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Protocol for Neutralizing Antibody Assay for HIV-1 in A3R5 Cells

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I. Introduction

This assay measures neutralization in 96-well microdilution plates as a function of a reduction in luciferase reporter gene expression. A3R5 cells (A3.01/R5.7) were provided by Col. Jerome Kim and Dr. Robert McLinden of the US Medical HIV Research Program (USMHRP). This is a derivative of the human lymphoblastoid cell line, CEM, that naturally expresses CD4 and CXCR4 and was engineered by Dr. Robert McLinden in Col. Kim's laboratory to express CCR5 [1, 2]. The cells are maintained in growth medium containing 1 mg/ml geneticin to preserve this receptor. The cells are moderately permissive to infection by most strains of HIV-1. DEAE-Dextran is used in the medium during neutralization assays to enhance infectivity. Because the cell line does not contain a reporter gene, molecularly cloned viruses that carry a reporter gene in the viral genome must be used. Env-expressing infectious molecular clones carrying a Renilla luciferase reporter gene (Env.IMC.LucR viruses) provide suitable infection for neutralization assays [3]. Expression of the reporter genes is induced in cis by viral Tat protein soon after infection. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inocula. 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 multiple rounds of infection with Env.IMC.LucR viruses produced by transfection in 293T/17 cells [4].

II. Definitions

GM:	Antibiotic-Free Growth Medium
Luc:	Luciferase
RLU:	Relative Luminescence Units
FBS:	Fetal Bovine Serum
DEAE-Dextran:	Diethylaminoethyl-Dextran
ID:	Identification
IMC:	Infectious Molecular Clone
Env.IMC.LucR:	Env-expressing infectious molecular clones carrying a Renilla luciferase reporter gene
TCID:	Tissue Culture Infectious Dose

III. Reagents and Materials

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

Complete Growth Medium for A3R5 Assay (see Protocol for Reagent Preparation for Use in Neutralizing Antibody Assay for HIV-1 in A3R5 Cells)

A3R5 Cells
Vendor: NIH AIDS Reagent Repository Program

DEAE-Dextran, hydrochloride, average Mol. Wt. 500,000
Vendor: Sigma

ViviRen Live Cell Substrate (see Protocol for Reagent Preparation for Use in Neutralizing Antibody Assay for HIV-1 in A3R5 Cells)
Vendor: Promega

Microliter pipettor tips, sterile
Vendor: VWR
Vendor: Rainin
Vendor: Fisher

Disposable pipettes, sterile, individually wrapped
Vendor: Fisher
1 ml pipettes
2 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes
100 ml pipettes

Flat-bottom culture plates, 96-well, low evaporation, sterile
Vendor: Fisher

Flat-bottom white solid plates, 96-well
Vendor: Costar/Fisher

Culture flasks with vented caps, sterile
Vendor: Fisher
T-25 flask
T-75 flask

Reagent reservoirs, 50 ml, 100 ml capacity
Vendor: Costar
Vendor: VWR

Trypan Blue (0.4%)
Vendor: Sigma

IV. Instrumentation

Recommended manufacturers are listed. Unless otherwise specified, equipment of equal or better quality than the recommended ones can be used whenever necessary.

Biological Safety Cabinet
Manufacturer: Baker Co.

Incubator
Manufacturer: Forma Scientific / NuAIRE/Panasonic
Water-jacketed (37°C, 5% CO_2 standard requirements)

Light Microscope
Manufacturer: Olympus

Centrifuge
Manufacturer: Jouan (low speed capable of up to 500 x g)

Luminometer
Manufacturer: PerkinElmer Life and Analytical Sciences

Water bath
Manufacturer: Precision Scientific

Countess Automated Cell Counter
Manufacturer: Invitrogen

Hemacytometer
Manufacturer: INCYTO

> ***NOTE 1:*** An automated cell counting device (i.e., Countess, Manufacturer: Invitrogen) may be used in lieu of a light microscope / hemacytometer for cell counting and viability calculation.

Pipettor
Manufacturer: ThermoLabsystem
12-channel pipetteman, 5-50 µl
12-channel pipetteman, 30-300 µl
Single channel pipetteman, 5-50 µl
Single channel pipetteman, 30-200 µl

Manufacturer: Drummond Scientific Co.
Pipette Aid XP

Manufacturer: BioHit
12-channel, 50-1200 µl Electronic Pipette
Single channel, 10-300 µl Electronic Pipette
Single channel, 5-120 ul

Manufacturer: Rainin
12-channel pipettor, 20-200 µl

V. Specimens

Samples should be heat-inactivated at 56°C as described in Protocol for Heat-Activation of Serum and Plasma Samples. Samples may be serum or plasma, although serum is preferred. Anticoagulants in plasma are problematic in the assay, especially when heparin is used. For example, some forms of heparin have potent and strain-specific antiviral activity. Also, all anticoagulants are toxic to the cells at plasma dilutions lower than 1:60.

VI. Protocol

1. Neutralization Assay

NOTE 2: All incubations are performed in a humidified 37ºC, 5% CO_2 incubator unless otherwise specified.

1.1 Using the format of a 96-well flat bottom culture plate as illustrated in Appendix A, place 150 µl of pre-warmed 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).

1.2 Centrifuge test samples in a centrifuge at 2,000 – 5,000 xg for one minute prior to adding them to the test plate.

NOTE 3: Since the goal is to collect the entire volume at the bottom of the tube and pellet any debris that might be present, samples may be centrifuged at speeds between 2,000 – 10,000 xg (centrifuge time should be increased to 10-15 minutes if centrifuging at 2,000xg).

1.3 Add 11 µl of test sample in duplicate to row H, columns 3-12 in following order: sample 1 – wells H3-H4, sample 2 – wells H5-H6, sample 3 – wells H7-H8, sample 4 – wells H9-H10, and sample 5 – wells H11-H12. Mix the samples in row H and transfer 50 µl to row G. Repeat the transfer and mixing 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 waste container.

NOTE 4: The above description is for a starting dilution of 1:20. 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 (refer to "Sample dilution charts:" in Appendix C). This format is designed to assay 5 samples in duplicate wells at each serum dilution per plate (Appendix A). 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 (Appendix B) and adding additional samples to row D.

NOTE 5: 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.

1.4 Thaw the required number of vials of virus by placing in an ambient temperature water bath placed in a biological safety cabinet. When completely thawed, dilute the virus in GM to achieve a TCID of approximately 50,000-150,000 relative luminescence units (RLU) equivalents. For viruses that have low infectivity, select a dose of virus that gives at least 10 times the average RLU of the cell control. For viruses that have a high infectivity, select a dose of virus that does not produce cytopathic effects on the cells (observed via light microscopy). See Protocol for Preparation and Titration of HIV-1 IMC Viruses for measurement of TCID in A3R5 cells.

NOTE 6: The RLU equivalents measured in the TCID assay will not necessarily match the RLUs obtained in the virus control of the neutralization plate. This difference is acceptable provided that the virus control is ≥10X the background (cell control) and the virus control is not toxic to the cells observed by light microscopy.

NOTE 7: Leftover virus may be refrozen and stored at -80°C but first should be marked with a "1X" on the lid and label of the vial. The "1X" notes that this particular vial has been thawed one time. When using "1X" vials of virus in the A3R5 assay, the technician must consult the virus database to obtain the optimal virus dilution for viruses that have been thawed one time. No virus should be used in the A3R5 assay if it has been thawed and refrozen more than once.

1.5 Dispense 50 µl of cell-free virus to all wells in columns 2-12.

Virus Calculations:

To calculate the total volume (vol.) of virus/GM mixture needed for the assay, multiply the total number of plates by the volume of virus/GM mixture to be used per plate. Then divide the total volume of virus/GM mixture by the optimal virus dilution to use (based on the TCID assay) to derive the volume of undiluted virus needed. Then subtract the volume of undiluted virus needed from the total volume of virus/GM mixture to derive the volume of GM needed.

Total number of plates X Vol. of virus/GM per plate = Total vol. virus/GM needed

Total vol. virus/GM needed ÷ Optimal virus dilution = Vol. of undiluted virus needed

Total vol. of virus/GM needed – Vol. of undiluted virus needed = Vol. of GM needed

1.6 Cover the plates and incubate for 45 – 90 minutes.

1.7 During the incubation, prepare a suspension of A3R5 cells at a concentration of 9.0×10^5 cells/ml in GM as described below:

1.7.1 Perform Cell Count (see laboratory specific protocol)

1.7.2 Cell Calculations:

To calculate the cell concentration, multiply the average number of cells per quadrant by the dilution factor to yield the cell concentration, "C1", in cells x 10^4. To calculate the total cell mixture volume, "V_2", that you need, multiply the number of plates by the total volume of cell mixture needed per plate. The concentration of cells desired is 900,000 cells/ml, "C_2". Thus, using the equation $C_1V_1 = C_2V_2$, one can solve for "V_1", the volume of cells needed.

For example:

Total number of viable cells counted = 60
Number of quadrants counted = 4
Dilution factor = 20
Number of plates = 1
Cell mixture needed per plate = 10 ml

60 cells ÷ 4 quadrants = 15 cells/quadrant

$15 \times 20 \times 10^4$ cells/ml = 30×10^5 cells/ml = C_1

1 plate x 10 ml/plate = 10 ml = V_2

Optimum final concentration of cells = 900,000/ml = C_2

Therefore: $C_1V_1 = C_2V_2$

V_1 = (900,000 X 10) ÷ 3,000,000 = 3.0 ml of cells

1.7.3 Addition of DEAE-Dextran to Cells:

The DEAE-Dextran concentration in the cell suspension will be 25 µg/ml. The final concentration of DEAE-Dextran in the assay will be 10 µg/ml.

To calculate the amount of DEAE-Dextran to use, first multiply the optimal concentration of DEAE-Dextran (see Protocol for Determination of Optimal Concentration of DEAE-Dextran) by 0.250 ml (the final volume in each well) to get the amount of DEAE-Dextran per well. Multiply the amount of DEAE-Dextran per well by 100 wells/plate (96 wells rounds to 100) to derive the amount of DEAE-Dextran per plate. Divide the amount of DEAE-Dextran needed per plate by the stock concentration of the DEAE-Dextran to yield the volume of DEAE-Dextran stock needed. Multiply this number by the number of plates to yield the total volume of DEAE-Dextran stock needed.

For example:

If the optimal concentration of DEAE-Dextran to use is 10 µg/ml and the DEAE-Dextran stock is at 5 mg/ml

10 µg/ml x 0.25 ml (volume in well) = 2.5 µg of DEAE-Dextran needed in each well

2.5 µg x 100 wells/plate = 250 µg of DEAE-Dextran needed per plate = 0.25 mg of DEAE-Dextran

0.25 mg of DEAE-Dextran per plate ÷ 5 mg/ml stock concentration = 0.05 ml of DEAE-Dextran stock needed per plate

To calculate the amount of Growth Medium to add, subtract the total volume of cells needed and the total volume of DEAE-Dextran stock needed from the total volume of cell mixture needed.

So, the total volume needed for one plate is 10 ml

10 ml – 3.0 ml cells – 0.05 ml DEAE-Dextran = 6.95 ml of GM

1.8 The GM/cells/DEAE-Dextran suspension should be prepared as follows: Add GM and DEAE-Dextran and mix; Add cells and thoroughly mix the prepared cell suspension immediately prior to plating. Dispense 100 µl of the prepared cell suspension (90,000 cells per well) to each well in columns 1-12, rows A through H.

NOTE 8: The concentrations of DEAE-Dextran shown above will vary by batch of DEAE-Dextran. The actual optimal concentration should be determined for each new batch of DEAE-Dextran prepared in accordance with Protocol for Determination of Optimal Concentration of DEAE-Dextran.

NOTE 9: The use of DEAE-Dextran is optional. When omitted, the TCID of the virus needs to have been measured in the absence of DEAE-Dextran.

1.9 Cover plates and incubate for approximately 4 days.

1.10 After incubation, remove 2-3 plates at a time from the incubator to examine for syncytia. Additionally, only 2-3 plates should be handled at a time when performing the luciferase reaction. Plates should not remain out of the incubator longer than 20-30 minutes prior to performing the luciferase reaction.

NOTE 10: 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 bottom row of the plate for the presence of toxicity. Cell toxicity could be erroneously interpreted as neutralization.

1.11 Carefully remove 90 µl of supernatant from the plate. Suspend cells in each well and transfer 75 µl to corresponding wells of a 96-well white solid plate (from row H to row A in the plate).

1.12 Dilute 10 µl of ViviRen Live Cell Substrate in 3.5 ml of GM. Substrate should be thawed immediately prior to use.

1.13 Dispense 30 µl of ViviRen Reagent to each well. Tap the plate lightly to mix.

1.14 Incubate at room temperature for 4 minutes to allow for maximum luminescence. Read the plate in a luminometer using the 0.5 sec/well protocol.

2. Analyzing and printing results

2.1 Prior to reading the plates in the luminometer, enter the assay protocol information in the Wallac Software of the luminometer.

2.2 Read the plates in a luminometer interfaced to a dedicated computer in the laboratory.

2.3 Use the software program associated with the luminometer to save the raw data onto the desired location, after each plate is read, using a unique file identification number (ID) for each plate.

2.4 Analyze and print the data using the appropriate Microsoft Excel "Luminescence" macro (provided by the Central Reference Laboratory). The data print-out must include: i) experiment number, ii) protocol and/or study 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.

NOTE 11: The "Luminescence" macro calculates the percent neutralization provided by each serum dilution. Percent neutralization is determined by calculating the difference in average RLU between virus control (cells + virus, column 2) and test wells (cells + serum sample + virus), dividing this result by the difference in average RLU between virus control (cell + virus, column 2) and cell control wells (column 1), and multiplying by 100. Neutralizing antibody titers are expressed as the reciprocal of the serum dilution required to reduce RLU by 50%. Failure to score at least 50% reduction of RLU at any serum dilution constitutes a negative test.

NOTE 12: Percent neutralization may also be determined by calculating the difference in average RLU between test wells containing post-immune sample and test wells containing pre-immune sample from the same individual. The pre-immune and post-immune samples must be assayed on the same assay plate (see Protocol for Neutralizing Antibody Screening Assay for HIV-1 in A3R5 Cells).

3. Acceptance Criteria

3.1 The average RLU of virus control wells is >10 times the average RLU of cell control wells.

3.2 The % CV of RLU in the virus control wells is ≤30%.

3.3 The % difference for duplicate wells is ≤30% for sample dilutions that yield at least 40% neutralization.

3.4 The neutralization curves are smooth and linear around the 50% neutralization cut-off.

3.5 When samples are positive in both the curve-based and point-based analyses, the neutralization titers are within 3-fold of each other.

3.6 The value of the positive control is within 3-fold with the average of the previous values for that particular control-virus combination.

NOTE 13: A final decision regarding the acceptability of the data will be made by the PI (or his/her Designee).

VII. References

1. Folks, T., Benn, S., Rabson, A., Theodore T., Hoggan, M.D., Martin, M., Lightfoote, M., and Sell, K. (1985) Characterization of a continuous T-cell susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci., 82:4539-4543.

2. McLinden, R.J.L., LaBranche, C.C., Chenine, A. L., Polonis, V.R., Eller, M.A., Wieczorek, L., Ochsenbauer, C., Kappes, J.C., Perfetto, S., Montefiori, D.C., Michael, N.L., and Kim, J.H. (2013) Detection of HIV-1 neutralizing antibodies in human CD4+ / CXCR4+ / CCR5+ T-lymphoblastoid cell assay system. PLoS One, (11): e77756.

3. Edmonds, T.G., Ding, H., Yuan, X., Wei, Q., Smith, K.S., Conway, J.A., Wieczorek, L., Brown, B., Polonis, V., West, J.T., Montefiori, D.C., Kappes, J.C., and Ochsenbauer, C. (2010) Replication competent molecular clones of HIV-1 expressing *Renilla* luciferase facilitate the analysis of antibody inhibition in PBMC. Virology, 408 (1), 1-13.

4. Sarzotti-Kelsoe, M., Daniell, X., Todd, C.A., Bilska, M., Martelli, A., LaBranche, C., Perez, L.G., Ochsenbauer, C., Kappes, J.C., Rountree, W., Denny, T.N., and Montefiori, D.C. (2014) Optimization and validation of a neutralizing antibody assay for HIV-1 in A3R5 cells. J. Immunol. Methods, pii: S0022-1759(14)00072-6.

VIII. Appendix

Appendix A: Assay template for measuring neutralization titers, 5 samples per plate

	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	

CC, Cell control wells (cells only).

VC, virus control wells (virus and cells but no serum sample are added here

Appendix B: Assay template for measuring neutralization titers, 10 samples per plate

	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
			Sample 1 (bottom) Sample 2 (top)		Sample 3(bottom) Sample 4 (top)		Sample 5 (bottom) Sample 6 (top)		Sample 7 (bottom) Sample 8 (top)		Sample 9 (bottom) Sample 10 (top)	

CC, Cell control wells (cells only).

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

Appendix C: Sample dilution charts

STANDARD DILUTION CHART FOR 2-FOLD SAMPLE DILUTIONS:

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 3-12. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 2 wells and do 2-fold dilutions (i.e., serial transfers of 100 µl).

STANDARD DILUTION CHART FOR 3-FOLD SAMPLE DILUTIONS

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 3-12. Add the extra amount of growth medium listed above, then add the desired sample volume to the first 2 wells and do 3-fold dilutions (i.e., serial transfers of 50 µl).