TZM-bl Neutralization Protocol

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This assay measures neutralization in TZM-bl cells as a function of a reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. The assay is performed in 96-well culture plates for high throughput capacity. Use of a clonal cell population provides enhanced precision and uniformity. The assay has been validated for single-round infection with either uncloned viruses grown in human lymphocytes or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T cells.

Components needed: 96-well round bottom plates, 12-channel pipette, complete DMEM (500ml DMEM + 50ml heat-inactivated FBS + 10ml L-Glutamine Pen-Strep, prewarmed to 37°C), heat-inactivated samples, pseudovirus stock, Steady-Glo substrate (Promega).

Considerations: All incubations are performed in a humidified 37°C, 5% CO_2 incubator. Plasma and serum samples should be heat-inactivated at 56°C for 60 minutes prior to first use. First, thaw samples at room temp or 37°C, mix thoroughly, spin down briefly, and then place at 56°C for 1 hour. After heat-inactivation, centrifuge samples at 13,000 rpm for 10 min and store at 4°C.

Protocol:

1. Using the format of a 96-well round bottom culture plate as illustrated in **Appendix A**, place 60 µl of cDMEM in all wells of column 12 (cell control). Place 30 µl in all wells of columns 1-11 (column 11 will be the virus control). Place an additional 24 µl in all wells of columns 1-10, row A (to receive test samples).
2. Spin test samples in a centrifuge at 13,000 rpm for one minute prior to adding them to the test plate.
3. Add 6 µl of test sample in duplicate to row A, columns 1-10 in following order: sample 1 – wells A1-A2, sample 2 – wells A3-A4, sample 3 – wells A5-A6, sample 4 – wells A7-A8, and sample 5 – wells A9-A10. Mix the samples in row A by pipetting up and down 5 times and transfer 50 µl to row B. Repeat the transfer, mixing, and dilution of samples through row H (these are serial 2-fold dilutions). After final transfer and mixing is complete, discard 50 µl from the wells in columns 1-10, row H into waste container.
   NOTE: If the desired final start dilution is 1:20, the samples are initially diluted at 1:10 in row A. This is so, because an equal volume of virus is added to the samples later on, which brings the dilution down to 1:20. Make sure to control for this for any dilutions that you make, including when calculating the amount of MAb of IgG to be used.
   NOTE: This format is designed to measure neutralizing antibody titers in the range of 1:20 to 1:2,560. The above description is for a starting dilution of 1:20. Appropriate adjustments may be made to test a different range of dilutions. 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. In addition, more than one viral isolate can be used per plate as long as a Virus Control is included for each virus.

4. It is advisable that 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.
5. Thaw the required number of vials of virus by placing in an ambient temperature water bath. When completely thawed, dilute the virus in cDMEM to achieve a TCID of approximately 150,000 – 200,000 RLU equivalents. For pseudoviruses that do not reach 150,000 RLU, pick a dose of virus that gives RLU equivalents of ≥10X the cell only background but is not toxic to the cells via light microscopy.
6. Dispense 30 µl of cell-free virus to all wells in columns 1-11.
   Virus Calculations: To calculate the total volume of virus/media mixture needed for the assay, multiply the total number of wells by the volume of virus/media mixture to be used per well (30 µl /well). Then multiply the total number of wells by the concentration of undiluted virus needed per well (as reported on the SharePoint Pseudovirus tracking website). Divide the total ng of virus needed by the stock concentration of that virus (also reported on SharePoint) to find the total volume of undiluted virus needed. Finally, subtract the volume of undiluted virus needed from the total volume of virus/media mixture to derive the volume of media needed.
   Example: Need to use Virus X at 2 ng/well, stock at 1000 ng/ml, and have a total of 2 plates for that virus. 2 plates x 100 wells/plate = 200 wells total x 30 µl /well = 6 ml of virus/media mixture needed.
   200 wells x 2 ng/well = 400 ng undiluted virus needed.
   400ng ÷ 1000ng/ml = 0.4 ml undiluted virus needed.
   6ml virus/media mixture – 0.4ml undiluted virus = 5.6ml media only needed
7. Cover plates and incubate for 90 minutes.
8. When the incubation has been going on for 60 minutes, polybrene the cells by adding 50 µl (2 µg/ml) polybrene in cDMEM per well, which brings the final dilution to 1 µg/ml. Stock is at 10 mg/ml, so dilute 1 µl / 5ml cDMEM to add to cells.
   NOTE: The cells have been plated the day before. Examine the cells by microscope for adequate confluency – they should be ~30% confluent. Avoid using cells that are >50% confluent.
9. Cover plates and incubate the cells for 30 minutes.
10. Aspirate the polybrene from the cells and transfer the sample/virus mixture from the round-bottom plates to the cells, starting with row H all the way to row A.
11. Cover plates and incubate for 72 hours if Env-pseudotyped viruses are used. If replication-competent virus is used, the plates should be incubated for 46-50 hours to minimize virus replication.

12. After incubation, remove plates from the incubator. Plates should not stay out of the incubator longer than one hour before running the luciferase reaction.
    NOTE: Examine at least 2 virus control wells for the presence of syncytia by microscopic examination. It is important to note the presence of syncytia as too many syncytia indicate cell killing and thus the validity of the assay is compromised. If cell killing is present, the assays should be repeated using a lower dose of the virus. Also check the top row of the plate for the presence of toxicity. Cell toxicity could be erroneously interpreted as neutralization.
13. Thaw Steady-Glo directly before use in an ambient temperature water bath away from light.
14. Aspirate all the culture medium from each well.
15. Dispense 100 µl of Steady-Glo Reagent to each well.
16. Incubate at room temperature away from light for 15 minutes (Tzm cells) or 5 minutes (U87 cells) to allow complete cell lysis. Mix by pipettor action (at least two strokes) and transfer 75 µl to a corresponding 96-well white plate. Read the RLU using a luminometer.

APPENDIX A:

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

CC, Cell control wells (cells only).
VC, virus control wells (virus and cells but no serum sample are added here).