$TCID_{50}$ (50% Tissue Culture Infectious Dose) Determination Quantitation of Viable HIV-1 Virions in Culture Supernatants

1. PRINCIPLE

1.1 In-vitro cultures of HIV-1 are typically monitored for viral replication by quantitative measure of p24 antigen production in lymphoid cells acutely infected with a viral isolate. While p24 antigen provides a relative measure of viral concentration, total p24 antigen in culture supernatants actually represents free antigen released from lysed mononuclear cells and p24 from the assembled intact virions. The $TCID_{50}$ assay measures the level of replication competent infectious virus from culture supernatants. This assay is performed prior to viral input-sensitive experimental procedures such as phenotypic drug susceptibility testing. The $TCID_{50}$ assay estimates viable virus using a streamlined endpoint dilution assay that is analyzed by the Spearman-Karber statistical method. The $TCID_{50}$ infectivity titration determination uses PHA-stimulated PBMC from normal donors (see culture methods for preparation of normal donor PBMCs). $TCID_{50}$ determinations are performed after high titer (assessed by p24 determination) culture supernatants are harvested, aliquoted and stored at –70 C or colder (preferably at liquid nitrogen temperatures).

2. SPECIMEN REQUIREMENTS

2.1 Cell-free supernatant is obtained from a positive HIV culture of patient PBMC, plasma, body fluid or tissue. This may originate from a qualitative macroculture, a qualitative microculture or a quantitative microculture. This supernatant is referred to as an “isolate” or “viral stock.” The stocks from microcultures are likely to require some expansion to reach adequate levels of infectivity for most experimental procedures (typically, supernatants containing greater than 25 ngs/mL of p24 antigen will have high viral titers).

3. REAGENTS

3.1 All reagents are prepared using deionized water, reagent grade I.

3.1.1 Sterile Phosphate Buffered Saline (PBS) or sterile Hank’s Balanced Salt Solution (HBSS), both without calcium or magnesium. Store at room temperature. Note manufacturer’s outdate or discard one week after opening.

3.1.2 Penicillin/Streptomycin – available in 100X concentration bottles. Store at frozen @-20C. Final concentrations are 100 Units Penicillin/mL and 100 micrograms/mL Streptomycin. Observe manufacturer’s outdate.

3.1.3 Gentamicin – available in 10 or 50 mg/mL bottles. Use at 50 micrograms/mL when combined with penicillin or 10 micrograms/mL when used with Pen/Strep (optional). Observe manufacturer's outdate.

3.1.4 Fetal Bovine Serum (FBS), available in 500mL sterile bottles from various manufacturers. (NOTE: HIV Culture tested FBS products are available, check with ACTG ops office for current source and contract information.) Store frozen at -20˚C. Note manufacturers outdate. When needed, rapidly thaw a bottle in a 37˚C water bath, then heat-inactivate in a 56˚C water bath for 30 minutes with occasional shaking. (NOTE: some FCS preparations have been heat inactivated by the manufacturer) The level of H_2O in the water bath should be as high as the level of serum in the bottle during inactivation. Store at 4˚C after thawing. Thawed, heat-inactivated FBS has a one-month outdate.

3.1.5 RPMI 1640 medium with L-glutamine (2 mM) – Store at 4˚C and observe manufacturer's outdate.

3.1.6 IL-2 (interleukin-2) recombinant, available in 50mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Alternatively, obtain natural IL-2 from Zeptometrix (formally Cellular products). Store at -20˚C. Note manufacturer's outdate and storage recommendations after opening.

Basic Medium: (**For 1 Liter**)

3.1.7 Add 200mL heat-inactivated FBS to 775mL of RPMI 1640 medium with L-glutamine. Final FBS concentration is approximately 20%.

3.1.8 Add 10mL stock (100X) penicillin or Pen/Strep.

3.1.9 Add 1.0mL of 50 mg/mL Gentamicin when combined with Penicillin only or 200µL of 50 mg/mL Gentamicin (1.0mL of 10 mg/mL Gent.) when using Pen/Strep.

Store Basic Medium at 4˚C for up to 1 month.

Growth Medium (culture medium) – also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium: To make 500mL:

3.1.10 475mL Basic Medium.

3.1.11 25mL IL-2. (Final concentration = 25mL / 500mL = 5%).

3.1.12 Store Growth Medium at 4˚C for up to 1 month. Growth Medium should be warmed before use.

3.1.13 Trypan Blue Stain – this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4gm Trypan Blue (available from Sigma) and 1mL Glacial Acetic Acid to 99mLs distilled H_2O or Saline. After dissolving, filter solution through Whatman filter paper or a 0.45µ filter.

3.1.14 PHA-stimulated uninfected donor PBMCs – see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

4. SUPPLIES AND EQUIPMENT

4.1 Gloves.
4.2 Disposable lab coat.
4.3 Laminar flow hood (Class 2 biosafety hood).
4.4 Sterile 2, 5, and 10 mL pipettes.
4.5 Hemacytometer or automated cell counter.
4.6 96-well, flat-bottomed tissue culture plates.
4.7 Sterile 1.5 and 0.5 mL microcentrifuge tubes.
4.8 20 µL, 200 µL, and 1000 µL micropipettors.
4.9 Sterile 20 µL, 200 µL, and 1000 µL pipette tips.
4.10 Multichannel 50 µL, 200 µL micropipettors.
4.11 Repeat pipettor and sterile tips.
4.12 Borosilicate glass tubes.
4.13 1% bleach or suitable disinfectant.
4.14 Low speed centrifuge with O-ring sealed safety cups.
4.15 Compound microscope.
4.16 CO_2 incubator (37 ± 1˚C with humidity).
4.17 37˚C and 56˚C water baths.

5. PROCEDURE

5.1 Virus Stocks: Collect cell-free, virus-containing supernatants from positive HIV cocultures (patient or control isolates) according to standard methods found in other sections of the Manual (**Tip**: Make sure that you are starting with relatively high titer supernatants i.e. undiluted supernatants with p24 values in the linear range of the p24 assay are destined to have very low $TCID_{50}$ values whereas supernatants with significant p24 values [>30 pg/mL] at 1:1000 or 1:10000 dilution will yield acceptable $TCID_{50}$ values)

NOTE: **SUBSEQUENT PROCEDURE SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).**

5.2 **Virus Stock Infectivity Titration**: Seven serial four-fold dilutions of virus stock, ranging from 1:16 through 1:65, 635, are titrated in 96-well flat-bottomed tissue culture plates.

5.2.1 Centrifuge 1 to 3 day old PHA-stimulated donor PBMC at 400 x g for 10 minutes at 20˚C to 24˚C.

5.2.2 Remove and discard supernatant, then resuspend cells in Growth Medium and enumerate cells

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5.2.3 Determine viability with 0.4% trypan blue exclusion dye. Do not use cells if viability is less than 85%.

5.2.4 Adjust sample with Growth Medium to a concentration of 4 x 10^6 cells/mL (exactly 4.2 million cells are needed per plate as currently formatted; adjust volume accordingly).

5.2.5 Keep in CO_2 incubator at 37˚C until step 6.

5.3 Add 200µL PBS or HBSS to all wells labeled P; see "Plate Format" below.

PLATE FORMAT FOR HIV TITRATION

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
D	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
E	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

5.3.1 Add 150µL Growth Medium to the wells labeled 4^{-3} to 4^{-8} (rows C to E, columns 4 to 9) with a multi-channel micropipettor.

5.3.2 Rapidly thaw an aliquot of the virus stock at 37˚C in a water bath until only a small crystal of ice remains. Immediately dilute the sample 1:12 in Growth Medium (e.g., 0.1mL of virus stock to 1.1mL of culture medium) and transfer 200µL to each well labeled 4^{-2} (column 3 in rows C to E).

5.3.3 With a multi-channel pipette, transfer 50µL from the wells labeled 4^{-2} to wells labeled 4^{-3} (column 3 to column 4 in rows C to F). Continue such transfers, moving from ***left to right***, changing tips prior to mixing contents of the next column of wells. Discard 50µL from the wells labeled 4^{-8} (column 9).

5.3.4 Dispense 50µL of PBMC (200,000 cells) from step 1 to all wells containing viral stock, moving from ***right to left*** (to prevent carry-over of higher concentration virus.

5.3.5 Cover the plate and incubate at 37˚C, 5% CO_2 with humidity.

5.3.6 On day 4, with a multi-channel pipette, moving from ***right*** to ***left*** across the plate, resuspend the cells in each culture well by mixing and remove and discard 125µL of the cell suspension. Add 150µL of fresh Growth Medium back to each well, again moving from *right* to *left*. Return the plate to the incubator.

5.3.7 On day 7, the HIV titration assay is terminated and the appropriate supernatants are tested for HIV p24 antigen as follows:

5.3.8 Transfer 100µL of supernatant from the titration wells to wells of a HIV p24 antigen plate that contains 100µL of Growth or Basic Medium and 20µL of the manufacturer's disruption buffer containing Triton X-100.

5.3.9 The assay is performed according to the recommendations of the manufacturer using VQA reference standard and QC check samples as described in "Standard HIV p24 Antigen Assay." A well is scored "positive' if the VQA corrected value is 50 pg/mL.

6 Interpretation of Results

6.1 The $TCID_{50}$ is calculated by the Spearman-Karber method. An example of this calculation follows:

6.2 Scoring the HIV p24 antigen plate:

	1	2	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	10	11	12
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	+	+	+	+	-	-	-	P	P	P
D	P	P	+	+	-	-	-	-	-	P	P	P
E	P	P	+	+	+	-	-	-	-	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

6.3 Calculating $TCID_{50}$/mL:

xk = dose of the highest dilution.
r = sum of the number of "-" responses.
d = spacing between dilutions.
n = wells per dilution.

Spearman-Karber formula: $M = xk + d\,[0.5 - (1/n)\,(r)]$

= 8 + 1 [0.5 – (1/3) (12)]
= 8 + 1 (0.5 – 4.0)

The 50% endpoint is $4^{-4.5}$

Converting to 10^x:
x = 4.5 * log 4
= 4.5 * 0.602
= 2.7 the titer is $10^{2.7}$

To calculate the $TCID_{50}$/mL of virus stock, the original dilution must be corrected by multiplying by 5 (1000 µL ÷ 200 µL):

$TCID_{50}$/mL = 5 * $10^{2.7}$
= $10^{0.70}$ * $10^{2.7}$
= $10^{3.4}$

$TCID_{50}$/mL – 2.51 x 10^3

6.4 The appendix following this procedure has calculated $TCID_{50}$ values for all possible assay outcomes for 3 or 4 replicate assays (1-21 positive wells/3 replicates or 1-28 wells/4 replicates). An electronic $TCID_{50}$ calculator is also available and is posted with this procedure on the ACTG website. http//:www.actg@nih.gov.

Notes:

- For extremely high tittered virus, the value for "xk" will increase by 1 unit for each additional 4-fold dilution of culture supernatants prepared.
- Adding replicates refines the degree of assay outcomes, which may be of importance for very inoculum dependent procedures. For example, 6 replicates will approximately double the accuracy of $TCID_{50}$ outcomes in when compared to 3 replicates.

7. QUALITY CONTROL

7.1 Most low $TCID_{50}$ titers are related to inadequate expansion of the primary viral culture. This is to be expected for isolates from patients that are known to harbor HIV that has many mutations related to prolonged antiretroviral exposure. While most laboratory strains of HIV will produce high titer viral supernatants in 10 -14 days, clinical isolates may require additional time in expansion to reach suitable viable virus levels.

7.2 Remember to harvest, aliquot and freeze supernatants prior to attempting $TCID_{50}$ determination since viable virus titers will decrease after freezing. Frozen viral isolates will retain their viability for long periods when stored in liquid nitrogen.

7.3 Remember to carefully identify and document each viral isolate stock with test date and viral titer.

8. REFERENCES

8.1 Chou TC and Talalay P. Quantitative Analysis of Dose-effect Relationships: the Combined Effects of Multiple Drugs or enzyme inhibitors. Adv Enzyme Regul 22:27-55, 1984.

8.2 Chou TC. The Median-effect Principle and the Combination index for Quantitation of synergism and antagonism. Synergism and Antagonism in Chemotherapy, Chou TC and Rideout DC, eds., Academic Press, San Diego, 1991.

Appendix I:

Calculated TCID 50 values for 3 or 4 Replicate Plate Configuration.:

3 Replicate Plate Configuration 4 Replicate Plate Configuration

Appendix II; The TCID$_{50}$ Electronic Calculator:

TCID50

M=xk + d [0.5 - (1/n)r]

Constants	Value
d	1
Variables	Value
xk	8
r	7
n	3

Calculations	
M	Titer
0.333333	3.713
0.33	3.7
-1.833333	4.4
-1.8333	
6.167	
6.2	

TCID50/ml	Log 10
2.6E+04	

TCID50/ml
25854.0

# of p24 Neg Wells	Log10 TCID50	TCID50
0	656.1E+3	>=656100
1	413.0E+3	413000
2	261.0E+3	261000
3	164.0E+3	164000
4	103.5E+3	103500
5	657.7E+2	65770
6	412.0E+2	41200
7	325.1E+2	32510
8	162.2E+2	16220
9	102.6E+2	10260
10	645.7E+1	6457
11	407.4E+1	4074
12	251.0E+1	2510
13	161.4E+1	1614
14	101.9E+1	1019
15	641.0E-0	641
16	405.0E-0	405
17	254.0E-0	254
18	160.0E-0	160
19	101.0E-0	101
20	640.0E-1	64
21	400.0E-1	<=40

# of p24 Neg Wells	Log10 TCID50	TCID50
0	650.1E+3	>=650100
1	464.0E+3	464000
2	328.1E+3	328100
3	232.0E+3	232000
4	164.0E+3	164000
5	116.0E+3	116010
6	820.3E+2	82030
7	580.1E+2	58010
8	410.2E+2	41020
9	290.0E+2	29000
10	205.7E+2	20570
11	145.0E+2	14500
12	102.6E+2	10260
13	725.3E+1	7253
14	512.8E+1	5128
15	362.6E+1	3626
16	256.4E+1	2564
17	181.3E+1	1813
18	127.0E+1	1270
19	907.0E-0	907
20	641.0E-0	641
21	453.0E-0	453
22	321.0E-0	321
23	227.0E-0	227
24	160.0E-0	160
25	113.0E-0	113
26	802.0E-1	80.2
27	567.0E-1	56.7
28	401.0E-1	<=40.1

Instructions for use:

In order to calculate the TCID50 first double click on the above calculator. Then change the values for the Spearman-Karber formula variables you used in your particular procedure. The calculator will calculate your TCID50 results for you.

The TCID50 Calculator was prepared by Jason Rippe, at Children's Memorial Hospital, Chicago, IL 1/2004.

The TCID$_{50}$ Calculator on the next page can be used as both an assay worksheet and calculated TCID$_{50}$ values. The file below was contributed by Cheryl Jennings at the VQA, Rush University, Chicago.

TCID50 Estimations for Viral Stocks

Example:

4^ -2	4^ -3	4^ -4	4^ -5	4^ -6	4^ -7	4^ -8
+	+	+	+	-	-	-
+	+	+	+	-	-	-
+	+	+	+	-	-	-

xk	d	n	r	spearman-Karber formula	conversion to log 10	TCID50/mL
8	1	3	9	**5.50**	**3.31**	**10,232**

xk= dose of the highest dilution
d= the spacing between dilutions
n= wells per dilution
r= sum of the negative responses

Instructions:

1. Fill in Table 1 with the culture results obtained for the TCID50
2. Fill in Table 2 using the results from the TCID50 culture (definitions of each variable is listed below the table)
3. The algorithm will automatically calculate the TCID50 per mL of viral stock

Table 1:

4^ -2	4^ -3	4^ -4	4^ -5	4^ -6	4^ -7	4^ -8

Table 2:

xk	d	n	r	spearman-Karber formula	conversion to log 10	TCID50/mL
				#DIV/0!	#DIV/0!	#DIV/0!

xk= dose of the highest dilution
d= the spacing between dilutions
n= wells per dilution
r= sum of the negative responses

Procedure: $TCID_{50}$ Determination of Viable HIV-1

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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Supersedes Archived Protocol: DAIDS Virology Manual for HIV Laboratories, Version January 1997 (Within Phenotyipic AZT Resistance Assay Procedure)