC, virus control wells (virus and cells but no serum sample are added here); BLK, blank wells; S#, sample number; Pre, pre-immune sample; Post, post-immune sample.

STANDARD DILUTION CHART FOR 2-FOLD DILUTIONS

DESIRED START DILUTION	GM VOLUME (μl)	SAMPLE VOLUME (μl)
1:5	40	60
1:10	70	30
1:15	80	20
1:20	85	15
1:25	90	12
1:30	90	10
1:50	95	6

First place 100 μl of growth medium in all wells of columns 3-11. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 3 wells and do 2-fold dilutions (i.e., serial transfers of 100 μl).

STANDARD DILUTION CHART FOR 3-FOLD DILUTIONS

DESIRED START DILUTION	GM VOLUME (μl)	SAMPLE VOLUME (μl)
1:5	5	45
1:8	25	28
1:10	30	22
1:15	35	15
1:20	40	11
1:24	50	10
1:45	45	5

First place 100 μl of growth medium in all wells of columns 3-11. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 3 wells and do 3-fold dilutions (i.e., serial transfers of 50 μl).

EQUIPMENT, SUPPLIES AND REAGENTS

Item	Source	Cat. No.	Unit	Price
Fetal Bovine Serum, Hyclone, heat-inact.	Duke CCC	AME 16299	500 ml	$138.20
D-MEM (4.5 g/l D-glucose, 110 mg/ml sodium pyruvate, L-glutamine)	Invitrogen	11995-065	500 ml	15.10
HEPES	Gibco	15630	100 ml	
Gentamicin	Sigma	G1272	10 ml	8.45
Trypsin-EDTA (0.25% trypsin, 1 mM EDTA tetrasodium salt)	Invitrogen	25200-056	100 ml	7.95
DEAE-dextran, hydrochloride, avg. mol. wt. 500,000	Sigma	D-9885	10 g	24.60
Microliter Pipet Tips	ICN	77-988-H2	10 bxs	66.30
Sterile Disposable Reagent Reservoirs	Falcon/VWR	4870	200/CS	62.93
1 ml Sterile Disposable Pipets	Falcon/VWR	357521	1000/CS	124.10
5 ml Sterile Disposable Pipets	Falcon/VWR	357543	200/CS	31.64
10 ml Sterile Disposable Pipets	Falcon/VWR	357551	200/CS	32.53
25 ml Sterile Disposable Pipets	Falcon/VWR	357535	200/CS	74.74
Sterile Disposable Culture Flasks, T-25	Costar/VWR	3056	200/CS	139.52
Sterile Disposable Culture Flasks, T-75	Costar/VWR	3375	100/CS	75.52
96-Well Flat-Bottom Culture Plates	Costar/VWR	3595	50/CS	68.82
96-Well Black Solid Plates	Costar/VWR	3915	100/CS	260.30
Britelite Luminescence Reporter Gene Assay System Contact: Julie.Ginsler@perkinelmer.com	PerkinElmer Life Sciences	6016979	1 L	1,250
Surgical Gowns (Trimax)	Allegiance	39010	CS	217.82
Latex Gloves, Perry Surgical	Allegiance	22537-570	100/bx	15.29
Shoe Covers	Allegiance	AT01-3701	CS	44.16
Biohazard Autoclave Bags	VWR	14220-52	200/CS	126.18

Major Equipment:				
Luminometer, Victor for FI and LUM Contact: Julie.Ginsler@perkinelmer.com	PerkinElmer Life Sciences	1420-032	Each	15,999.00