Henry Jackson Foundation, USA

Neutnet code: 5A
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1.0 PURPOSE

To measure a reduction in virus infectious titer mediated by exposure to antibody. Antibodies protect against virus diseases. This *in vivo* protective immunity is often associated with *in vitro* detection of neutralizing antibodies (NAb). Based on experience with other viruses, it is reasonable to assume that NAb will play important role in protection against HIV-1. Viral neutralization assays are designed to measure a reduction in virus infectious titer mediated by exposure to antibody. In this microtiter plate neutralization assay, virus infection in donor PHA-stimulated PBMC is assessed by a quantitative ELISA measurement of HIV-1 p24 antigen expressed in PBMC culture supernatants.

2.0 SCOPE

This procedure applies to all PBMC Neutralization assays in the Humoral Immunology Laboratory.

3.0 RESPONSIBILITIES

3.1 It is the responsibility of all laboratory personnel performing this SOP to read and fully understand this procedure before commencing. Laboratory personnel are responsible for the integrity of the assay and results.

4.0 REFERENCES

4.1 Reagent Preparation SOP

5.0 ATTACHMENTS (NONE)

6.0 MATERIALS AND EQUIPMENT

6.1 Biological safety cabinet class II

6.2 Humidified 37˚C incubator with 5% CO_2.

6.3 Centrifuge equipped with microplate carriers

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6.4 Electric Pipettor

6.5 Adjustable pipetters

6.6 Multichannel pipetters

6.7 Twelve-channel manual ELISA aspirator for washing cells in PGC microtiter box (Drummond Scientific)

6.8 *V*max ELISA plate reader with Soft Max Pro software

6.9 96 well plate washer

6.10 Vacuum trap suction

6.11 Microscope

6.12 Hemocytometer

6.13 Disposable plastic pipettes

6.14 1-250µl pipette tips

6.15 50ml conical centrifuge tube

6.16 96-well flat bottom plates

6.17 96-well round bottom plates

6.18 96-well microtiter (yellow) boxes

6.19 Plate covers

6.20 Micotiter plate sealers

6.21 Sterile reagent reservoirs

6.22 IL-2 media

For details on media preparation refer to Reagent Preparation SOP. Medium should be warmed to 37°C before use in all procedures described below.

6.23 Wash media

6.24 PBS-Dulbecco's Phosphate-buffered saline without calcium and magnesium

6.25 PHA-P prepared in sterile distilled water to 1 mg/ml.

6.26 Target cells: Ficoll gradient separated PBMC from an HIV-1-seronegative donor.

6.27 HIV-1 p24 antigen ELISA kit (Advanced Bioscience Laboratories, ABL)

6.28 Pooled normal human serum (NHS)

6.29 Control pool of serum from HIV-1 positive individuals

6.30 Trypan blue: 0.4 %

6.31 70 % ethanol

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7.0 HEALTH AND SAFETY

7.1 Personnel should read the applicable MSDS sheets for all chemicals and reagents used in the procedure.

7.2 The appropriate PPE should be worn at all times. Exercise caution when handling chemical and biological hazardous materials.

8.0 DEFINITIONS AND ABBREVIATIONS

8.1 PPE Personal Protective Equipment

8.2 PBS Phosphate Buffered Saline

8.3 BSC Biological Safety Cabinet

8.4 PBMC Peripheral Blood Mononuclear Cell

9.0 PROCEDURE

9.1 **Day 0**

9.1.1 Count the PHA-stimulated PBMC, those stimulated three to four days prior to the assay. The viability should be greater than 90%. Resuspend cells in IL-2 medium at $3x10^6$ cells/ml.

9.1.2 Prior to use, ensure that all test sera and control sera have been complement depleted by heat inactivation for 45 minutes at 56°C. Purified antibodies or peptide reagents do not need to be heat inactivated.

9.1.3 Dilute sera in a 96-well flat bottom plate (<200ul/well) or titer tube box (>200ul/well) using IL-2 medium as a diluent. Calculate amounts for number of operators and triplicate wells with 20 % extra. Aliquot diluted serum into the deep-well plate (25 µl/well). To avoid air bubbles in the wells, when aliquoting place the tips of the multichannel pipette at the bottom of wells.

9.1.4 Set up one row (at least 6 wells) with IL-2 medium only, this will serve as a baseline for virus growth (it is referred to as the virus only or regular medium row).

9.1.5 Thaw quickly in a 37°C water bath one aliquot of virus stock. Dilute it with IL-2 medium to the concentration calculated from the viral titration. Add 25µl of diluted viral stock to the side of each well of the deep-well plate. Be careful not to touch the tips to the plate or the serum at the bottom of the wells. After dispensing virus gently tap the deep-well plate to ensure that serum and virus are well mixed in the bottom of the wells. Incubate plates at 37°C for thirty minutes.

9.1.6 Add 50µl of PHA-stimulated PBMC, resuspended at $3x10^6$ cells/ml, to each well, gently tap the plate to mix PBMC with sera/virus. Cover the plate with loose fitting plate lid and incubate overnight (at least 18 hours) at 37˚C, 5%CO_2.

9.2 **Day 1**

9.2.1 The next day, wash the deep well plates three times by filling wells to 500µl with Wash medium (first 2 washes) and IL-2 medium (last wash). Seal plate with adherent plastic plate sealer and centrifuge for 10 minutes at 1200 rpm.

9.2.2 After each wash aspirate supernatants from each well with an aspirator set to leave 50µl well. Bring the volume up to 500µl in each well and then centrifuge again. Repeat with IL-2.

9.2.3 After the last wash add 200ml of IL-2 medium to each well. Thoroughly resuspend cell pellets and transfer 220ml from the deep-well plate to a round bottom 96-well microplate. Inspect plates daily for uniformity in size of cell pellets and pH of culture media. Incubate at 37˚C, 5%CO_2 for 3 days.

9.3 **Day 4/Day6**

9.3.1 Measure p24 antigen production on days 4 or 6. Remove 50µl of culture supernatant from each well and place into a corresponding 96-well flat bottom plate with 150ul of 1:4 Disruption buffer (provided in the ABL ELISA kit), diluted in PBS (final overall dilution 1:4).

9.3.2 Using ABL p24 ELISA kit, determine p24 concentration in Virus Only row of the experiment plate (see p24 ELISA SOP). If the concentration is $\geq$ 10 ng/ml four days after infection, analyze corresponding sera samples. The harvest p24 plate can be stored at -20˚C until p24 determination is complete. If the experiment needs to be continued, $\leq$10 ng/ml p24, add 100ml of fresh IL-2 media to each well of the plate and incubate at 37˚C. Repeat collection of culture supernatants six days after infection and check the p24 concentration.

9.3.3 Calculate percent neutralization and IC50,80, 90. Record data.

REVISION HISTORY

05Dec08- Anita Gillis

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