Protocol for Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells

(October 2021)

1. Introduction

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. TZM-bl cells (also called JC57BL-13) may be obtained from the NIH AIDS Reagent Program. This is a HeLa cell clone that was engineered to express CD4 and CCR5 [7.1] and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR [7.2], permitting sensitive and accurate measurements of infection. The cells are highly permissive to infection by most strains of HIV, SIV and SHIV, including primary HIV-1 isolates and molecularly cloned Env-pseudotyped viruses. DEAE-Dextran is used in the medium during neutralization assays to enhance infectivity. Expression of the reporter genes is induced in trans 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 inoculum. 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 virus quaispecies or molecularly cloned Env-pseudotyped viruses [7.3].

2. Definitions

%CV: Percent coefficient of variation

DEAE-Dextran: Diethylaminoethyl-Dextran

DPBS: Dulbecco's Phosphate Buffered Saline

EDTA: Ethylenediaminetetraacetic acid

GM: Complete Growth Medium

ID: Identification

ID50: 50 percent inhibitory dose

Luc: Luciferase

NIH: National Institutes of Health

PI: Principal Investigator

RLU: Relative Luminescence Units

TCID: Tissue Culture Infectious Dose

Vol: Volume

3. 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.

TZM-bl Cells
Supplier: NIH AIDS Reagent Program

Complete Growth Medium (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells**)**

DEAE-Dextran, hydrochloride, average Mol. Wt. 500,000 (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells**)**
Manufacturer: Sigma

Trypsin-EDTA (0.25% trypsin, 1 mM EDTA) (see Protocol for Thawing, Expanding, Maintaining, and Cryopreserving Adherent Cell Lines)
Manufacturer: Thermo Fisher Scientific

Trypan Blue (0.4%)
Manufacturer: Thermo Fisher Scientific

Dulbecco's Phosphate Buffered Saline (DPBS)
Manufacturer: Thermo Fisher Scientific

Britelite™ Plus Reporter Gene Assay System (see Protocol for Reagent Preparation for Use in the Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells**)**
Manufacturer: PerkinElmer

NOTE 1: The lyophilized britelite plus substrate is not classified as hazardous.

Bright-Glo™
Manufacturer: Promega

NOTE 2: Bright-Glo Luciferase Assay System from Promega Corporation is an acceptable substitute for britelite plus. Please follow manufacturer's guidelines for preparation and use. Bright-Glo is classified as hazardous. Personal Protective Equipment (PPE) is required when working with this reagent.

Microliter pipettor tips, sterile
Manufacturer: Rainin or Sartorius

Disposable pipettes, sterile, individually wrapped
1 ml pipettes
2 ml pipettes
5 ml pipettes
10 ml pipettes
25 ml pipettes
50 ml pipettes
100 ml pipettes
Manufacturer: Corning

Flat-bottom culture plates, 96-well, low evaporation, sterile
Manufacturer: Corning

Flat-bottom black solid plates, 96-well
Manufacturer: PerkinElmer, Inc.

Culture flasks with vented caps, sterile
T-25 flask
T-75 flask
Manufacturer: Corning

Reagent reservoirs, 50 ml, 100 ml capacity
Manufacturer: Corning

Conical tubes, sterile
15 ml capacity
50 ml capacity
Manufacturer: Corning

4. 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. or LabConco

Incubator (37°C, 5% CO2 standard requirements)
Manufacturer: Panasonic

Centrifuge and Microcentrifuge
(low speed centrifuge capable of up to 500 x g)
50 ml tube holder
15 ml tube holder
Manufacturer: Jouan

(high speed microcentrifuge)
18 place standard rotor F-45-18-11 for 1.5 ml microcentrifuge tubes
Manufacturer: Eppendorf

Luminometer
Manufacturer: PerkinElmer or Promega

Computer
Manufacturer: Dell

Water bath
Manufacturer: VWR International

Light Microscope
Manufacturer: Olympus

Hemacytometer
Manufacturer: INCYTO

NOTE 3: An automated cell counting device (e.g., Countess, Manufacturer: Invitrogen; Luna, Manufacturer: Logos Biosystems, Inc) may be used in lieu of a light microscope / hemacytometer for cell counting and viability calculation.

Pipettor
Single channel electronic pipettor, 10-300 µl
12-channel electronic pipettor, 50-1200 µl
12-channel electronic pipettor, 10-300 µl
Single channel manual, 0.5-10 µl
Single channel manual, 2-20 µl
Single channel manual, 20-200 µl
Single channel manual, 100-1000 µl
Manufacturer: Sartorius

PipetteAid XP
Manufacturer: Drummond Scientific Co.

12 channel pipettor, 20-200 µl
Manufacturer: Rainin

Ultra Low Temperature Freezer (−70°C or lower)
Manufacturer: Harris or Thermo Fisher Scientific

4°C Refrigerator
Manufacturer: LABREPCO, Inc.

−20°C Freezer
Manufacturer: LABREPCO, Inc.

5. Specimens

1. Serum and plasma

1.1. Serum and plasma samples should be heat-inactivated at 56°C as described in Protocol for Heat-Inactivation of Serum and Plasma Samples.

1.2. Serum is preferred to over plasma because the anticoagulants in plasma are problematic in the assay, especially when heparin is used because some forms of heparin have potent and strain-specific antiviral activity. All anticoagulants are toxic to the cells at low plasma dilutions.

2. Purified antibodies

2.1. Do not heat inactivate purified antibodies

3. Mucosal and other samples

3.1. Mucosal samples should be filter sterilized before use in the assay.

6. Protocol

1. Neutralization Assay Setup

NOTE 4: 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 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 15 µl in all wells of columns 3-12, row H (to receive test samples).

1.2 Centrifuge the test samples at the appropriate time and speed in order to pellet any debris that might be present.

1.3 Add 9.5 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 (at least 5 times) in row H and transfer 25 µl to row G. Repeat the mixing and transferring of samples through row A (these are serial 5-fold dilutions). After final transfer and mixing is complete, discard 25 µl from the wells in columns 3-12, row A into waste container.

NOTE 5: This format is designed to assay 5 samples in duplicate wells at each serum dilution per plate (Appendix A). The above description is for a starting sample dilution of 1:20, and do 5-fold serial dilutions. This format is designed to measure neutralizing antibody titers in the range of 1:20 to 1:1,562,500.

NOTE 6: 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 (see Appendix B).

NOTE 7: Appropriate adjustments may be made to test a different range of dilutions (refer to "Sample dilution charts" in Appendix C).

NOTE 8: 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. When completely thawed, mix the virus in the tube (**do not vortex),** and dilute the virus in GM to achieve a TCID range of approximately 50,000-150,000 Relative Luminescence Units (RLU) equivalents (+/- 15,000 RLU). For pseudoviruses that do not reach 50,000-150,000 RLU, pick a dose of virus that gives at least 10 times background RLU but is not toxic to the cells (observed via light microscopy). See Protocol for Preparation and Titration of HIV-1 Env-pseudotyped Viruses for measurement of TCID in TZM-bl cells.

NOTE 9: The RLU equivalents measured in the TCID assay may not match the RLUs in the virus control of the neutralization plate. This difference is acceptable provided that the virus control is ≥10X the background and the virus control is not toxic to the cells observed by light microscopy.

NOTE 10: Leftover virus may be refrozen in an ultra-low temperature freezer and 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 TZM-bl assay, the technician must consult the virus database to obtain the optimal virus dilution for viruses that have been thawed one time. No pseudovirus should be used in the TZM-bl assay if it has been thawed and refrozen more than once.

NOTE 11: For viruses with high dilution (i.e., 1:100 or higher), it is recommended to aliquot and freeze leftover viruses into smaller volumes. The aliquots should be based on the recommended dilution determined from the TCID assay of a 1X thawed virus aliquot (See Protocol for Preparation and Titration of Env-Pseudotyped Viruses). If no dilution is given for a 1X thawed virus, make 150-300 µl aliquots. The following information should be written on the 1X tube: name of virus, harvest date, and volume of virus. A "1X" should be written on the cap of the tube and the tube to denote that the virus has been thawed and refrozen once.

1.5 Prepare virus/GM suspension at the recommended dilution as described below:

1.5.1 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 The virus/GM suspension should be prepared as follows: Add GM and virus to a conical tube or bottle and thoroughly mix immediately prior to plating. Dispense 50 µl of the virus/GM suspension to all wells in columns 2-12. Virus should be added from row A to row H.

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

1.8. During the incubation, prepare a suspension of TZM-bl cells at a concentration of 100,000 cells/ml in GM as described below.

1.8.1. Perform Viable Cell Count (See laboratory specific protocol)

1.8.2. Cell Calculations (if using a hemacytomer):

To calculate the cell concentration, multiple the average number of cells per quadrant, the dilution factor, and 10,000 to yield the cell concentration, "C_1", in cells/ml. 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 100,000 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 = 10
Number of plates = 1
Cell mixture needed per plate = 10 ml

60 cells ÷ 4 quadrants = 15 cells/quadrant

15 cells/quadrant x dilution factor of 10 x 10,000 cells/ml = 1,500,000 cells/ml = C_1

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

Optimum final concentration of cells = 100,000/ml = C_2

Therefore: $C_1V_1=C_2V_2$

V_1 = (100,000 cells/ml x 10 ml) ÷ 1,500,000 cells/ml = 0.67 ml of cells

Addition of DEAE-Dextran to Cells

NOTE 12: The concentrations of DEAE-Dextran shown 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 the Protocol for the Determination of Optimal Concentration of DEAE-Dextran.

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 0.250ml (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 in the assay 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 GM 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.

The total volume needed for one plate is 10 ml.

10 ml – 0.67 ml cells – 0.05 ml DEAE-Dextran = 9.3 ml of GM

1.9. The GM/cells/DEAE-Dextran suspension should be prepared as follows: Add GM and DEAE-Dextran then mix. Add cells and thoroughly mix the prepared cell suspension immediately prior to plating. Dispense 100 µl of the prepared cell suspension (10,000 cells per well) to each well in columns 1-12. Cells should be added to cell control wells first (on all plates) and then from row A to H.

NOTE 13: The use of DEAE-Dextran is optional. When omitted, the TCID of the virus should be measured in the absence of DEAE-Dextran.

1.10. Cover plates and incubate for 44-72 hours if Env-pseudotyped viruses are used. If replication-competent virus is used, the plates should be incubated for 44-50 hours to minimize virus replication.

1.11. 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 14: Using a microscope, examine at least 2 virus control wells for the presence of syncytia. 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 plate for cell toxicity in the presence of samples at the lowest dilution (and at higher dilutions if toxicity is observed). Cell toxicity could be erroneously interpreted as neutralization.

1.12. Thaw Britelite™ plus or Bright-Glo™ directly before use in an ambient temperature water bath away from the light. Once thawed, invert tube a few times to mix.

1.13. Remove 150 µl of culture medium from each well and discard, leaving approximately 100 µl in the well.

1.14. Dispense 100 µl of Britelite™ plus or Bright-Glo™ to each well.

1.15. Incubate away from light at room temperature for 2 minutes (but no longer than 15 minutes) to allow complete cell lysis then mix by pipettor action (at least two strokes) and transfer 150 µl to a corresponding 96-well black plate. Read the plate in a luminometer.

NOTE 15: Bright-Glo™ and Britelite™ plus reagents can be subjected to 7 to 10 freeze thaw cycles respectively with no effect on potency.

2. Analyzing and printing results

2.1. Prior to reading the plates in the luminometer, enter the assay protocol information in the luminometer software program.

2.2. Read the plates in a luminometer interfaced to a dedicated computer in the laboratory. Two data files will be generated from the luminometer for each plate read: an Excel file and a non-modifiable file.

2.3. The software program associated with the luminometer automatically saves the raw data in Excel format in a desire location, after each plate is read, using a unique file identification number (ID) for each plate.

2.4. Save the original plate data file directly from the luminometer software as a PDF file or another non-modifiable file. This file should also be saved automatically in a read only folder, for archival purpose.

2.5. Analyze and print the data using the appropriate Microsoft Excel "Luminescence" macro (provided by the Central Reference Laboratory).

NOTE 16: 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 (ID50 values) 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 17: 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.

2.6. Prepare a printed data packet to provide to the date reviewer(s) and /or PI. The data packet should include, at a minimum, the following items:

2.7. The data run summary, which must include: i) experiment number, ii) protocol and/or study number, iii) cells used in the assay, iv) length of incubation in hours, v) name, date, lot number and dilution of the virus stock used, vi) all pertinent sample information and vii) signature of technician who performed the assay.

2.8. Include a copy of the original plate data file from the luminometer (saved as a non-modifiable file).

NOTE 18: Based upon the PI's request, a copy of the original plate data (saved as a non-modifiable file) may be kept in electronic form.

3. Pass/Fail Criteria

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

3.2. The percent coefficient of variation (%CV) between RLU in the virus control wells is ≤30%.

3.3. The percent coefficient of variation (%CV) between RLU in the cell control wells is ≤30%.

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

3.5. Neutralization curves cross the 50% neutralization cut-off 0-1 times

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

7. References

1. Platt, E.J., K. Wehrly, S.E. Kuhmann, B. Chesebro, and D. Kabat. 1998. Effects of CCR5 and CD4 cell surface concentrations on infection by macrophage tropic isolates of human immunodeficiency virus type 1. J. Virol. 72:2855-2864.

2. Wei, X., J.M. Decker, H. Liu, Z. Zhang, R.B. Arani, J.M. Kilby, M.S. Saag, X. Wu, G.M. Shaw, and J.C. Kappes. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46:1896-1905.

3. Sarzotti-Kelsoe, M., R.T. Bailer, E. Turk, C.L. Lin, M. Bilska, K.M. Greene, H. Gao, C.A. Todd, D.A. Ozaki, M.S. Seaman, J.R. Mascola, and DC. Montefiori. 2013. Optimization and Validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1. J. Immunol Methods. Doi:pii: S0022-1759(13)00359-1.10.1016/j.jim.2013.110.22.

8. Appendices:

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.

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).

C: Sample Dilution Charts

STANDARD DILUTION CHART FOR 2-FOLD SAMPLE 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 3-12. Add the extra amount of growth medium listed above to wells where samples will be added, then add the corresponding 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

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

STANDARD DILUTION CHART FOR 5-FOLD SAMPLE DILUTIONS

Desired Start Dilution	GM Volume (µl)	Sample Volume (µl)
1:10	7	18.75
1:15	12.5	12.5
1:20	15	9.5
1:25	17.5	7.5
1:30	20	6.25
1:50	21	3.75

First place 100 µl of growth medium in all wells of columns 3-12. Add the extra amount of growth medium listed above to wells where samples will be added, then add the corresponding sample volume to the first 2 wells and do 5-fold dilutions (i.e., serial transfers of 25 µl).