Optimization and Validation of a Neutralizing Antibody Assay for HIV-1 in TZM-bl Cells Using Luciferase Reporter Gene Expression

Report
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1.0 INTRODUCTION

A neutralizing antibody response is detected in nearly every HIV-1-infected individual. The value of this response after infection is compromised by neutralization-escape variants that emerge soon after neutralizing antibodies are produced. Neutralizing antibodies might be of greater importance for prophylaxis and are given a high priority for AIDS vaccine development (Mascola, 2003; Letvin et al., 2002). Antibodies neutralize HIV-1 by binding either the surface gp120 or transmembrane gp41 envelope glycoproteins to prevent virus entry (Wyatt & Sodroski, 1998) **(Fig. 1)**. All vaccines containing these envelope glycoproteins have the potential to generate neutralizing antibodies.

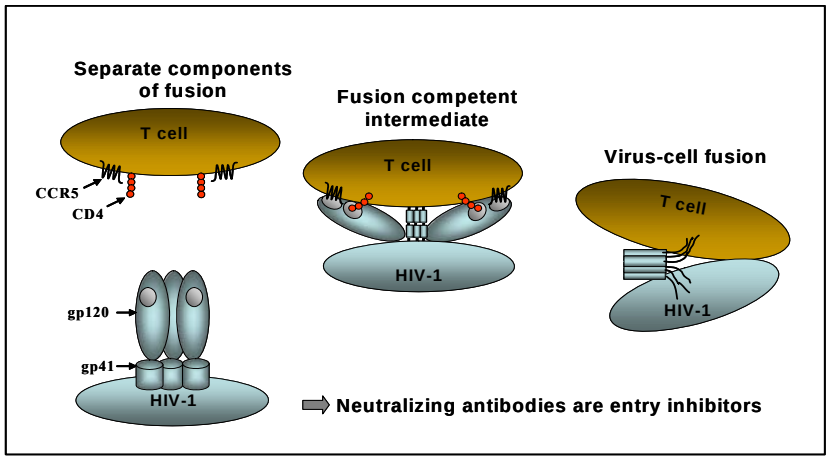

Figure 1. Stages of HIV-1 entry as targets for antibody-mediated neutralization.

Antibody-mediated neutralization of HIV-1 is best evaluated by measuring reductions in virus infectivity in cells that express receptor (CD4) and coreceptor (CCR5, CXCR4) molecules. Neutralization is assessed by pre-incubating cell-free virus with multiple dilutions of test sample, adding cells and continuing the incubation until linear measurements of infection can be recorded at each dilution of test sample. Titers of neutralizing antibodies are usually defined as the sample dilution that reduces infection by 50% (this cut-off may differ depending on assay conditions and the background activity of control samples). Alternatively, neutralization may be recorded as the % reduction in virus infectivity at a single sample dilution. This latter format is particularly useful when screening large numbers of samples for neutralizing activity against multiple strains of virus.

This document describes the optimization and validation of an assay that measures HIV-1 neutralization in TZM-bl cells as a function of reductions in luciferase (Luc) reporter gene expression after a single round of virus infection **(Fig. 2)**. The assay may be used with either uncloned virus prepared in human lymphocytes or Env-pseudotyped virus prepared by transfection in 293T cells. The HIV-1 protease inhibitor, indinavir, may be added to the medium at a final concentration of 1 μM to prevent replication of uncloned viruses. Indinavir is not needed for assays with pseudoviruses, since these viruses are replication-defective by design. The assay is preferred for use with pseudoviruses, since sensitivity is lacking in some cases with uncloned viruses.

TZM-bl cells (also called JC53BL-13) may be obtained from the NIH AIDS Research and Reference Reagent Program (Cat. No. 8129). This is a HeLa cell clone that was engineered to express CD4, CCR5 and CXCR4 (Platt et al., 1998) and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR (Wei et al., 2002), 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. The polycation, DEAE dextran, is added to the medium during neutralization assays to enhance virus 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 and uses a clonal cell population for enhanced precision and uniformity.

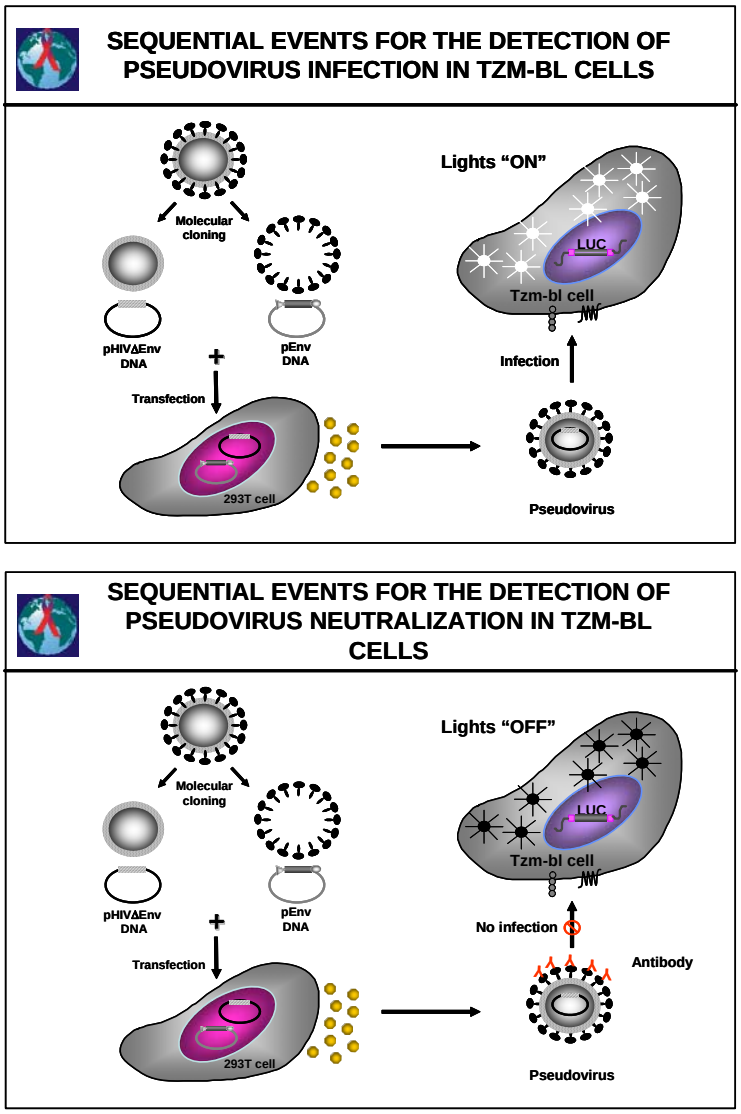

Figure 2. Sequence of events for detecting infection and neutralization of HIV-1 Env-pseudotyped viruses in TZM-bl cells using luciferase reporter gene expression. *Pseudoviruses are prepared by using two plasmid transfection in 293T cells. One plasmid expresses full-length molecularly cloned gp160 from the HIV-1 strain of choice. This plasmid is co-transfected with a backbone plasmid containing a complete HIV-1 genome deleted in a portion of Env that does not eliminate Tat. Transfection yields pseudovirus virus particles capable of a single round of infection (the pseudoviruses do not carry a functional env gene and therefore are unable to produce infectious progeny virions). Luc reporter gene expression is induced in trans by viral Tat protein soon after infection and is quantified by luminescence (top panel). Luminescence is diminished in the presence of neutralizing antibodies (lower panel).*

2.0 ASSAY DEVELOPMENT AND OPTIMIZATION

This section describes experiments that led to the identification of optimal conditions for infection and detecting neutralizing antibodies in TZM-bl cells. All experiments were performed in 96-well culture plates. Unless otherwise specified, all incubations were at 37°C in a humidified, 5% CO_2/95% air environment. Also, all serum samples were heat-inactivated at 56°C for 1 hour.

2.1 Establishing the susceptibility of TZM-bl cells to infection by a wide range of HIV-1 strains

The suitability of any cell line for neutralization assays with HIV-1 is highly dependent on an ability to support infection by a wide spectrum of genetic variants. The suitability of using TZM-bl cells was first demonstrated in studies of antiretroviral drugs (Wei et al., 2002) and later in a study of neutralizing antibodies (Wei et al., 2003). It became apparent from these studies that TZM-bl cells were susceptible to infection by a broad range of HIV-1 variants. We aimed to confirm and extend this observation by testing uncloned and molecularly cloned variants of the virus. Conditions for infection were based on published procedures (Wei et al., 2003) with some modifications. Briefly, cells were obtained from the NIH AIDS Research and Reference Reagent Program (Lot Number 206/01/2003), thawed and cultured in a T-75 flask in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (heat-inactivated) and 50 μg gentamicin/ml. At confluency the cells were trypsinized and expanded in multiple T-75 culture flasks. When the expanded cultures reached confluency, they were trypsinized and a portion of the cells was cryopreserved in multiple aliquots as future seed stocks. The remaining portion of cells was continued in culture for subsequent assay development.

Tests for infection-susceptibility were performed by using molecularly cloned pseudoviruses of clade B HIV-1 produced by transfection in 293T cells. Based on the experience of other investigators, DEAE dextran (20 μg/ml final concentration) was added to the medium to facilitate infection. This polycation counters the repulsive electrostatic forces between the virus and cells surface without affecting antibody binding and neutralization. Cells were typsinized, washed once with growth medium, re-suspended in growth medium and seeded at 1 x 10^4 cells/100 μl growth medium/well in 96-well culture plates. In some cases (test 1) cells were permitted to attach for 24 hours before adding 50 μl of virus to 3 wells for each virus tested. In other cases (tests 2-4) freshly trypsinized cells were washed with growth medium, suspended in fresh growth medium and added directly to wells containing virus (50 μl virus, 100 μl cells). Luciferase activity was measured by luminescence 2 days later. To measure luciferase, 100 μl of cell suspension was transferred to corresponding wells of either white solid plates or black solid plates containing lysis buffer and substrate (BrightGlo, Promega). Luminescence was measured 2 minutes later by using a PerkinElmer Life Sciences, Model Victor2 luminometer.

TZM-bl cells were highly susceptible to infection by all pseudoviruses tested. Relative luminescence units (RLU) in the presence of virus were approximately 100-times greater than the background in wells containing uninfected cells **(Table 1, test 1)**. Background activity resulting from low levels of constitutive luciferase expression was 4.7 x 10^3 RLU. This compared to approximately 1 x 10^6 RLU in the presence of pseudovirus. This signal-to-noise ratio is well-suited to measurements of neutralizing antibodies; however, it was noted that strong luminescence in positive wells scattered to adjacent wells, producing artifactual positive readings that could interfere with accurate measurements of infection. To eliminate light scattering, solid black plates were investigated in a subsequent series of infections. Use of solid black plates reduced RLU approximately 5-10 fold compared to white plates **(Table 1, tests 2 & 3)**. It also completely eliminated light scatter to adjacent wells. Background luminescence in cell control wells and virus-containing wells was reduced proportionately such that the signal-to noise ratio was not affected.

We next performed a preliminary test of the infectivity of uncloned, PBMC-grown primary isolates representing clades A, B, C and E. These experiments were performed as above but used only 1 x 10^4 cells/well. Luciferase activity was assessed 3 days post virus inoculation. As shown in **Table 1** (tests 3 and 4), all strains tested were highly infectious regardless of clade.

These tests established a wide and useful range of infection of different HIV-1 strains in TZM-bl cells, much the same as described previously (Wei et al., 2002, 2003). They also established an advantage of using solid black plates rather than white solid plates for reducing background and eliminating light scatter. Finally, the results indicated that freshly trypsinized cells could be added directly to the virus without the need for overnight adherence prior to infection.

Table 1. Range of infectivity of different HIV-1 strains in TZM-bl cells.

HIV-1 strain	Clade	Produced in:	RLU in white plate	RLU in black plate
Test 1: Molecularly cloned pseudoviruses				
6101.1	B	293T	1,116,244	not tested
QH0692.42	B	293T	913,784	not tested
QH0515.1	B	293T	1,387,576	not tested
AR1.29	B	293T	782,784	not tested
5768.4	B	293T	940,660	not tested
SS1196.1	B	293T	1,421,216	not tested
BG1168.1	B	293T	967,150	not tested
PVO.4	B	293T	1,839,574	not tested
NL4-3	B	293T	2,098,686	not tested
Background	--	--	4,658	not tested
Test 2: Molecularly cloned pseudoviruses				
6101.1	B	293T	858,835	173,579
QH0692.42	B	293T	1,023,156	175,861
QH0515.1	B	293T	1,425,795	224,269
AR1.29	B	293T	1,660,842	246,619
5768.4	B	293T	1,547,557	214,681
SS1196.1	B	293T	1,405,500	190,079
BG1168.1	B	293T	1,226,373	184,208
PVO.4	B	293T	1,271,193	230,872
Background	--	--	2,200	330
Test 3: Uncloned viruses				
SF162	B	PBMC	373,592	59,931
MN	B	H9	161,183	23,770
Bal	B	PBMC	740,527	139,475
JR-FL	B	PBMC	731,599	124,027
6101	B	PBMC	819,825	134,135
Background	--	--	2,400	260
Test 4: Uncloned viruses				
PVO	B	PBMC	not tested	552,715
6101	B	PBMC	not tested	722,084
ADA	B	PBMC	not tested	70,588
Du422	C	PBMC	not tested	821,743
S021	C	PBMC	not tested	1,064,819
92RW020	A	PBMC	not tested	194,796
CM244	E	PBMC	not tested	592,080
Background	--	--	not tested	1,135

2.2 Optimization of DEAE-dextran usage

In preparation for neutralization assays, we next determined the minimum concentration of DEAE dextran to maximize virus infectivity without causing cell toxicity. To test for cell toxicity, freshly trypsinized cells (1 x 10^4/100 µl/well) were added directly to multiple concentrations of DEAE dextran in 96-well plates (triplicate wells for each concentration of DEAE dextran tested). Cell viability was assessed by neutral red staining (Montefiori et al., 1988) at DEAE dextran doses of 2.5 - 80 µg/ml. All concentrations were non-toxic after two days of incubation **(Table 2)**.

Table 2. Test of DEAE-dextran toxicity in TZM-bl cells.

DEAE dextran concentration	A540
0 µg/ml	0.597
2.5 µg/ml	0.790
5 µg/ml	0.797
10 µg/ml	0.806
20 µg/ml	0.805
40 µg/ml	0.787
80 µg/ml	0.650

To determine the minimum concentration of DEAE dextran needed for maximum levels of infectivity, four different molecularly cloned pseudoviruses were tested for infectivity in the presence and absence of DEAE dextran at doses of 5 - 40 µg/ml. Equal amounts of each virus were added to each dose of DEAE dextran. RLU were measured 2 days later. Peak infectivity occurred at 20 - 40 µg/ml **(Fig. 3)**. We chose 30 µg/ml as a standard dose for future assays.

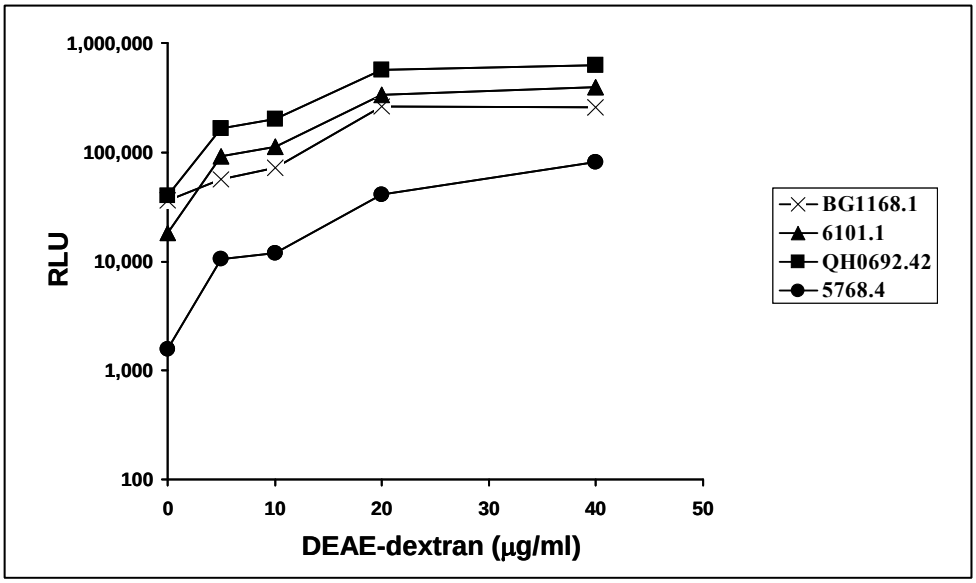

Figure 3. Minimum concentration of DEAE-dextran needed for maximum infection in TZM-bl cells.

2.3 Optimization of TZM-bl cell numbers for infection

It was necessary to identify the optimal inoculum density of TZM-bl cells that permitted linear measurements of infection. In this experiment, freshly trypsinized and washed cells were added at various densities to a 96-well plate containing equal amounts of pseudovirus QH0692.42. DEAE dextran was present at 30 μg/ml. Luminescence was measured in solid black plates after 48 hours. Infection increased linearly over a range of 17 to 37,037 cells added/well **(Fig. 4)**. A sharp decrease in infection was observed at higher cell numbers. Background RLU in cell control wells also diminished at high cell densities, suggesting either a loss of cell viability or that density-dependent down-regulation of LTR-Luc had occurred.

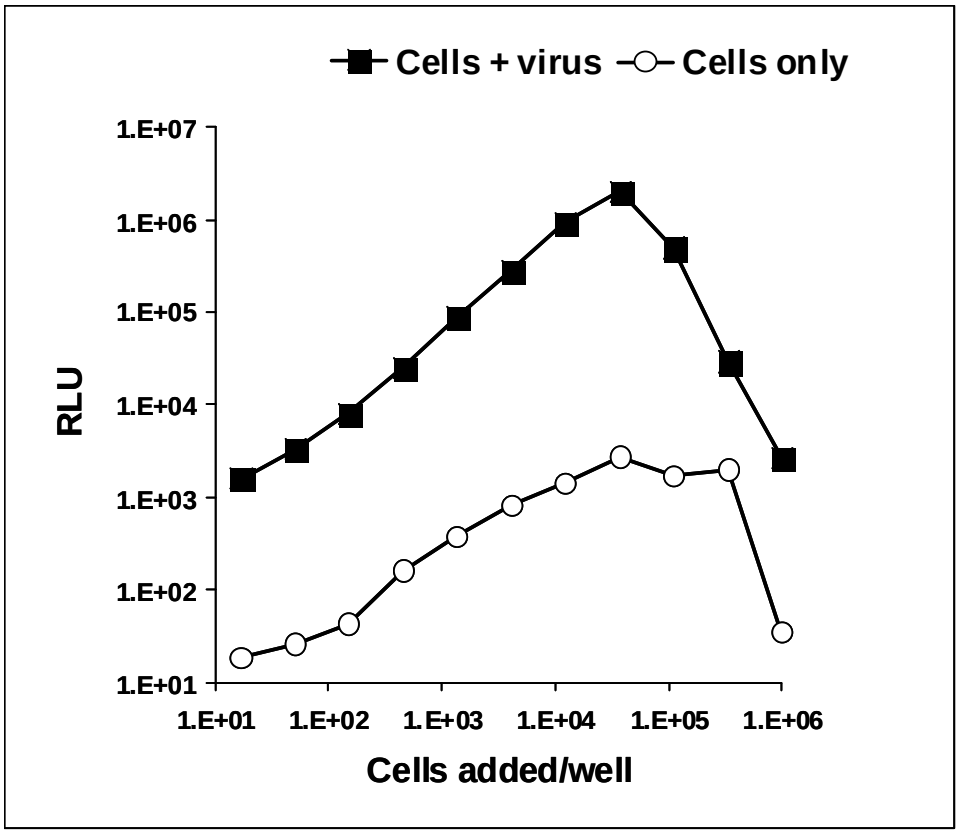

Figure 4. Linear range of input TZM-bl cell numbers that support HIV-1 infection.

2.4 Linear relationship between virus inoculum size and RLU

Having defined an optimal number of cells to use for inoculation, a final experiment prior to performing a neutralization assay was to establish the linear relationship between input virus dose and RLU. In this experiment, freshly trypsinized TZM-bl cells (10,000/100 μl/well) were added to serially diluted pseudoviruses in 96-well plates. Eleven 5-fold dilutions of virus ranging from 1:10 to 1:97,656,250 were made in quadruplicate. DEAE dextran was present at 30 μg/ml after the addition of cells. Luciferase activity was quantified by luminescence 48 hours later. The tissue culture infectious dose 50 (TCID50) of the three pseudoviruses used in this experiment was calculated according to the Reed and Muench equation (Johnson and Byington, 1990). An RLU value of ≥2.5x background was used to score a positive. Curves were constructed by plotting TCID50 against corresponding RLU on a log-log scale **(Fig. 5)**. RLU increased in a

linear fashion over a wide range of infectious input virus doses. This linear range extended from approximately 2,500 - 500,000 RLU, corresponding to approximately 1 - 2,500 input TCID50/well.

These curves were revealing in two additional ways. First, although the slopes were similar, the magnitude of RLU was low for strain SS1196.01 compared to the other two strains, indicating that equal doses of different strains may not be expected to yield equivalent absolute RLU values. Second, RLU values for strain QH0692.42 peaked and then declined. This decline in RLU was associated with virus-induced syncytium formation and associated cell killing effects. We have observed even greater cytopathic effects with other strains of virus at high input doses but these cytopathic effects are rare at input TCID50 values ≤200. Thus, a 200 TCID50 inoculum avoids unwanted cytopathic effects in a majority of cases. A 200 TCID50 also provides a means to measure 2-log reductions in virus infectivity over a linear range.

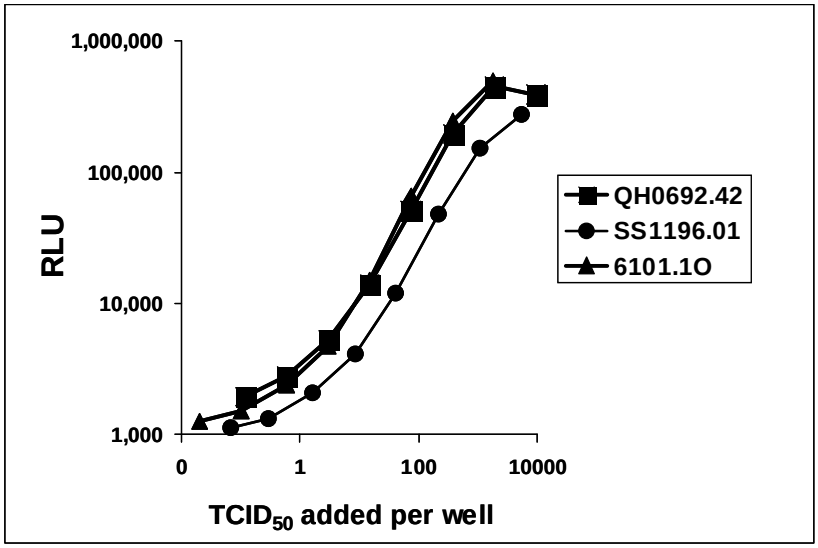

Figure 5. Linear range of infection in TZM-bl cells.

2.5 Effect of virus dose in the neutralization assay

A neutralization assay was designed **(Fig. 6)** in which 50, 200, 1,000 and 5,000 TCID50 of pseudovirus SS1196.01 were incubated with serial dilutions of antibody or sCD4 in a total volume of 150 µl of growth medium. SS1196.01 was chosen for this experiment because little or no cytopathic effects were observed at doses up to 5,000 TCID50. Each dilution of antibody or sCD4 was tested in triplicate wells of a 96-well plate. Freshly trypsinized TZM-bl cells in 100 µl growth medium containing DEAE dextran (75 µg/ml; 30 µg/ml final concentration) were added to each well at a density of 10,000 cells/well. One set of 8 control wells received cells only (cell control). Another set of 8 wells received cells and virus but no antibody (virus control). The final volume of control wells was adjusted to 250 µl with growth medium. Luciferase activity was quantified by luminescence 48 hours later. Percent reduction in RLU (% neutralization) was determined by calculating the difference in average RLU between test wells (cells + sample + virus) and cell control wells (cells only), dividing this result by the difference in average RLU between virus control (cell + virus) and cell control wells, subtracting from 1 and multiplying by 100.

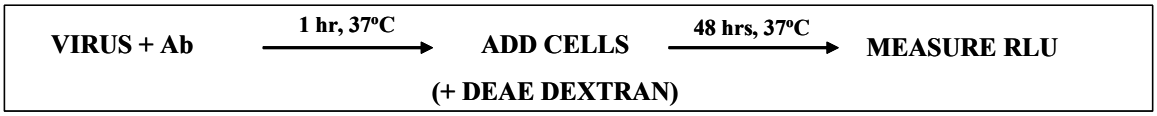

Figure 6. Sequential steps in the neutralizing antibody assay.

Positive neutralization was detected with all four reagents tested regardless of input virus dose **(Fig. 7)**. Test reagents included a pooled serum sample from HIV-1-infected individuals, sCD4 and the human monoclonal antibodies IgG1b12 and 2G12. Dose-response curves were linear between approximately 20% and 85% reductions in RLU in all cases.

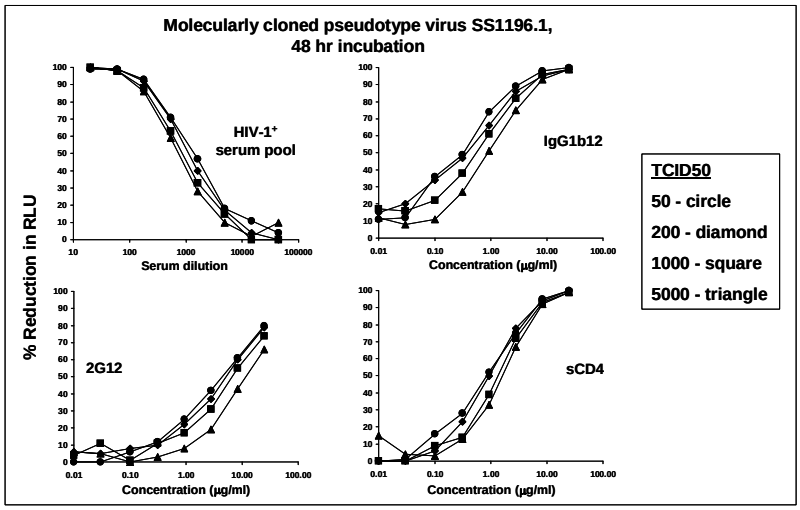

Figure 7. Neutralization curves in TZM-bl cells at different input TCID50. *Average RLU in cell control and virus control wells, respectively: 50 TCID50 (926 vs 11,813; range = 10,887); 200 TCID50 (997 vs 55,285; range = 54,287); 1,000 TCID50 (1,059 vs 242,013; range = 240,954); 5,000 TCID50 (1,194 vs 551,651; range = 550,457).*

From the neutralization curves shown in **Figure 7**, 50% inhibitory doses (ID50) were calculated for each test reagent at each dose of input virus **(Fig. 8)**. The relative potency of each test reagent increased with decreasing input virus, indicating that assay sensitivity is dependent on virus dose, where greater sensitivity is achieved with lower virus doses. This effect on assay sensitivity was not dramatic, however, since the ID50 values of each serologic reagent differed by <3-fold over a 100-fold range of virus doses. Thus, minor differences in the dose of virus used in this neutralization assay are unlikely to alter the results significantly.

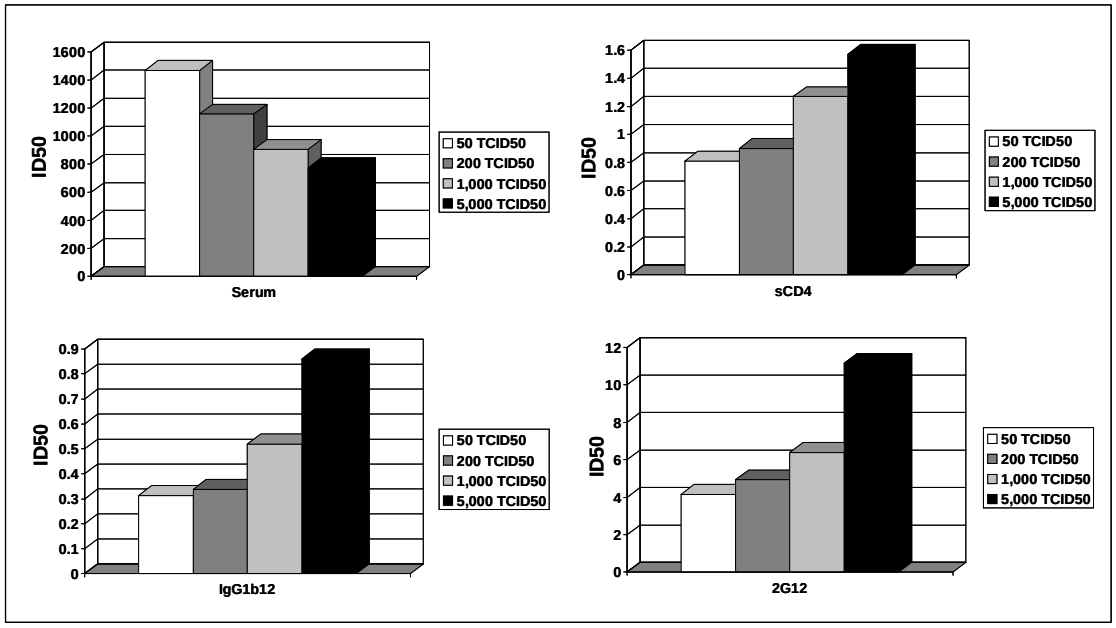

Figure 8. Effect of virus dose on assay sensitivity as judged by ID50.

2.6 Effect of input TZM-bl cell number on assay sensitivity

We had previously determined that linear measurements of virus infectivity in a 48 hour assay were only possible when <40,000 cells were added per well in 96-well plates **(Fig. 4)**. Using these results as a guide, we aimed to determine the effect of input cell number on the sensitivity of the assay for detecting neutralization. In this experiment, 200 TCID50 of pseudovirus SS1196.01 was assayed with two HIV-1-positive serum samples (DUMC-3 and LW-0013) and sCD4 as described for the experiment in **Figure 7** with the exception that three different densities of TZM-bl cells were used (5,000, 10,000 and 20,000 cells/well). Separate assay plates were set-up for each cell density. Neutralization curves **(Fig. 9)** and ID50 values **(Fig. 10)** were nearly identical for all three serologic reagents when 5,000 and 10,000 cells were used in the assay. A small loss in assay sensitivity was seen when 20,000 cells were used.

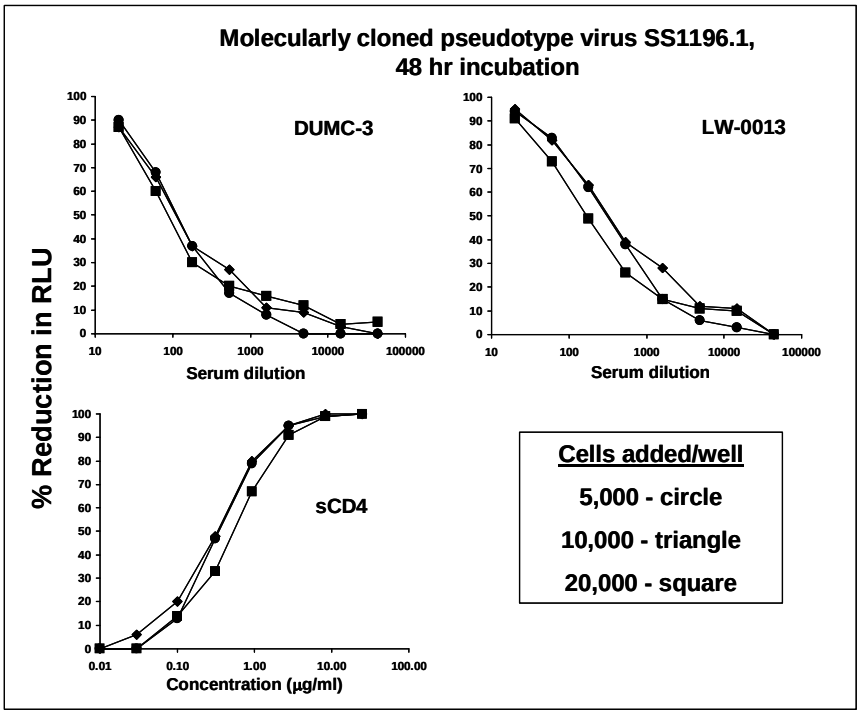

Figure 9. Effect of input cell number on assay sensitivity as judged by dose-response curves.

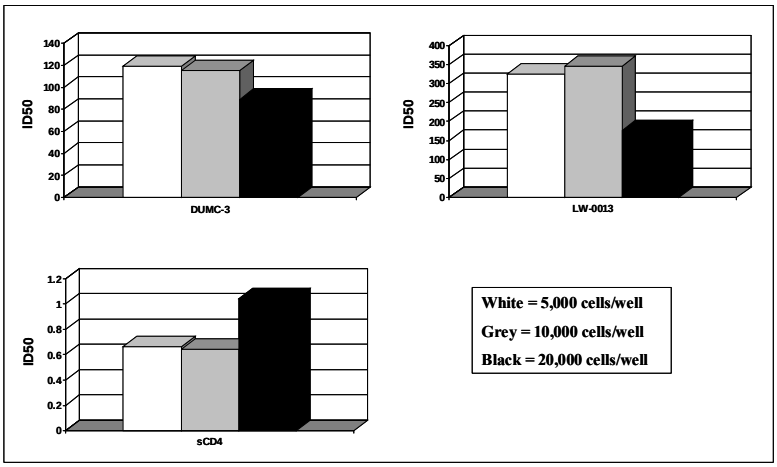

Figure 10. Effect of input cell number on assay sensitivity as judged by ID50.

2.7 Neutralization assays performed under optimal conditions

In this experiment, pseudovirus QH0692.42 was assayed with the human monoclonal antibodies IgG1b12, 2G12 and 2F5 under conditions that were determined to be optimal in the above experiments. Each dilution of monoclonal antibody was tested in triplicate wells of a 96-well plate. Dilutions of antibodies were made in 100 µl of growth medium/well. Fifty microliters of pseudovirus (200 TCID50) were added to each well and incubated for 1 hr. Freshly trypsinized and washed TZM-bl cells were suspended in growth medium containing DEAE dextran (75 µg/ml) and 100 µl of this cell suspension (10,000 cells) was added to each well (final concentration of DEAE dextran = 30 µg/ml). One set of 8 control wells received cells only (cell control). Another set of 8 wells received cells and virus but no antibody (virus control). The final volume of control wells was adjusted to 250 µl with growth medium. Luciferase activity was quantified by luminescence 48 hours later. Percent reduction in RLU (% neutralization) was determined by calculating the difference in average RLU between test wells (cells + sample + virus) and cell control wells (cells only), dividing this result by the difference in average RLU between virus control (cell + virus) and cell control wells, subtracting from 1 and multiplying by 100.

As expected, each monoclonal antibody exhibited positive neutralizing activity. Dose-response curves were linear between 20% and 90% reductions in RLU **(Fig. 11)**. The range of RLU between cell control wells (background) and virus control wells was sufficient to measure a minimum 2-log reduction in virus infectivity.

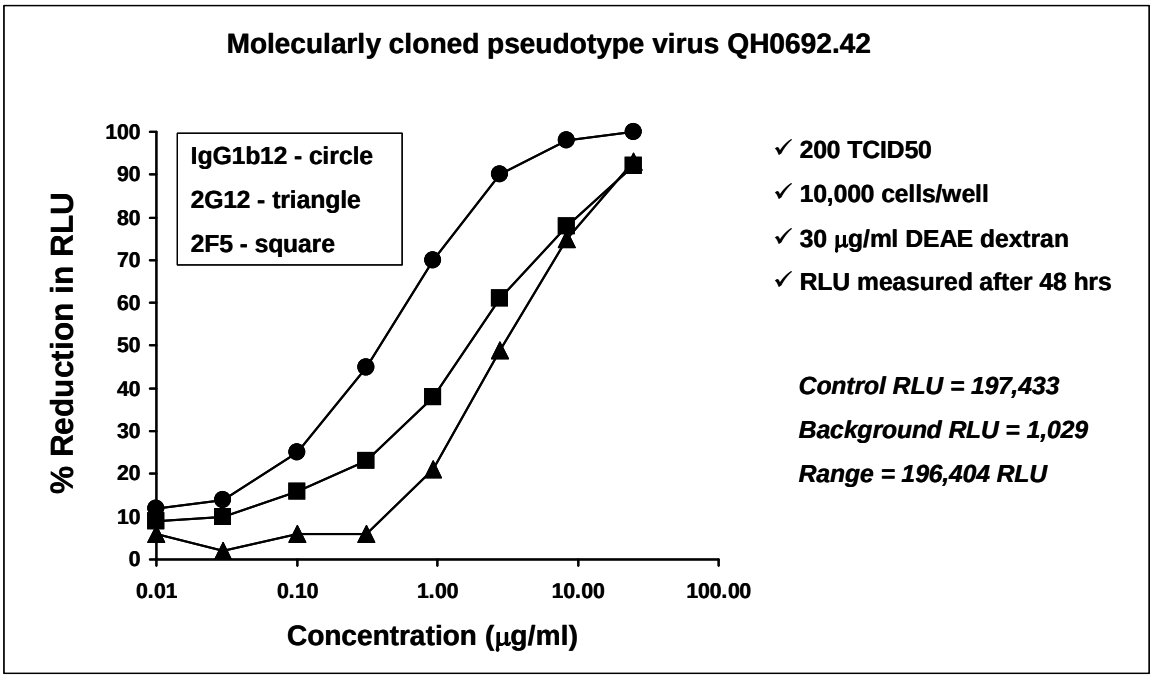

Figure 11. Dose-response curves in the TZM-bl neutralization assay under optimal conditions.

2.8 Optimization of indinavir for single round infection with replication competent viruses

The experiment depicted in **Figure 5** determined the linear relationship between input virus dose and RLU after a single round of infection with molecularly cloned pseudoviruses. To preserve this linear relationship with uncloned viruses, an HIV-1 protease inhibitor (indinavir) was used to disrupt virus assembly, thereby blocking the production of infectious progeny virions after a single round of infection. It was necessary to determine an optimal concentration of indinavir that prevented virus replication without causing cell toxicity. To test for cell toxicity, freshly trypsinized cells (1 x 10^4/100 μl/well) were added directly to multiple concentrations of indinavir in triplicate wells of a 96-well plate. Cell viability was assessed by neutral red staining (Montefiori et al., 1988) at indinavir doses of 0.0051 - 100 μM. All concentrations were non-toxic after 68 hours of incubation **(Table 3)**.

Table 3. Indinavir toxicity in TZM-bl cells.

Indinavir (μM)	A540
0	1.12
0.0051	1.10
0.0152	1.10
0.0457	1.10
0.1372	1.12
0.4115	1.11
1.235	1.05
3.704	1.09
11.11	1.14
33.33	1.13
100	1.34

To determine the optimal concentration of indinavir that is needed to prevent virus replication, six different strains of uncloned HIV-1 grown in PBMC were tested for infectivity in the presence and absence of indinavir (0.005 - 50 μM). Equal amounts of each virus were added to each dose of indinavir. RLU was measured after 72 hours. Although little replication occurred during this 72 hour period, maximum inhibition of replication of each virus was achieved at ≥0.5 μM indinavir **(Fig. 12)**. We chose 1 μM as a standard dose for future assays.

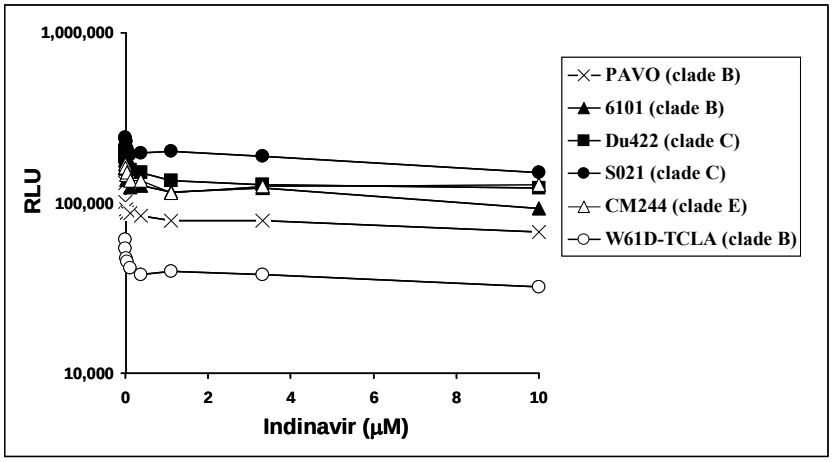

Figure 12. Dose-response curve for inhibition of virus replication by indinavir in TZM-bl cells.

2.9 Neutralization phenotype of molecularly cloned pseudoviruses compared to the uncloned parental virus

Both uncloned and molecularly cloned viruses may be used in this assay. Molecularly cloned pseudoviruses have the advantage of being easier and quicker to produce and titrate compared to uncloned viruses that are produced and titrated in PBMC. Moreover, molecular clones have greater stability and a precisely known sequence that facilitate GLP assay validation. Since most studies in the past have relied on uncloned viruses, it was necessary to establish the neutralization phenotype of molecular clones compared to uncloned parental viruses. This was done is a large series of assays with multiple strains of virus and multiple antibody specificities that included sera from HIV-1-infected individuals, sCD4 and monoclonal antibodies IgG1b12, 2G12, 2F5 and 4E10. Pseudoviruses were prepared from full-length gp160 derived from infected PBMC. Culture supernatants from these infected PBMC were harvested, filtered and stored in aliquots as the immediate uncloned parental virus **(Fig. 13)**.

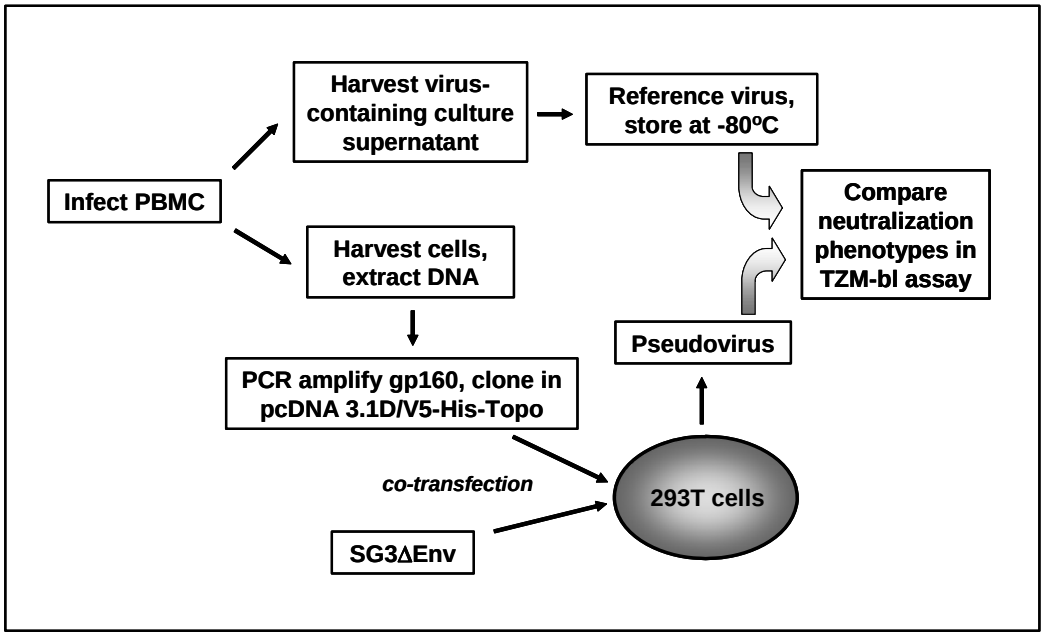

Figure 13. Construction of molecularly cloned pseudoviruses for comparison to uncloned parental viruses in neutralization assays.

Seventeen pseudoviruses and their immediate uncloned parental virus (12 clade B, 5 clade C) were assayed in TZM-bl cells as described in Duke SOP # HVTN02-A0009. All uncloned viruses were assayed in the presence of 1 μM indinavir to prevent replication. A summary of ID50 values is shown in **Figure 14**. In general, the pseudoviruses were more sensitive to neutralization compared to their parental uncloned virus. Difference in neutralization-sensitivity were most dramatic with monoclonal antibody 4E10. This monoclonal antibody possessed little or no detectable neutralizing activity against all 17 uncloned viruses (ID50 17 - >50 μg/ml) but was extremely potent against most pseudoviruses (all pseudoviruses neutralized; ID50 <1 μg/ml in 11/17 cases). Despite this dichotomy in neutralization-sensitivity, there were many cases where neither virus was neutralized by the HIV-1-positive serum samples. Since these serum samples all contained high titer neutralizing antibodies against T cell line adapted strains of HIV-1, we do not believe the pseudoviruses are unusually sensitive to neutralization. Rather, the uncloned PBMC-grown viruses appear to lack sensitivity in the TZM-bl assay. This sensitivity is regained by the use of molecularly cloned pseudoviruses that are produced by transfection in 293T cells. Results of this experiment have been published (Li et al., 2005).

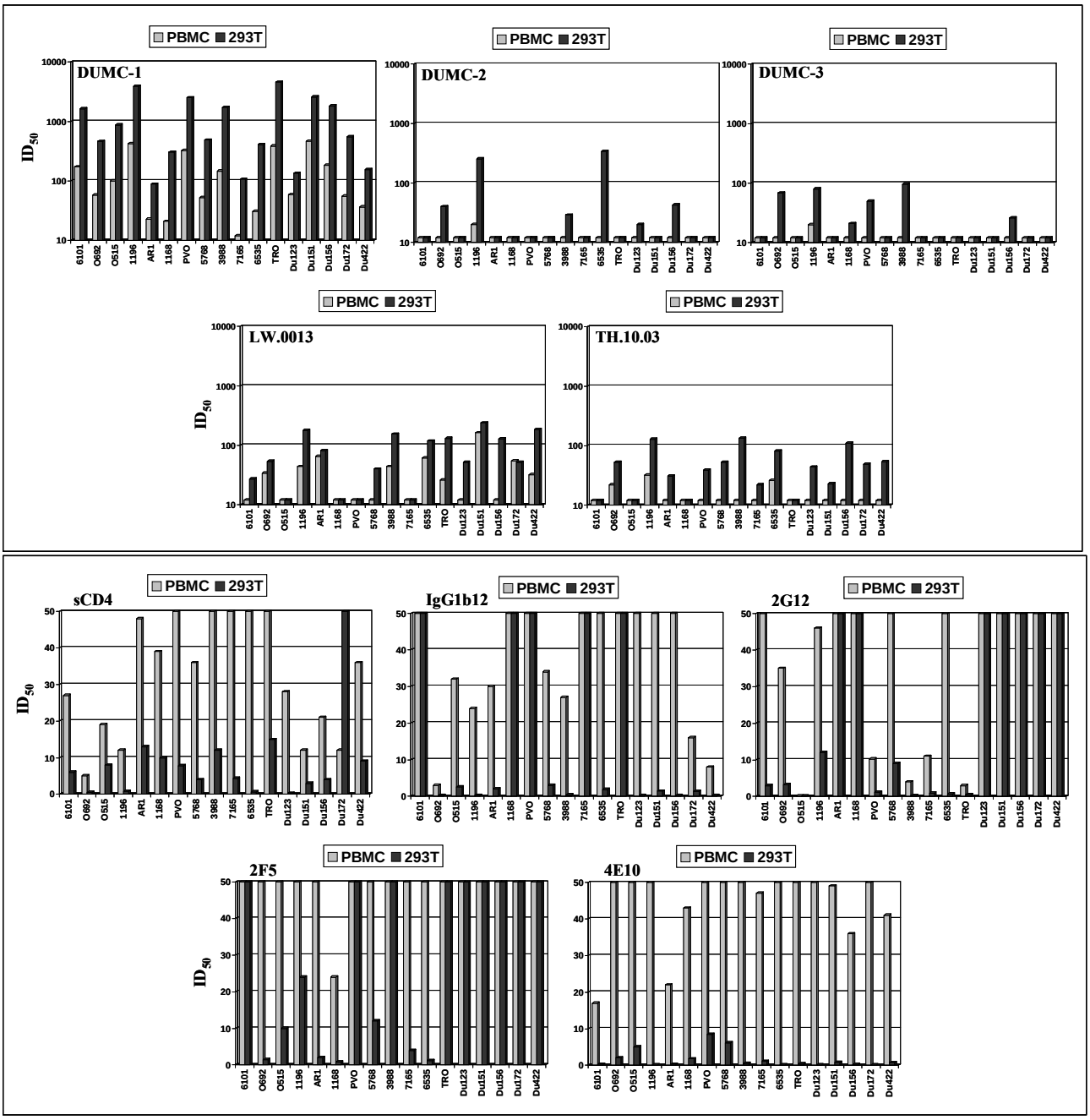

Figure 14. Neutralization assays comparing molecularly cloned pseudoviruses to parental PBMC viruses in TZM-bl cells. *Top panel: HIV-1-positive serum samples (clade B). Bottom panel: sCD4 and monoclonal antibodies.*

2.10 Confirmation of the need to use DEAE-dextran to achieve adequate levels of infection

We aimed to determine whether it was necessary to complicate the analyte medium by having DEAE-dextran present. Pseudovirus stocks must contain at least 4,000 TCID50/ml to achieve a 200 TCID50 inoculum in the assay (50 µl inoculum size). To conserve supplies, it is desirable that pseudovirus stocks contain at least 40,000 TCID50/ml. The infectivity of 12 Env-pseudotyped strains of HIV-1 was measured in TCID50 assays in the presence and absence of DEAE-dextran (30 µg/ml). As shown in **Table 4**, the presence of DEAE-dextran resulted in a substantial increase in TCID50 in every case. The mean increase was 82-times the TCID50 in the absence of DEAE dextran. An adequate level of infectivity for use in neutralization assays was seen in only 3 of 12 cases in the absence of DEAE-dextran. This compared to 10 of 12 pseudovirus stocks that had an adequate titer in the presence of DEAE dextran. The 3 pseudoviruses that had an adequate TCID50 in the absence of DEAE-dextran may be used to assess the effect of DEAE-dextran on neutralization.

Notably, TCID50 was unrelated to p24 Gag antigen concentration. The concentration of this antigen is therefore not useful for assay standardization purposes.

Table 4. TCID50 in the presence and absence of DEAE-dextran

			TCID50/ml		
Pseudovirus	**Lot**	**p24 (ng/ml)**	**-DEAE dextran**	**+DEAE dextran**	**Increase**
SS1196.1	ID#509	39	559	91,376	163x
6101.1	ID#503	175	6,250	91,376	15x
QH0692.42	ID#506	120	18,275	456,878	25x
PVO.4	ID#538	228	2,795	267,184	96x
5768.4	ID#524	172	146	2,795	19x
3988.25	ID#554	198	559	69,877	125x
6535.3	ID#552	393	22	3,655	166x
BG1168.1	ID#530	358	13,975	781,250	56x
TORNO.11	ID#584	164	559	53,437	96x
RHPA.7	ID#562	613	1,250	91,376	73x
REJO.67	ID#636	206	559	69,877	125x
THRO.18	ID#564	484	2,795	267,181	25x
					mean = 82x

2.11 Optimization of transfection in 293T/17 cells

A standard transfection protocol was used to compare the performance of various transfection kits: Effectene (QIAGEN), Polyfect (QIAGEN), SuperFect (QIAGEN), JetPEI (Polyplus) and FuGENE-6 (Roche). Each kit was used according to the manuufacturer's instructions to generate Env-pseudotyped viruses using the HIV-1 Env plasmid, ZM19584M.4, and the backbone plasmid, SG3Δenv. In some cases, multiple tests were performed to examine different concentrations of transfection reagent and plasmid DNA as recommended by the manufacturer. Based on TCID50 values, FuGENE-6 was the superior transfection kit **(Table 5)**.

Table 5. Comparison of different transfection reagent kits.

Transfection kit	Reagent (µl)	Env plasmid (µg)	Backbone plasmid (µg)	p24 (ng/ml)	TCID50 in TZM-bl cells
Effectene (QIAGEN)	4	0.125	0.275	50	69,877
Effectene (QIAGEN)	10	0.125	0.275	47	69,877
Effectene (QIAGEN)	20	0.125	0.275	53	53,437
Effectene (QIAGEN)	8	0.25	0.55	68	69,877
Effectene (QIAGEN)	20	0.25	0.55	62	53,437
Effectene (QIAGEN)	40	0.25	0.55	79	69,877
PolyFect (QIAGEN)	20	0.5	1.0	100	6,250
PolyFect (QIAGEN)	20	1.0	3.0	143	13,975
PolyFect (QIAGEN)	20	1.0	none	0	<50
PolyFect (QIAGEN)	20	none	1.0	387	<50
JetPEI (Polyplus)	2.4	1.0	2.0	0	<50
JetPEI (Polyplus)	4	1.0	2.0	0	<50
JetPEI (Polyplus)	6.4	1.0	2.0	43	6,250
JetPEI (Polyplus)	8	1.0	2.0	61	13,975
SuperFect (QIAGEN)	2	0.25	0.5	14	2,795
SuperFect (QIAGEN)	5	0.25	0.5	87	13,975
SuperFect (QIAGEN)	10	0.25	0.5	156	13,975
SuperFect (QIAGEN)	4	0.75	1.25	37	2,795
SuperFect (QIAGEN)	10	0.75	1.25	136	13,975
SuperFect (QIAGEN)	20	0.75	1.25	169	13,975
SuperFect (QIAGEN)	8	1.25	2.75	65	2,795
SuperFect (QIAGEN)	20	1.25	2.75	84	3,655
SuperFect (QIAGEN)	40	1.25	2.75	58	2,795
FuGENE-6 (Roche)	3	0.5	1.0	94	69,877
FuGENE-6 (Roche)	**6**	**0.5**	**1.0**	**201**	**267,184**
FuGENE-6 (Roche)	9	1.0	3.0	250	91,376

To further optimize the FuGENE-6 transfection protocol, we performed a series of tests in which different ratios and concentrations of plasmid DNAs used to make four Env-pseudotyped viruses: SF162.LS, 6535.3, WITO.33 and H077.31. Each pseudovirus was prepared using the pSG3Δenv backbone plasmid and tested for infectivity in TZM-bl cells. We discovered that optimium conditions vary depending on the Env plasmid **(Table 6)**. Thus, a 1:2 DNA ratio (4 µg Env : 8 µg SG3Δenv) worked well for 3 or 4 viruses tested but was suboptimal for SF162.LS, where a 4:1 ratio (8 µg Env : 2 µg pSG3Δenv) worked best.

Table 6. Optimization of the FuGENE-6 transfection protocol.

Env-pseudotyped virus	Env/backbone ratio	Plasmid/FuGENE ratio	Env plasmid (µg)	Backbone plasmid (µg)	Total plasmid (µg)	FuGENE (µl)	TCID50 in TZM-bl cells
SF162.LS	4:1	1:2.4	16	4	20	48	53,437
SF162.LS	3:1	1:3	12	4	16	48	69,877
SF162.LS	2:1	1:4	8	4	12	48	91,376
SF162.LS	1:1	1:4	6	6	12	48	13,975
SF162.LS	**1:2**	**1:4**	**4**	**8**	**12**	**48**	**13,975**
SF162.LS	1:3	1:4	3	9	12	48	13,975
SF162.LS	1:4	1:3.2	3	12	15	48	13,975
SF162.LS	2:1	1:2	16	8	24	48	349,386
SF162.LS	2:1	1:4	8	4	12	48	349,386
SF162.LS	2:1	1:8	4	2	6	48	349,386
SF162.LS	2:1	1:12	2	1	3	48	69,877
SF162.LS	2:1	1:32	1	0.5	1.5	48	3,655
SF162.LS	**4:1**	**1:4.8**	**8**	**2**	**10**	**48**	**456,878**
SF162.LS	8:1	1:5.3	8	1	9	48	69,877
SF162.LS	4:1	1:9.6	4	1	5	48	68,877
SF162.LS	8:1	1:10.6	4	0.5	4.5	48	18,275
SF162.LS	2:1	1:10	4	2	6	60	18,275
SF162.LS	2:1	1:8	4	2	6	48	31,250
SF162.LS	2:1	1:6	4	2	6	36	53,437
SF162.LS	2:1	1:3	4	2	6	18	53,437
SF162.LS	2:1	1:2	4	2	6	12	18,275
6535.3	**1:2**	**1:4**	**4**	**8**	**12**	**48**	**13,975**
6535.3	2:1	1:6	4	2	6	48	13,975
WITO.33	**1:2**	**1:4**	**4**	**8**	**12**	**48**	**91,376**
WITO.33	2:1	1:6	4	2	6	48	13,975
H077.31	**1:2**	**1:4**	**4**	**8**	**12**	**48**	**100,513**
H077.31	2:1	1:6	4	2	6	48	100,513
H077.31	4:1	1:5	8	2	10	48	76,865

3.0 ASSAY VALIDATION

Unless otherwise specified, the experiments described in this section were performed in TZM-bl cells as described in Duke SOP # HVTN02-A0009. Molecularly cloned pseudoviruses were prepared and titrated as described in Duke SOP # HVTN02-A0010. PBMC-grown virus stocks were prepared and titrated as described in Duke SOP # HVTN02-A0002. All serum samples were heat-inactivated at 56°C for 1 hour as described in Duke SOP # HVTN02-A0008.

3.1 SPECIFICITY

3.1.1 Nonspecific background activity of normal human serum

The specificity of an assay is its ability to assess unequivocally the analyte in the presence of components that might be expected to be present. The specificity of this assay may be affected by cellular toxicity and/or non-specific antiviral activity of normal serum components that produce false-positive artifacts. To determine the ability of the assay to discriminate between true neutralizing antibody activity and possible artifacts, the nonspecific background activity of normal human serum samples was assessed with multiple strains of virus. Serum samples from six healthy HIV-1-negative subjects were assayed against 18 strains of Env-pseudotyped HIV-1 **(Table 7)**. Serum samples were assayed at 3-fold dilutions of 1:20 to 1:43,740. Only 1 of 108 serum/virus combinations tested positive using 50% reduction in RLU as cut-off (ID50). The ID50 titer of this one positive test result was just above the lowest serum dilution tested. **Figure 15** ilustrates the background activity of the lowest serum dilutions tested (1:20). Considerable variation was seen between samples and viruses but, as mentioned above, the values rarely reached 50% reductions in RLU.

Table 7. Nonspecific activity of normal human serum samples.

				ID50 in TZM-bl Cells[1]					
Virus	**Clade**	**CoR**	**Source[2]**	**DUMC-C1**	**DUMC-C2**	**DUMC-C3**	**DUMC-C4**	**DUMC-C5**	**DUMC-C6**
6101.10	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
QH0692.42	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
QH0515.1	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
SS1196.1	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
AR1.29	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
BG1168.1	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
PVO.4	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
5768.4	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
3988.25	B	R5	ccPBMC	<20	<20	21	<20	<20	<20
7165.18	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
6535.3	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
TRO.11	B	R5	ccPBMC	<20	<20	<20	<20	<20	<20
RHPA.7	B	R5	Plasma	<20	<20	<20	<20	<20	<20
REJO.67	B	R5	Plasma	<20	<20	<20	<20	<20	<20
THRO.18	B	R5	Plasma	<20	<20	<20	<20	<20	<20
TRJO.58	B	R5	Plasma	<20	<20	<20	<20	<20	<20
SC42.8	B	R5	Plasma	<20	<20	<20	<20	<20	<20
WITO.33	B	R5	Plasma	<20	<20	<20	<20	<20	<20

[1]*Values are the serum dilution at which RLU were reduced 50% compared to virus control wells.*

[2]*ccPBMC, co-cultured PBMC; ucPBMC, uncultured PBMC. All viruses are molecularly cloned pseudoviruses producued by transfection in 293T cells.*

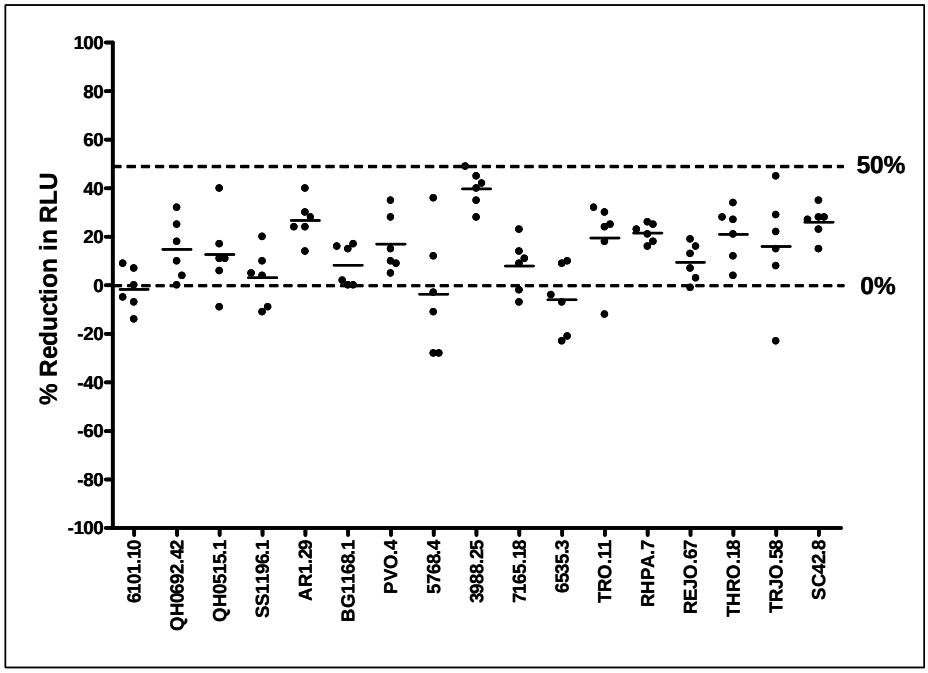

Figure 15. Nonspecific activity of normal human serum samples assayed at a 1:20 dilution.

We also tested the background activity of pre-immune serum samples from normal healthy uninfected subjects in HVTN 203. Serum samples were assayed at a 1:8 dilution in triplicate against four Env-pseudodtyped strains of HIV-1 and one uncloned strain (HIV-1 5768). The results are shown in **Figure 16**. The background activity again varied between samples and virus strain. Notably, greater background activity was seen in this experiment compared to the normal serum samples tested in **Figure 15**. In some cases the background ativity exceeded 50% reductions in RLU, especially in the case of pseudovirus QH0692.42.

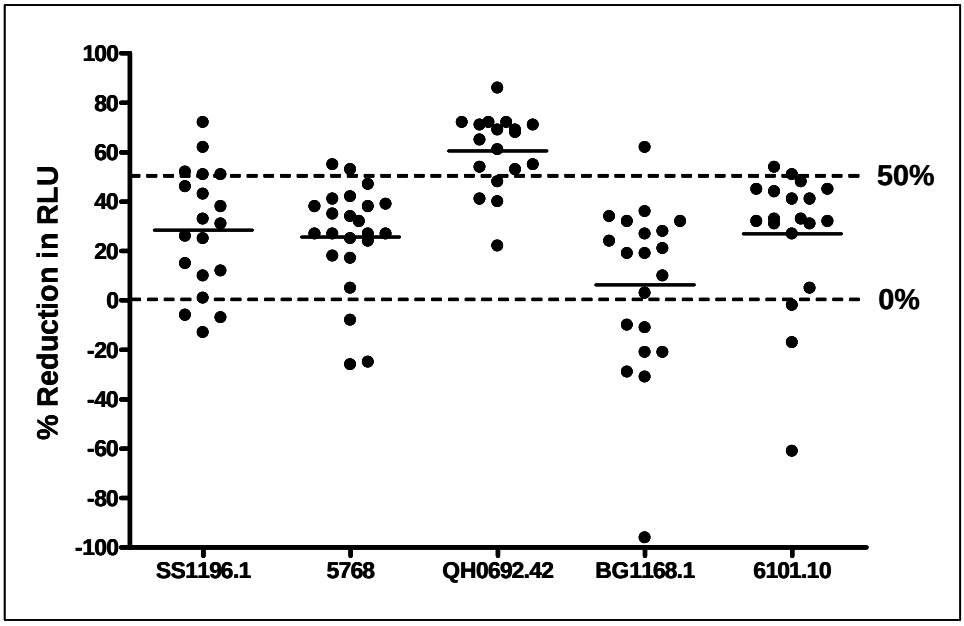

Figure 16. Nonspecific activity of 1:8-diluted pre-immune serum samples from HVTN 203.

Specificity was examined in greater detail by assaying five HIV-1-negative serum samples at dilutions as low as 1:4 against three strains of Env-pseudotyped viruses and one uncloned TCLA strain (HIV-1 MN). HIV-1 MN was assayed in the presence of 1 μM indinavir to prevent virus replication. TriMab was included as a positive control. As shown in **Figure 17**, little or no reduction in RLU was seen for any negative serum sample when assayed against three of the four viruses. Positive deflections ranging from 10-40% reductions in RLU were seen against the fourth virus (THRO.18) at low serum dilutions. In the case of the other three viruses, most negative serum samples exhibited a mild to moderate enhancement of infection. Infection-enhancement was most dramatic with HIV-1 MN but this enhancement does not appear to reduce the sensitivity of the assay for detecting neutralization compared to the MT-2 assay **(Table 8)**.

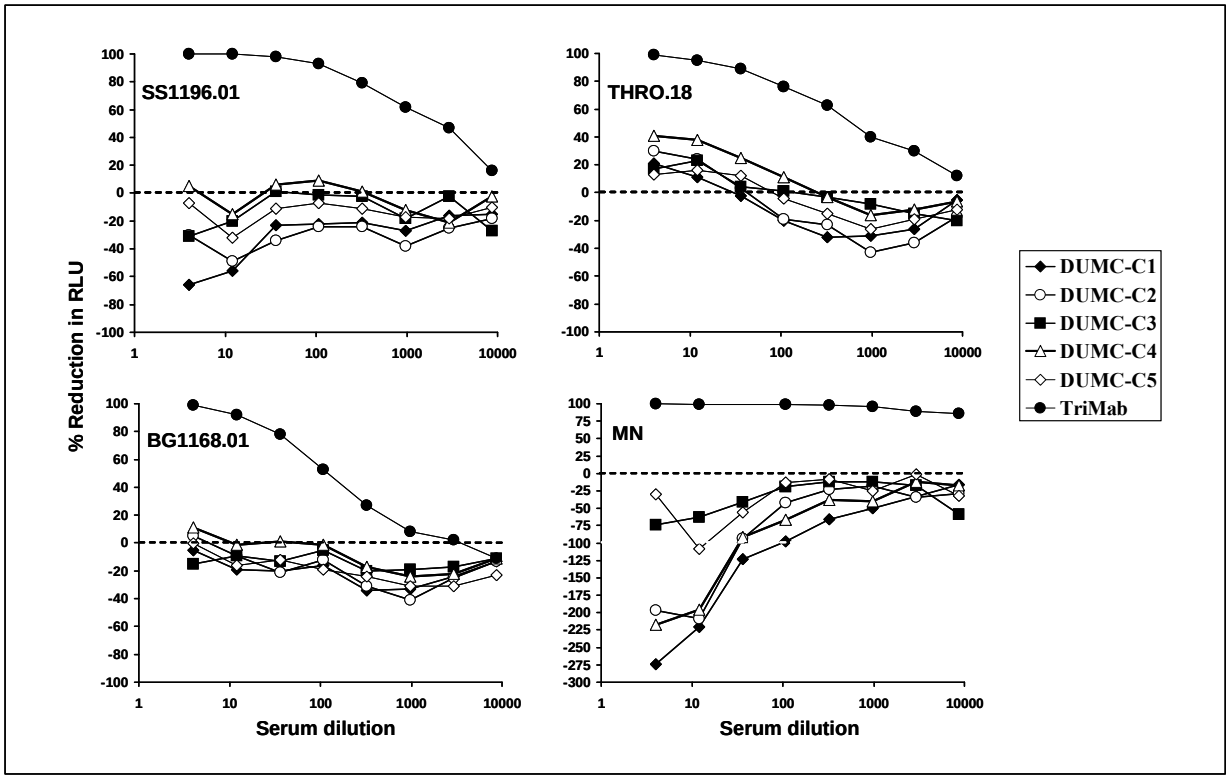

Figure 17. Nonspecific activity of normal human serum samples and TriMab. *Serum samples were assayed at multiple dilutions. TriMab was assayed at 0.06 - 125 μg/ml (values not shown in figure).*

We also assessed the specificity of the assay by using sera from recipients of a recombinant HIV-1 Env vaccine. Participants in this clinical trial (HVTN 039) received four inoculations of a recombinant canarypox vaccine (vCP1452) that expressed membrane anchored gp120 among other HIV-1 antigens and epitopes. Sera obtained pre-immunization (visit 2) and two weeks post final boosting (visit 8) were assayed against HIV-1 MN in TZM-bl cells the presence of indinavir. These same samples had been assayed previously with HIV-1 MN in an MT-2 cell-killing assay (Duke SOP # HVTN02-A0005). The virus in both cases was grown in H9 cells. As seen in **Table 6**, all pre-immune serum samples tested negative in both assays, whereas all post-immune samples tested positive. As evidence for very low background activity, no positive reductions in RLU were seen at a 1:10 dilution of pre-immune serum.

Table 8. Neutralization of HIV-1 MN in TZM-bl cells with sera from vaccine recipients.

		ID50 in MT-2	ID50 in TZM-bl[1]
Sample	**Visit**	**HIV-1 MN**	**HIV-1 MN**
DUMC-1	--	7,511	15,260
DUMC-2	--	1,815	8,083
DUMC-3	--	387	1,541
LW-0013	--	1,462	5,972
TH-10-03	--	3,831	11,325
TriMab	--	0.01 µg/ml	0.02 µg/ml
039-A	8	283	401
039-B	8	326	736
039-C	8	414	676
039-D	8	324	3,787
039-E	8	265	6,520
039-F	8	220	952
039-G	8	231	1,820
039-H	8	446	2,547
039-I	8	489	3,192
039-J	8	276	4,833
039-K	8	587	16,722
039-L	8	366	4,438
039-M	8	253	2,005
039-N	8	275	1,520
039-A	2	<10	<10 (-83)
039-B	2	<10	<10 (-91)
039-E	2	<10	<10 (-48)
039-G	2	<10	<10 (-19)
039-H	2	<10	<10 (-27)
039-I	2	<10	<10 (-18)
039-J	2	<10	<10 (-9)
039-L	2	<10	<10 (-16)
039-M	2	<10	<10 (1)
039-N	2	<10	<10 (-14)

[1]*Values in parentheses are the % reduction in RLU at a 1:10 dilution of serum sample. Negative values in parentheses indicate mild infection-enhancement (i.e., RLU were higher than virus control wells).*

Overall, the results in **Figures 15-17** and **Tables 7 and 8** indicate that use of ID50 is a specific measurement of neutralization at serum dilutions ≥1:10 and at lower serum dilutions in many cases. Due to variation between serum samples and virus strains, specificity should be re-examined in each study by including corresponding pre-immune samples as controls.

3.1.2 Effect of heat-inactivation and the use of serum vs plasma

Heat-inactivation is a routine practice that is applied when preparing serum samples for neutralization assays. Heat-inactivation is needed to destroy complement, which might otherwise enhance virus infection in cells that express appropriate complement receptors. The infection-enhancing effect of complement is facilitated by the presence of Env-specific antibodies and may mask the activity of neutralizing antibodies. TZM-bl cells are not known to express complement receptors and, therefore, might not be susceptible to these complement effects. Nonetheless, if heat-inactivation is used, it is necessary to establish the effect it has on neutralizing activity of serum samples as measured in the TZM-bl assay. We assessed the neutralizing activity of serum samples from HIV-1-infected individuals before and after heat-inactivation at 56°C for 1 hour (Duke SOP #HVTN02-A0008). These serum samples were collected from whole blood that was allowed to coagulate overnight at 4°C. Serum was collected and stored at -80°C. The

serum samples were thawed and a portion heat-inactivated prior to assay. As shown in **Table 9**, a minor decrease in neutralizing activity was detected after heat-inactivation in each case examined (16% - 28%). This could be due to either a minor enhancing effect of complement under non-heat-inactivated conditions or a small loss in antibody stability under conditions of heat-inactivation. Effects were minor and should have little or no significant impact on the specificity of the assay.

Table 9. Effect of heat-inactivation on serum neutralizing antibody activity.

	ID50 against SS1196.1		
Serum Sample	**Heat-inactivated**	**Non-heat-inactivated**	**Change**
DUMC-2	1,267	1,062	16 %
DUMC-3	163	127	22 %
LW.0013	599	431	28 %
TH.10.03	353	268	24 %

We also assessed the effect of heat-inactivation on serum and plasma samples from 6 healthy uninfected subjects who received a pentavalent HIV-1 gp120 vaccine (U. Mass clinical trial DP6-001). As shown in **Figure 18**, neutralizing activities were compared between fresh and heat-inactvated serum and plasma against two strains of HIV-1 in TZM-bl cells (SOP HVTN02-A0009). Greatest sensitivity and specificity were achieved with heat-inactivated serum. Of note, background levels of nonspecific activity were elevated with fresh and heat-inactivated plasma compared to fresh or heat-inactivated serum. Heat-inactivated plasma has less background than fresh plasma.

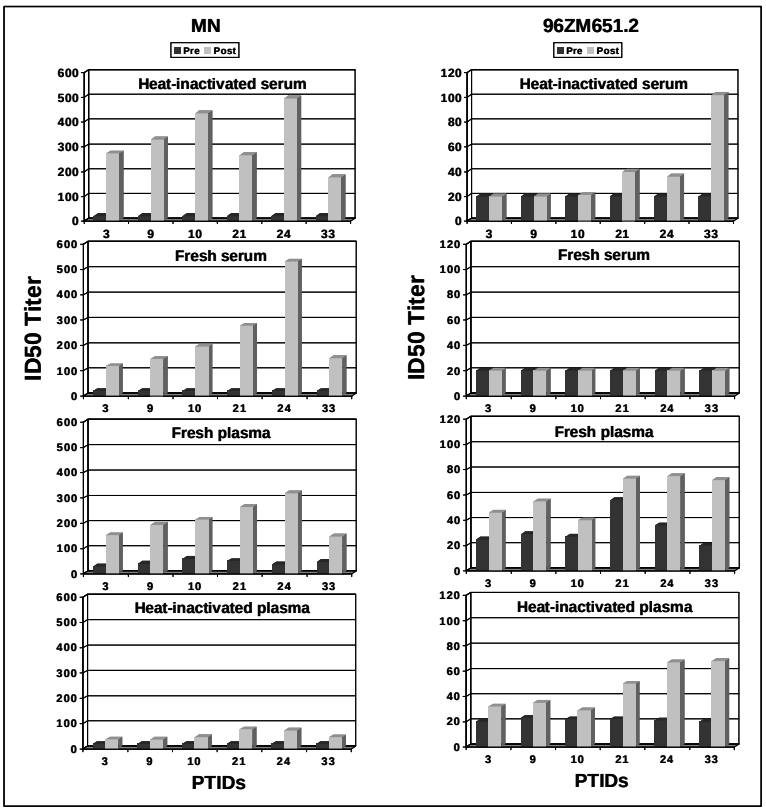

Figure 18. Neutralizing activity of fresh serum, heat-inactivated serum and fresh plasma.

3.1.3 Effect of DEAE dextran

It is necessary to supplement the assay medium with the polycation, DEAE dextran, in order to achieve adequate levels of infection. The possibility that DEAE-dextran might alter the activity of neutralizing antibodies was examined by performing neutralization assays with two pseudoviruses that exhibit adequate infectivity in the absence of the polycation. In this experiment, sCD4, IgG1b12, 2G12, 2F5, 4E10, TriMab and serum samples from 5 HIV-1-infected subjects were assayed against pseudoviruses BG1168.1 and QH0692.42 in the presence and absence of 30 μg/ml DEAE-dextran. As shown in **Table 10**, minor differences in assay sensitivity were seen in most cases. In no case was there a significant loss of sensitivity when DEAE-dextran was present. The largest difference was a 340% increase in sensitivity in the presence of DEAE-dextran when HIV-1-positive serum DUMC-1 was tested against pseudovirus QH0692.42. All remaining comparisons resulted in differences <200%. It may be concluded that DEAE-dextran has little effect on the neutralizing activity of many different antibody specificities.

Table 10. Effect of DEAE-dextran.

	ID50 against BG1168.1			ID50 against QH0692.42		
Serum Sample	**+DEAE dextran**	**-DEAE dextran**	**Change**	**+DEAE dextran**	**-DEAE dextran**	**Change**
DUMC-1	305	127	140%	462	603	23%
DUMC-2	<20	<20	0%	40	<20	≥ 100%
DUMC-3	21	<20	≥ 1%	68	<20	≥ 340%
LW.0013	<20	<20	0%	54	57	5%
TH.10.03	<20	<20	0%	53	33	38%
sCD4	10.0 μg/ml	22.7 μg/ml	127%	0.5 μg/ml	1.0 μg/ml	100%
IgG1b12	>50 μg/ml	>50 μg/ml	0%	0.3 μg/ml	0.3 μg/ml	0%
2G12	>50 μg/ml	>50 μg/ml	0%	3.2 μg/ml	2.7 μg/ml	16%
2F5	0.8 μg/ml	1.7 μg/ml	113%	1.5 μg/ml	1.2 μg/ml	20%
4E10	1.8 μg/ml	1.5 μg/ml	17%	2.1 μg/ml	1.8 μg/ml	14%
TriMab	2.4 μg/ml	5.7 μg/ml	138%	0.3 μg/ml	0.6 μg/ml	100%

3.1.4 Lack of residual infectivity of the SG3Δenv backbone plasmid

Our SOP for Env-pseudotyped virus production calls for the use of a presumed Env-defective backbone plasmid, designated SG3Δenv. Although this plasmid should be incapable of producing infectious virus, the fact that only a portion of *env* is deleted in the construct raises the possibility that a mutated form of Env might give rise to residual infectivity. Residual amounts of infectious Env-mutant virus could complicate the interpretation of neutralization assays, as it would represent an unspecifiied target for neutralization. We therefore conducted an experiment in which 293T/17 cells were transfected with the backbone vector alone according to our standard protocol. The transfection generated high concentrations of p24 Gag antigen in the culture supernatants as evidence that transfection was effecient. When these supernatants were tested for infectivity, no luminescence was present in TZM-bl cells after 2 and 3 days of infection. Thus, under our standard conditions, the SG3Δenv backbone plasmid by itself does not produce detectable levels of infectious virus in the absence of an Env-expressing helper plasmid.

3.1.5 Use of an RT-resistant backbone vector to circumvent antiretroviral drugs in serum and plasma

Studies of patients who are taking antiretroviral drugs are complicated by the presence of residual amounts of the drugs in serum and plasma samples that can inhibit the virus and be mistaken for the presence of neutralizing antibodies. This is a major concern for nucleoside and non-nucleoside reverse transcriptase (RT) inhibitors because they can prevent Tat-induced Luce reporter gene expression in TZM-bl cells. A third class of antiretroviral drugs, the protease inhibitors, act at a late stage of viral assembly and thus do not interfer with Luc reporter gene expression in a single cycle infection assay. One way to circumvent the activity of RT inhibitors would be to utilize an RT-resistant backbone vector when making Env-pseudotyped viruses. We investigated one such backbone vector that, in addition to carrying a deletion that inactivated Env, additional mutations were introduced in the Pol gene that confer resistance to nucleoside and non-nucleoside reverse transcriptase inhibitors. This backbone vector, 1617RT/K103N, was obtained from Dr. Ronald Swanstrom (UNC-Chapel Hill) and was built using the HIV-1 NL4-3 backbone vector (accession number AF003887) in which a short *pol* gene fragment containing multiple nucleoside RT inhibitor resistance mutations (fragment 1617-1, accession number AY351767) was used to replace the corresponding region of NL4-3. In addition, an amino acid substitution (K103N) was introduced to confer resistance to non-nucleoside RT inhibitors. The parental virus was isolated prior to the introduction of antiretroviral therapy (J. Acquir. Immune defic. Syndr. Hum. Retrovirol. 12: 352-357, 1996). The 1617RT/K103N backbone vector was used to generate a MLV Env-pseudotyped virus that was subsequently tested with serum samples from individuals who were on standard antiretroviral therapy. As a control, the same MLV Env plasmid was used to generate pseudovirus with our standard pSG3Δenv backbone plasmid. Because the pseudoviruses contain an irrelevant MLV Env, neutralizing antibodies in sera from HIV-1-infected individuals should have no activity. Any activity against this pseudovirus would be attributed to the antiretroviral drugs.

As shown in Fig. 18.1, each serum sample exhibited potent antiviral activity against MLV Env-pseudotyped virus built with the SG3Δenv backbone (open bars), demonstrating how serious a problem the drugs are for specific measurements of neutralizing antibdies. Antiviral activity was greatly diminished but not entirely eliminated with the use of the RT-resistant backbone plasmid (solid bars). Additional improvements will be needed to increase the RT resistance of the 1617RT/K103N backbone plasmid before it is acceptable for neutralizing antibody assays with serum and plasma from patients on ART.

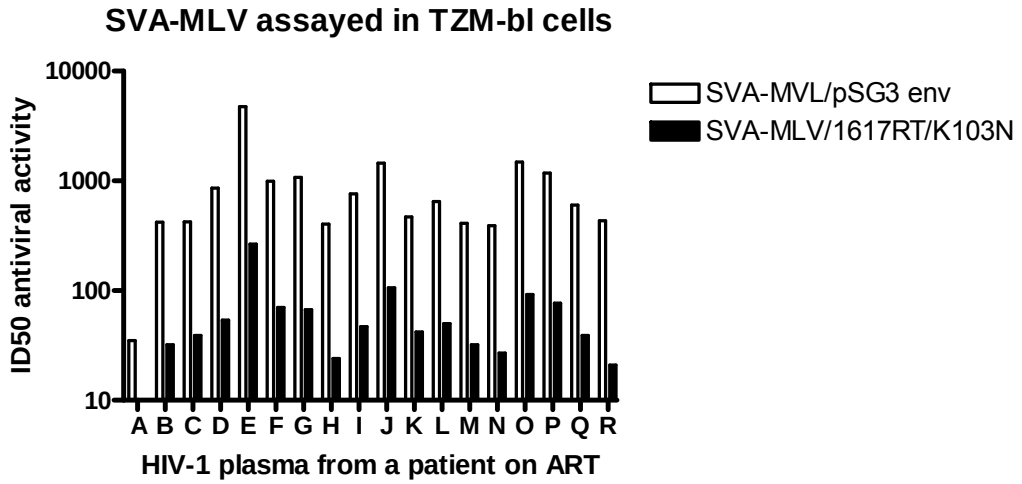

Figure 18.1. Antiviral activity of ART plasma samples againstvirus speudotyped with murine leukemia virus env

3.2 ACCURACY

Efforts to assess the accuracy of HIV-1 neutralizing antibody assays are hindered by the absence of gold standard reference reagents. In the absence of these reagents, we have compared the performance of the TZM-bl assay to the performance of other assays conducted in our laboratory and in other laboratories.

3.2.1 Comparison to the 5.25.EGFP.Luc.M7 assay using TriMab as a standard

Performance of the TZM-bl assay was compared to the performance of a similar Luc reporter gene assay in 5.25.EGFP.Luc.M7 cells. This latter assay is based on multiple rounds of replication of PBMC-grown stocks of HIV-1 (Duke SOP # HVTN02-A0006). The 5.25.EGFP.Luc.M7 assay performs comparably to other assays used in different laboratories, including PBMC assays and a single-round infection assay with pseudotyped viruses (manuscript in preparation).

In this experiment, TriMab was assayed against seven different strains of virus. TriMab is a serologic reagent that is comprised of equal concentrations of the three human monoclonal antibodies IgG1b12, 2G12 and 2F5. TriMab was assayed at least 3 times against each strain of virus in each assay. Results were fairly uniform, agreeing within 3-fold in 4 of 5 cases **(Table 11)**. In the fifth case, the TZM-bl assay was 8-times more sensitive in detecting neutralization of strain SS1196.

Table 11. Neutralization potency of TriMab in TZM-bl compared to 5.25.EGFP.Luc.M7 cells.

Virus	Clade	Mean ID50 in TZM-bl cells[1] (μg/ml)	Mean ID50 in M7-Luc cells[2] (μg/ml)	Fold difference
6101	B	9.6 ± 3.5 (n= 3)	4.1 ± 1.9 (n= 8)	2.3
QH0692	B	0.3 ± 0.1 (n= 6)	0.8 ± 0.2 (n= 9)	2.7
SS1196	B	0.5 ± 0.1 (n= 6)	4.3 ± 3.1 (n= 3)	8.6
BG1168	B	3.6 ± 1.2 (n= 5)	2.8 ± 1.1 (n= 4)	1.3
Pavo	B	4.1 ± 1.4 (n= 5)	1.8 ± 0.8 (n= 4)	2.3

[1]*Assays in TZM-bl cells were performed with molecularly cloned pseudoviruses produced by transfection in 293T cells.*

[2]*Assays in 5.25.EGFP.Luc.M7 cells were performed with replication competent viruses produced in human PBMC.*

3.2.2 Comparison to the ViroLogic and PBMC assays using 2G12 and 2F5 as standards

In this experiment, monoclonal antibodies 2G12 and 2F5 were assayed against eight different strains of clade B HIV-1 in three different assays. The Virologics assay (Richman et al., 2003) utilized pseudoviruses prepared by ViroLogic by using PBMC-grown stocks of viruses provided by Dr. Montefiori; pseudoviruses were prepared by transfection in 293T cells using molecular quasispecies plasmid DNA and a Luc-containing backbone plasmid. The PBMC assay utilized PBMC-grown stocks of virus and was performed in Dr. Montefiori's laboratory as described in Duke SOP # HVTN02-A0003. The TZM-bl assay was performed in Dr. Montefiori's laboratory as described in Duke SOP # HVTN02-A0009) and utilized molecularly cloned pseudoviruses prepared by transfection in 293T cells (Duke SOP # HVNT-A0010). As shown in **Figure 19**, relative potencies were similar in all three assays. In particular, the TZM-bl and Virologic assays exhibited striking equivalency in 14 of 16 cases, where the titers in each assay were within 2-fold agreement. Both assays were more sensitive than the PBMC assay. This difference in sensitivity might be related to the ID50 used for TZM-bl and ViroLogic assays compared to ID80 used for the PBMC assay. Because of greater variation in the PBMC assay, an ID50 was not recommended.

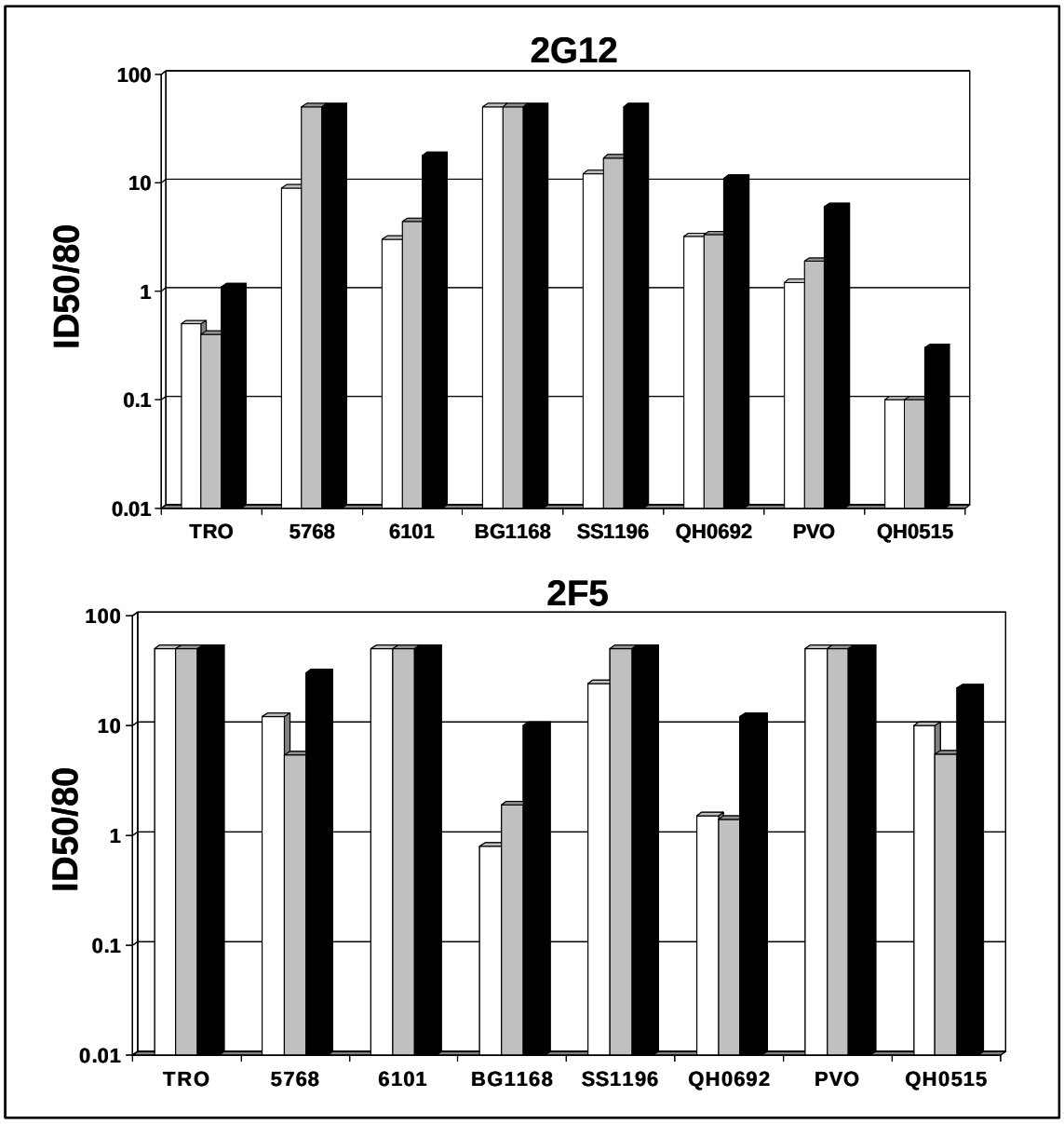

Figure 19. Comparison of the TZM-bl assay to the ViroLogic and PBMC assays using 2G12 and 2F5 as standards. *Values on the Y-axis are μg/ml. An ID50 was used for the TZM-bl and ViroLogic assay. An ID80 was used for the PBMC assay. TZM-bl assay (white); ViroLogic assay (grey); PBMC assay (black).*

3.2.3 Comparison to the 5.25.EGFP.Luc.M7 assay using serum samples from vaccinated rabbits and macaques

Serum samples from SF162 Env-immunized rabbits and macaques (Chiron) were assayed against uncloned PBMC-grown SF162 in M7-Luc cells as part of routine end-point assays performed for the Chiron preclinical HIV-1 vaccine development program. These same samples were later assayed against molecularly cloned SF162.LS pseudovirus in TZM-bl cells. As shown in **Figure 20**, results were similar in both assays. Neutralization titers for the rabbit serum samples were uniformly higher in the TZM-bl assay (average 2.5x higher, range 0.1 - 4.4x higher). Neutralization titers for the macaque serum samples were nearly identical in both assays for each serum sample (all differences ≤ 2x). These results indicate that the TZM-bl and M7-Luc assays perform similarly.

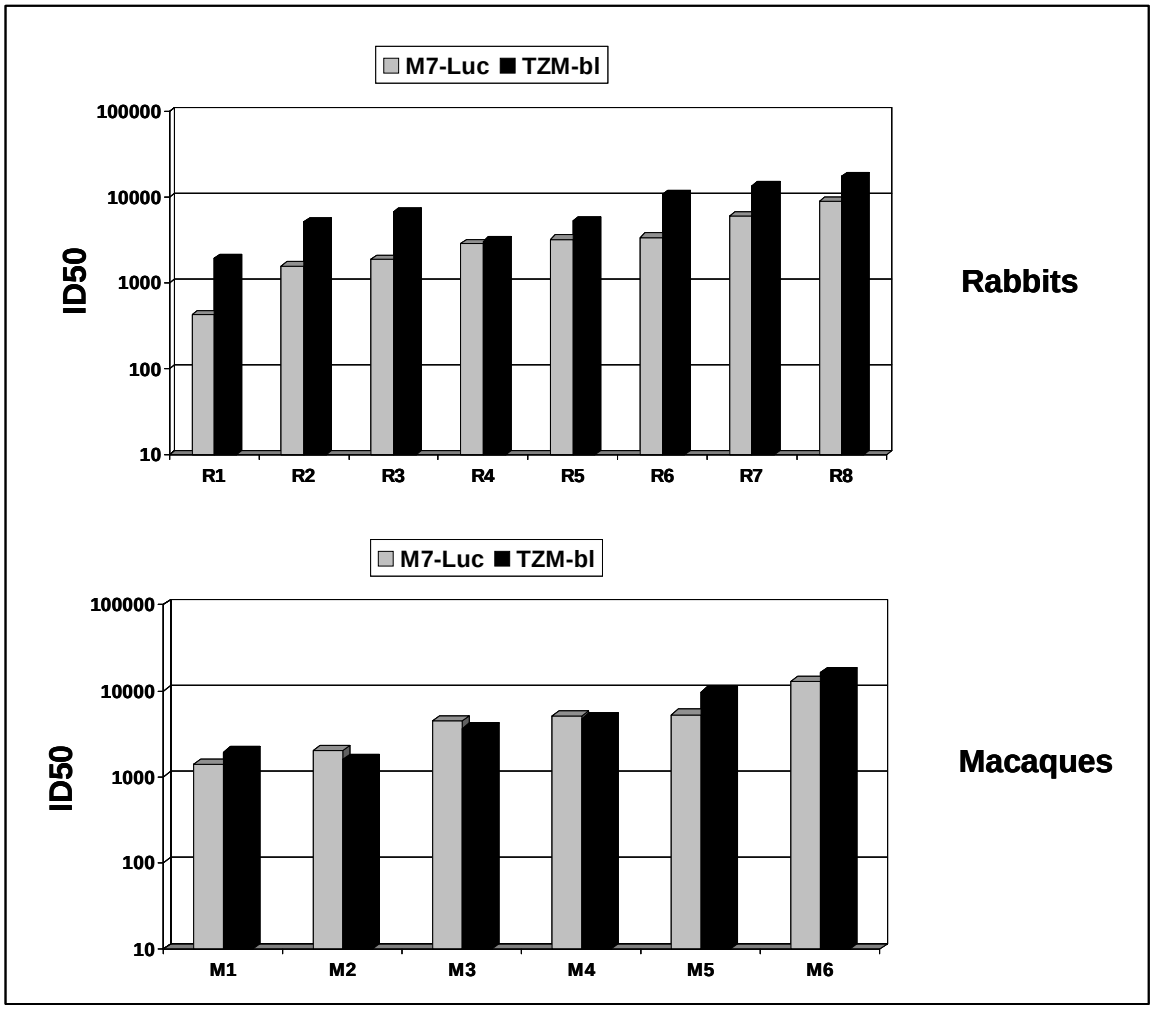

Figure 20. Comparison of the TZM-bl assay to the M7-Luc assay using sera from Env-immunized animals.

3.3 PRECISION

3.3.1 Well-to-well variability in a single assay

As a representation of intra-assay variation, standard deviations of triplicate values for each dilution of an HIV-1-positive serum sample (DUMC-1) assayed against three different Env-pseudotyped strain of HIV-1 are shown in **Figure 21**. The range of standard deviations was 0 - 11% of experimental values. The average standard deviation was <5% of experimental values. Data points with no error bars are cases where the standard deviation was ≤3 (error bar is too small to be visible in the figure).

Examination of large data sets has revealed that standard deviations increase as % reductions in RLU decrease (i.e., standard deviations are greatest at low neutralization potencies). Standard deviations rarely exceed 8% in the linear portion of the neutralization curve.

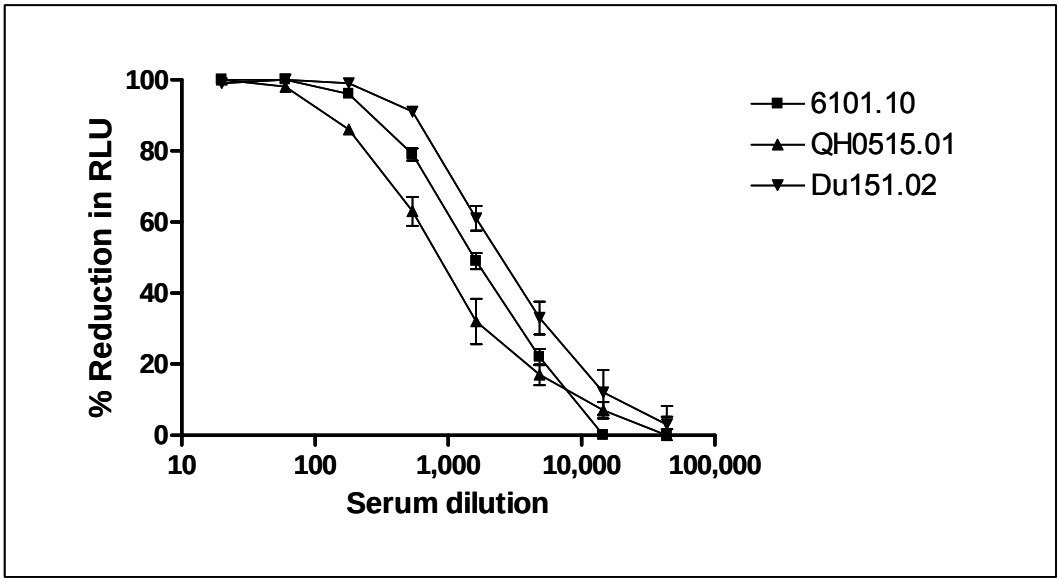

Figure 21. Neutralization curves with standard deviation bars. *HIV-1-positive serum DUMC-1 was assayed against molecularly cloned pseudoviruses 6101.10, QH0515.01 and Du151.02 in triplicate wells for each dilution of serum sample.*

3.3.2 Intra-operator variability

Intra-operator variability was examined by assaying serum samples from three vaccinated individuals and one HIV-1-infected individual multiple times on different days by the same operator. In this experiment, samples were assayed with an uncloned stock of W61D-TCLA produced in SupT1 cells. This strain of HIV-1 exhibited an unacceptable level of variation on other assays and, as such, should provide a stringent test of variability in the TZM-bl assay. Indinavir (1 μM) was added to the medium to prevent virus replication. Serum samples from vaccinees came from healthy, non-infected subjects in HVTN 041. These subjects received three inoculations of NefTat and $gp120_{W61D}$ protein in AS02A adjuvant (MPL, QS-21 and an oil-water emulsion). The serum samples were obtained two-weeks post final boosting (visit 7). All serum samples were assayed in serial 3-fold dilutions ranging from 1:20 to 1:43,740.

As shown in **Table 12**, each serum sample was assayed at least 8 times on a different date by the same operator. A high level of concordance was achieved in each case, ranging from 1 - 109% variation from the mean. Mean variation ranged from 20 - 43%.

Table 12. Intra-operator variability in repeat neutralization assays with sera from vaccinated subjects and an HIV-1-infected individual.

Vaccinated subject	Experiment Number	Assay Date	ID50	Variation from the mean
041-A	AG15-41	07/14/04	3,853	109%
	AG15-45	07/22/04	6,587	22%
	AG15-46	07/23/04	7,848	3%
	AG15-47	07/28/04	9,275	13%
	AG15-48	07/29/04	9,165	12%
	AG15-49	07/30/04	9,234	13%
	AG15-50	08/04/04	9,081	11%
	AG15-52	08/05/04	12,409	54%
	AG15-53	08/06/04	6,113	24%
	AG15-54	08/11/04	6,230	23%
	AG15-55	08/12/04	8,907	9%
Mean:			**8,064 ± 2,276**	**27% ± 30%**
041-B	AG15-41	07/14/04	3,614	109%
	AG15-45	0722/04	6,180	22%
	AG15-46	07/23/04	5,871	29%
	AG15-47	07/28/04	9,628	27%
	AG15-48	07/29/04	14,273	89%
	AG15-49	07/30/04	8,611	14%
	AG15-50	08/04/04	8,405	11%
	AG15-52	08/05/04	8,939	18%
	AG15-53	08/06/04	6,372	19%
	AG15-54	08/11/04	5,347	41%
	AG15-55	08/12/04	5,957	27%
Mean:			**7,563 ± 2,868**	**37% ± 32%**
041-C	AG15-42	07/15/04	3,846	50%
	AG15-45	07/22/04	4,276	35%
	AG15-46	07/23/04	5,144	12%
	AG15-47	07/28/04	7,228	25%
	AG15-48	07/29/04	7,780	35%
	AG15-49	07/30/04	5,832	1%
	AG15-50	08/04/04	6,713	16%
	AG15-52	08/05/04	5,597	3%
	AG15-53	08/06/04	6,273	9%
	AG15-54	08/11/04	4,580	26%
	AG15-55	08/12/04	6,237	8%
Mean:			**5,773 ± 1,237**	**20% ± 16%**
HIV-1-Positive Serum	**Experiment Number**	**Assay Date**	**ID50**	**Variation from the mean**
DUMC-3	AG15-47	07/28/04	7,016	59%
	AG15-48	07/29/04	4,834	9%
	AG15-49	07/30/04	3,450	28%
	AG15-50	08/04/04	7,563	71%
	AG15-52	08/05/04	2,920	51%
	AG15-53	08/06/04	4,060	9%
	AG15-54	08/11/04	2,782	59%
	AG15-55	08/12/04	2,754	61%
Mean:			**4,422 ± 1,911**	**43% ± 25%**

3.3.3 Inter-assay and inter-operator variability

Inter-assay variation was assessed by assaying a positive control reagent (TriMab) multiple times by different operators against different strains of Env-pseudotyped viruses. Each assay was performed on a separate date as the positive control for test samples in independent experiments. Results are shown in **Figure 22**. Mean ID50 values were 0.56 µg/ml (pseudovirus 3988.25), 0.36 µg/ml (pseudovirus QH0692.42), 0.51 µg/ml (pseudovirus SS1196.01) and 3.88 µg/ml (pseudovirus BG1168.01). ID50 values of replicate assays agreed within 2-fold of these means in each case. Standard deviations for triplicate wells of single TriMab concentrations did not exceed 17% of the mean.

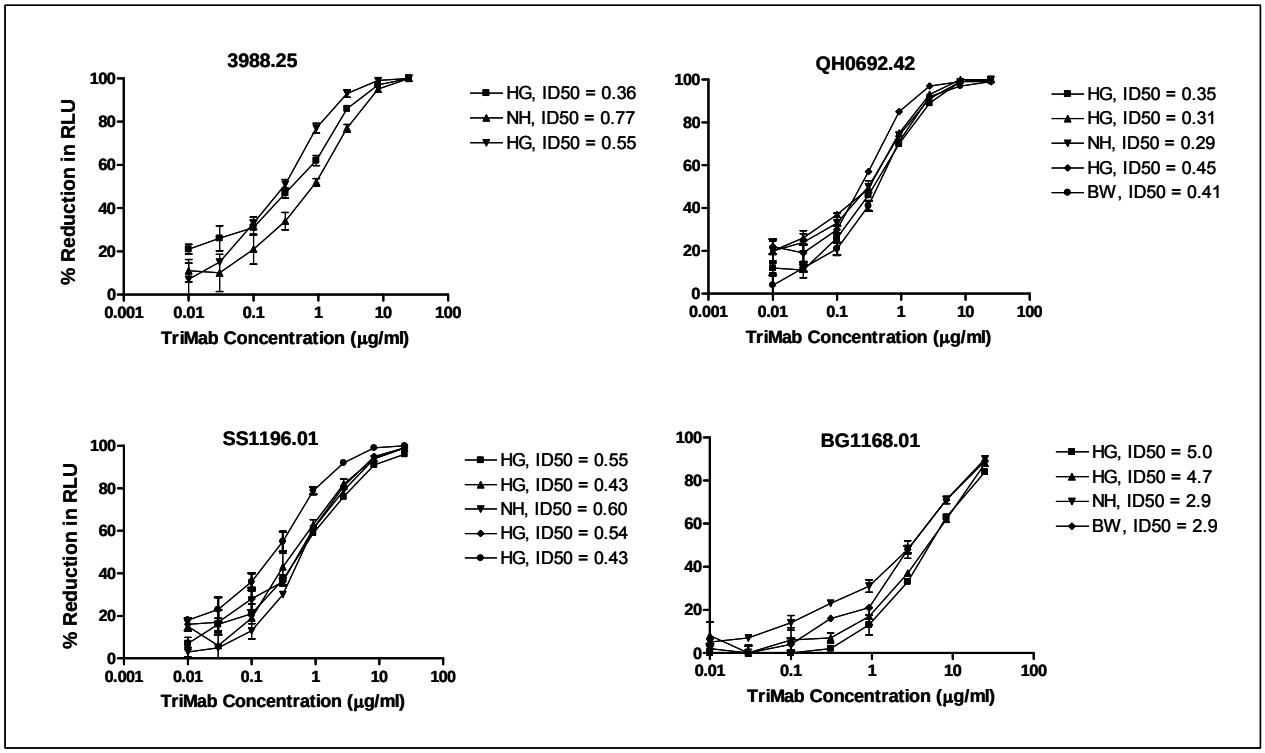

Figure 22. Inter-assay and inter-operator variability of TriMab. *TriMab was assayed multiple times against pseudoviruses 3988.25, QH0692.42, SS1196.01 and BG1168.01. ID50 values of each curve are shown next to the operator who performed the assay. Each operator is identified by their initials (HG, NH, BW).*

As an additional example of inter-operator variability, Env-pseudotyped strain Du152.02 was assayed with sera from HIV-1-infected individuals, sCD4, four monoclonal antibodies and TriMab at different times by two different operators. The results are expressed as ID50 values in **Table 13**. A high level of concordance was achieved between operators. Only one test was discordant (8-fold difference for monoclonal antibody 4E10). All other repeat assays agreed within 2-fold.

Table 13. Neutralization of Du151.2 in TZM-bl cells with sera from HIV-1-infected individuals, sCD4 and MAbs.

	ID50 in TZM-bl Cells		
Reagent	**Operator 1**	**Operator 2**	**Fold-difference**
DUMC-1	2,555	2,821	1.1
DUMC-2	<20	<20	n/a[1]
DUMC-3	<20	<20	n/a
LW.0013	238	367	1.5
TH.10.03	23	33	1.4
Pool A	347	346	1.0
Pool B	518	333	1.6
Pool C	527	825	1.6
Pool D	123	198	1.6
sCD4	3.0	1.8	1.7
IgG1b12	1.4	1.5	1.1
2G12	>50	>50	n/a
2F5	>50	>50	n/a
4E10	0.8	0.1	8.0
TriMab	6.2	4.3	1.4

[1]*n/a, not applicable.*

Inter-operator variability also was examined with sCD4 and monoclonal antibodies, which were assayed against 12 strains of Env-pseudotyped virus by two independent operators approximately 6 month apart. As shown in **Table 14**, total variation in ID50 values ranged from 1.0x to 5.5x differences between the two sets of assays. The average variation was ≤2.4x differences for sCD4 and each monoclonal antibody. We can conclude from these combined results that the normal variation in the assay with sCD4, monoclonal antibodies and HIV-1-positive serum samples is <3x differences in values.

Table 14. Reproducibility of assays with sCD4 and MAbs.

	ID50 in TZM-bl Cells (µg/ml)														
Virus	**sCD4 test 1**	**sCD4 test 2**	**sCD4 variation**	**IgG1b12 test 1**	**IgG1b12 test 2**	**IgG1b12 variation**	**2G12 test 1**	**2G12 test 2**	**2G12 variation**	**2F5 test 1**	**2F5 test 2**	**2F5 variation**	**4E10 test 1**	**4E10 test 2**	**4E10 variation**
6535.3	0.6	1.0	**1.7x**	1.9	0.9	**2.1x**	0.6	3.3	**5.5x**	1.2	2.5	**2.1x**	0.1	0.3	**3.0x**
QH0692.42	0.5	0.4	**1.3x**	0.3	0.2	**1.5x**	3.2	2.3	**1.4x**	1.5	0.4	**3.8x**	2.1	0.6	**3.5x**
SC422661.8	7.3	2.1	**3.5x**	0.2	0.1	**2.0x**	2.6	1.6	**1.6x**	1.0	0.4	**2.5x**	0.9	0.8	**1.1x**
PVO.4	7.8	5.6	**1.4x**	>50	>50	**n/a**	1.2	1.1	**1.1x**	>50	>50	**n/a**	8.5	4.4	**1.9x**
TRO.11	15.0	8.0	**1.9x**	>50	>50	**n/a**	0.5	0.2	**2.5x**	>50	>50	**n/a**	0.4	0.2	**2.0x**
AC10.0.29	13.0	4.0	**3.3x**	2.0	1.8	**1.1x**	>50	>50	**n/a**	2.0	0.5	**4.0x**	0.3	0.2	**1.5x**
RHPA4259.7	2.2	1.3	**1.7x**	0.1	0.1	**1.0x**	>50	>50	**n/a**	9.9	14.1	**1.4x**	8.3	5.5	**1.5x**
THRO4156.18	0.3	0.3	**1.0x**	0.4	0.6	**1.5x**	>50	>50	**n/a**	>50	>50	**n/a**	0.2	0.4	**2.0x**
REJO4541.67	0.3	0.8	**2.7x**	0.7	0.7	**1.0x**	>50	>50	**n/a**	0.2	1.0	**2.0x**	0.4	1.1	**2.8x**
TRJO4551.58	17.7	22.6	**1.3x**	>50	>50	**n/a**	>50	>50	**n/a**	>50	>50	**n/a**	4.1	4.8	**1.2x**
WITO4160.33	5.5	5.2	**1.1x**	2.9	3.4	**1.2x**	1.0	1.2	**1.1x**	0.6	0.7	**1.2x**	0.3	0.3	**1.0x**
CAAN5342.A2	15.4	16.6	**1.1x**	>50	>50	**n/a**	>50	>50	**n/a**	4.2	nt	**n/a**	1.9	3.4	**1.8x**
Avg. variation[1]	**--**	**--**	**1.8x**	**--**	**--**	**1.4x**	**--**	**--**	**2.2x**	**--**	**--**	**2.4x**	**--**	**--**	**1.9x**

[1]*Values that were either non-detectable or missing (not applicable, n/a) were not used in the calculation of average variation.*

3.3.4 Normal range of variation

The data in **Table 12** illustrated a high level of intra-operator reproducibility of assay results. Raw data from these assay plates was used to determine the range of variation in virus control wells, cell controls wells (background) and test wells (wells containing analyte) associated with this level of reproducibility **(Table 15)**. Since all 3 serum samples from vaccinated subjects were on the same plate in each of the 11 repeat assays, the same control values applied to all 3 samples and therefore are shown relative to a single sample. Additional control values are shown for the HIV-1 positive serum sample, since this sample was assayed on separate plates.

Plate-to-plate variation of virus control mean values was 2.5-fold (240,319 - 954,809 RLU) and 2.8-fold (232,795 - 662,998 RLU) for the vaccine serum and HIV-1-positive serum series of plates, respectively. Intra-plate variation (well-to-well variation) in virus control wells (8 wells/plate) was ≤12% in all cases. Plate-to-plate variation in cell control mean values was 1.9-fold (624 - 1,192 RLU) and 2.1-fold (588 - 1,259 RLU) for the two series of plates. Intra-plate variation in cell control wells (8 wells/plate) was ≤12% in all cases. This experiment tested the neutralizing activity of 8 different dilutions of each serum sample in triplicate wells. The range of variation in these triplicate wells was ≤17% in all cases. These ranges may be considered acceptable for a high level of quality assurance. The absolute range of variation for acceptable quality remains to be determined.

Table 15. Variation in virus control, cell control and test wells.

Vaccinated subject	Exp. No.	ID50	Variation from the mean	RLU in virus control (n=8)	SD	RLU in cell control (n=8)	SD	Range of SD in test wells (n=3)
041-A	AG15-41	3,853	109%	341,728	8%	725	7%	0-7%
	AG15-45	6,587	22%	358,309	6%	880	8%	0-2%
	AG15-46	7,848	3%	379,772	11%	835	7%	0-5%
	AG15-47	9,275	13%	437,620	9%	842	5%	0-11%
	AG15-48	9,165	12%	240,319	5%	624	7%	0-17%
	AG15-49	9,234	13%	574,735	9%	978	6%	0-5%
	AG15-50	9,081	11%	594,809	5%	1,192	3%	0-3%
	AG15-52	12,409	54%	435,208	7%	814	5%	0-10%
	AG15-53	6,113	24%	432,830	8%	859	12%	0-8%
	AG15-54	6,230	23%	379,854	7%	857	4%	0-3%
	AG15-55	8,907	9%	304,293	11%	770	8%	0-3%
HIV-1-Pos. Serum	**Exp. No.**	**ID50**	**Variation from the mean**	**RLU in virus control (n=8)**	**SD**	**RLU in cell control (n=8)**	**SD**	**Range of SD in test wells (n=3)**
DUMC-3	AG15-47	7,016	59%	437,348	8%	820	6%	0-11%
	AG15-48	4,834	9%	232,795	8%	588	6%	2-13%
	AG15-49	3,450	28%	600,862	6%	907	3%	0-8%
	AG15-50	7,563	71%	662,998	5%	1,259	4%	0-6%
	AG15-52	2,920	51%	443,441	9%	824	5%	0-8%
	AG15-53	4,060	9%	454,102	9%	907	6%	0-6%
	AG15-54	2,782	59%	404,798	14%	841	5%	0-9%
	AG15-55	2,754	61%	364,348	10%	882	7%	0-9%

3.4 LIMITS OF QUANTITATION

The lower limit of quantitation of neutralizing antibodies is the highest dilution of serum or lowest concentration of monoclonal antibody that reduces RLU by 50% relative to either the virus control wells or an appropriate negative test sample. This cut-off was chosen because it lies midway in the linear portion of neutralization curves (20-85% reductions in RLU) and is above the level of background noise in the assay.

The upper limit (lowest dilution) of quantitation of serum neutralizing antibodies is determined in large part by the nonspecific virus inhibitory activity and cell toxicity of normal serum samples. Referring to the data in **Figures 15-17 and Table 8** (Section 3.1), 50% reductions in RLU are rare for normal human serum diluted ≤1:10 and in some cases ≤1:4. However, Figure 16 shows that ≥50% reduction in RLU are occasionally seen at a 1:8 dilution of other normal human serum samples. We have performed numerous additional assays with 1:10-diluted normal human serum against different strains of HIV-1 and rarely detect reductions in RLU that are ≥50%. Based on these observations, the background activity of normal human serum (upper limit of quantitation) is usually <50% at a 1:10 serum dilution and possibly at lower dilutions. Since background activity may vary between serum samples and virus strains, it is strongly recommended that the upper limit of quantitation be confirmed with pre-immune serum samples in every study for each virus tested.

3.5 LINEARITY AND RANGE

Neutralization curves generated with positive serum samples and mAbs show a consistent pattern of linearity over an approximate range of 20-85% reductions in RLU **(Figs. 7, 9, 11, 21, 22)**. Values in this range are directly proportional to the concentration of neutralizing antibodies in the sample.

3.6 ROBUSTNESS

3.6.1 Varying the length of time of infection

Our preliminary tests had determined that RLU increased dramatically between 24 and 48 hours of infection, where 48 hours was adequate to measure as much as 2-log reductions in virus infectivity. This led to our use of a 48 hour infection period for assay development. Unexpected delays due to scheduling conflicts may sometimes require plates to be processed after a prolonged period of infection during routine assays. We therefore compared assay results after 48, 72 and 96 hours of infection. In this experiment, pseudovirus SS1196.01 was assayed as described in Duke SOP # HVTN02-A0009 except that RLU were measured after 48, 72 and 96 hours. Separate assay plates were set-up for each time point. Identical results were obtained at all three time points for each reagent **(Fig. 23)**.

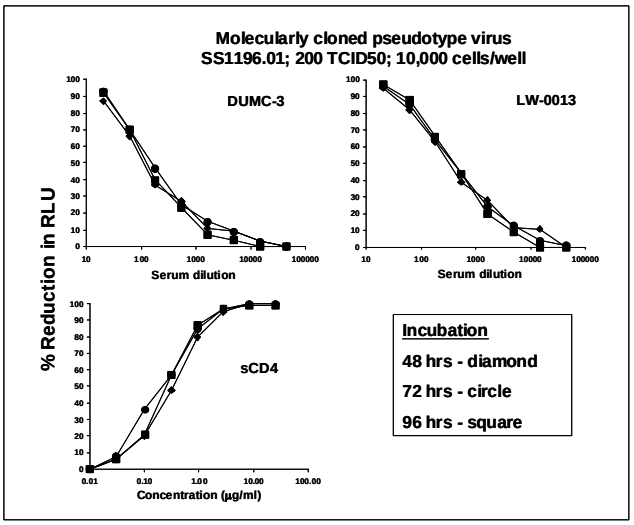

Figure 23. Neutralization curves: Effect of altering the length of time of infection prior to measuring luminescence. *RLU in cell control (background) and virus control wells were, respectively: 48 hrs = 1,068 vs 65,158; 72 hrs = 1,224 vs 70,756; 96 hrs = 1,604 vs 60,621.*

3.6.2 Stability of HIV-1 fusion receptor expression

Variation in the fraction of cells expressing HIV-1 fusion receptors (CD4 and either CCR5 or CXCR4) may affect the performance of the assay by altering the magnitude and kinetics of infection. To avoid this variation, we monitored the expression of CD4, CCR5 and CXCR4 over time by flow cytometry. Moreover, we measured the infectivity of TZM-bl cells after prolonged culture. Cells were stained for CD4, CCR5 and CXCR4 surface expression by using CD4-PE (Cat. No. 555347), CD195-PE (Cat. No. 556042) and CD184-PE (Cat. No. 555974), respectively. Mouse IgG_1, κ PE (Cat. No. 556650) was used for background staining in the case of CD4. Rat IgG2a, κ PE (Cat. No. 559317) was used for background staining of CCR5 and CXCR4. All antibodies were purchased from BD/Pharmingen. Approximately 10,000 events were acquired by using a FACSCalibur flow cytometer. As shown in **Table 16**, all three fusion receptors were stably expressed on TZM-bl cells for at least 24 weeks in culture (77 passages). A precipitous drop in CD4 and CCR5 expression, and more gradual decrease in CXCR4 expression, occurred soon afterward. Expression of all three fusion receptors on U87.CD4.CCR5.CXCR4 cells decreased dramatically after 10 weeks in culture in the absence of antibiotics, confirming the ability of our reagents to detect a reduction in the number of fusion receptor-positive cells.

Table 16. Flow cytometric analysis of CD4, CCR5 and CXCR4 cell surface expression.

Cells	Weeks in culture	Passage No.	% CD4 positive	% CCR5 positive	% CXCR4 positive
TZM-bl	2	17	99	100	99
without antibiotics	4	21	100	98	95
	6	26	98	98	89
	8	30	99	98	89
	10	34	99	100	90
	12	39	100	100	98
	14	44	99	99	98
	18	54	100	99	90
	22	64	99	99	83
	24	77	97	97	72
	28	89	**35**	**37**	**61**
	33	99	**1**	**22**	**65**
U87.CD4.CCR5.CXCR4	4	7	99	100	99
with antibiotics	6	11	80	79	78
	8	15	99	99	99
	10	19	100	100	99
	12	24	98	100	100
	14	29	99	100	99
	18	39	96	100	100
	22	48	97	99	100
U87.CD4.CCR5.CXCR4	4	3	100	96	94
without antibiotics	6	7	100	99	99
	8	11	78	79	78
	10	15	95	94	93
	12	19	**3**	**13**	**28**
	14	24	**0**	**11**	**61**
	18	29	**1**	**4**	**43**

3.6.3 Preservation of TZM-bl infectability after multiple passages

The infectivity of high passage TZM-bl cells (passage 64) was compared to low passage TZM-bl cells (passage 35) with the Env pseudotyped viruses WITO.12 (low yield virus) and Q768 (average yield virus). In this experiment, serial 5-fold dilution of each virus were incubated with TZM-bl cells of each passage number. Luminescence was measured after 48 hours. As shown in **Figure 24**, infectivity was remarkably preserved.

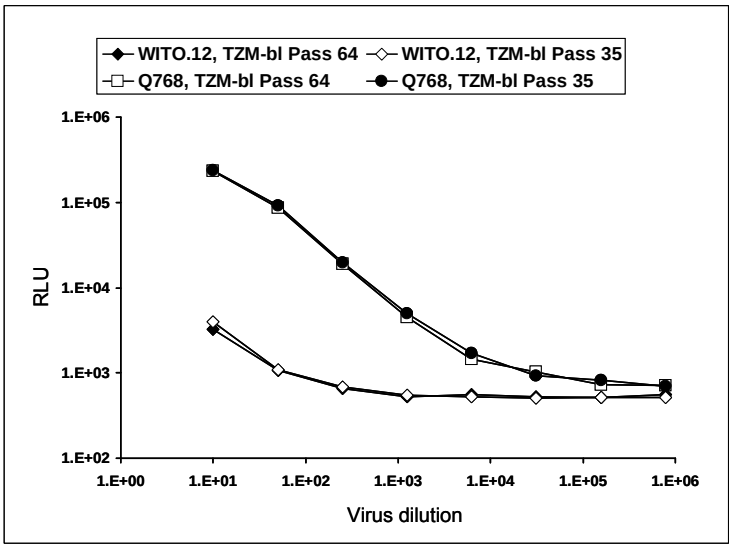

Figure 24. Preservation of TZM-bl infectability after multiple passages in culture.

3.6.4 Pseudovirus stability in -80°C storage and after multiple freeze-thaw cycles.

Pseudovirus preparations are routinely stored in aliquots at -80°C and thawed immediately before assay. The stability of these frozen pseudovirus aliquots was examined by thawing aliquots and measuring infectivity soon after the original stock was frozen and again 5-8 months later. Results varied between viruses and showed a loss in activity in some cases **(Table 17)**. In one case there was an increase in infectivity (BG1168.1). These results indicate that infectious viral titers need to be re-examined at regular intervals, preferably every 6 months. Additional data after longer storage times are needed to establish the best interval.

Table 17. Effect of prolonged storage at -80°C on pseudovirus infectivity.

Pseudovirus	TCID50/ml (<1 wk storage)	TCID50/ml (prolonged storage)	Storage time (months)
QH0692.42	2,284,389	781,250	8.0
QH0692.42	13,975	13,975	7.5
6101.10	456,878	91,376	8.0
SS1196.1	456,878	69,877	7.5
QH0515.1	13,975	31,250	7.0
5768.4	2,795	2,795	6.5
AR1.29	18,275	13,975	6.0
BG1168.1	156,250	456,878	6.0
6535.3	3,655	731	5.0

In cases where a frozen stock of pseudovirus has a high concentration of infectious particles, as a matter of convenience thawed aliquots may be refrozen for later use. We therefore exmined the infectivity of several different strains of pseudviruses after a second thaw cycle. As shown is **Table 18**, the infectivity of most pseudoviruses was reduced after the second thaw cycle. The amount of reduction varied between strains, where no reduction was seen in one case and a 10-fold reduction was seen in the most dramatic case. In 8 of 9 cases the infectivity remained at an adequate level (≥40,000 TCID50/ml) to be used in neutralization assays.

Table 18. Effect of multiple freeze-thaw cycles on pseudovirus infectivity.

Pseudovirus	TCID50/ml (1 thaw cycle)	TCID50/ml (2 thaw cycles)	TCID50 ratio (1 thaw/2 thaws)
QH0515.1	53,437	31,250	1.7
6101.10	156,250	156,250	1.0
THRO.18	349,386	91,376	3.8
BG1168.1	19,531,250	2,284,389	8.5
QH0692.42	1,021,610	119,488	8.5
DU151.2	2,694,383	267,184	10.0
SS1196.1	69,877	13,975	5.0
PVO.4	267,184	53,437	5.0
SC422661.8	349,386	69,877	5.0

As some stocks of our Env-pseudotyped viruses remained in storage for longer periods of time (Table 17 was only 5-8 months), additional stability testing of infectivity was conducted. A TCID50 assay was performed on five vials of Env-pseudotyped viruses that were in -80°C storage for approximately 2.6 years. Infectivity curves were compared to the curves that were generated at the time when the stocks were first generated 2.6 years early. Minor losses in infectivity (<1 log) were seen in each case but not to the extent that would alter neutralization results without adjusting the dose used for neutralization assays (2 logs, see section 2.5).

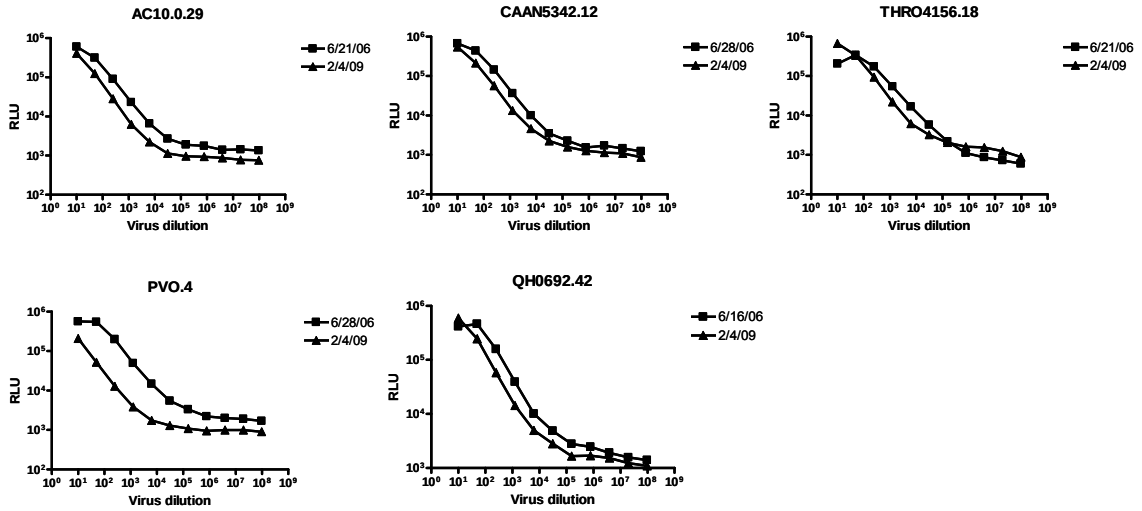

Figure 24A. Stability of Env-Pseudotyped Virus Infectivity After 2.6 Years of Storage at -80°C.

3.6.5 Performance of different lots of DEAE-dextran.

Two different lots of DEAE-dextran, both purchased from the same source (Sigma, Cat # D9885), were compared in terms of their toxicity to TZM-bl cells and their optimal concentration for pseudovirus infectivity in TZM-bl cells. Similar toxicity and infectivity curves were obtained with both lots of DEAE-dextran **(Fig. 25)**. Optimal infectivity was achieved at a dose of 30 μg/ml in each case. Both lots of DEAE-dextran exhibit dose-dependent cell toxicity with an associated dose-dependent decrease in infection at higher concentrations.

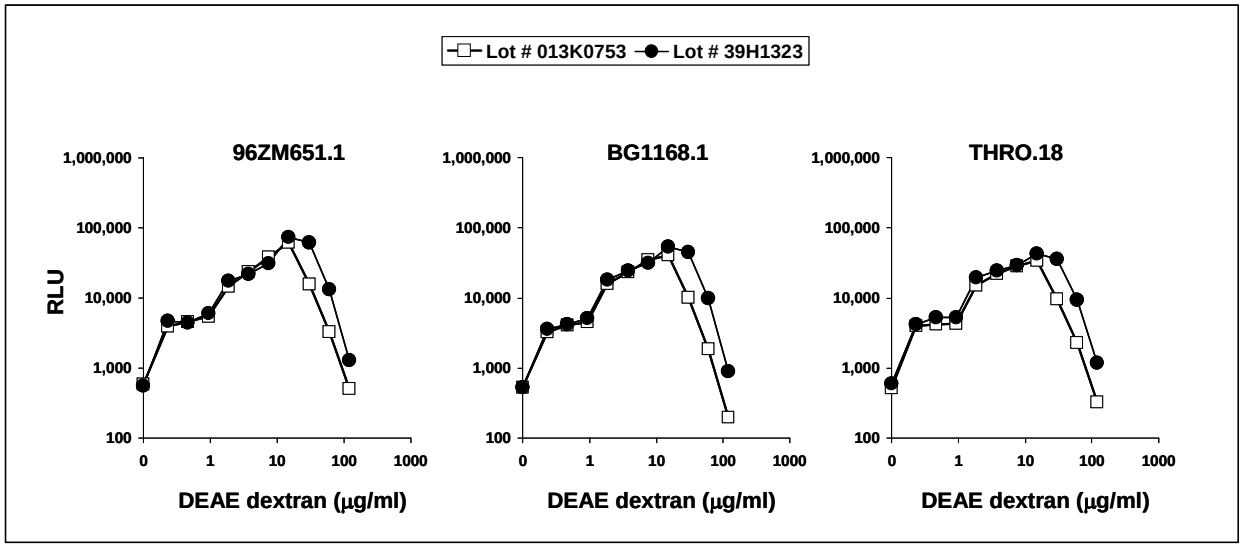

Figure 25. Cell toxicity and pseudovirus infectivity in the presence of two different lots of DEAE-dextran.

3.6.6 Varying the length of time of incubation in the luciferase reaction

Another variable condition of the assay is the length of time the luciferase reaction is incubated prior to reading in the luminometer. Adequate time is needed for the cells to be lysed by the substrate solution to release luciferase into the reaction mixture. The standard protocol in the instructions for the Promega Brigh-Glo kit calls for a 2 minute incubation at room temperature, which is what we routinely use. An experiment was performed in which TZM-bl cells were infected for 48 hours with Env pseudotyped strain BG1168.1 in 96-well plates. The dose of virus used in this experiment was determined previously to be well-within a linear range of RLU increase. Separate aliquots of cells were then processed for measurements of luciferase activity after increasing times of incubation with substrate buffer. RLU were roughly equivalent at each time point (0 min = 219,526 RLU; 3 min = 277,534 RLU; 6 min = 273,381 RLU; 9 min = 258,104 RLU; 12 min = 253,739 RLU).

3.6.7 Uniformity of luciferase reaction kits from different manufacturers

Performance of the Promega Bright-Glo kit (cat #E2650) was compared to the PerkinElmer Britelite kit (cat #6016979). We first assessed the Britelite kit after increasing times of incubation at room temperature. TZM-bl cells were infected with 200 TCID50 of SF162.LS in a mock of SOP HVTN02-A0009. Separate aliquots of cells were processed for measurements of luciferase activity after increasing times of incubation with substrate buffer. RLU were roughly equivalent at each time point **(Fig. 26)**. Our standard assay uses a 2 minute incubation time.

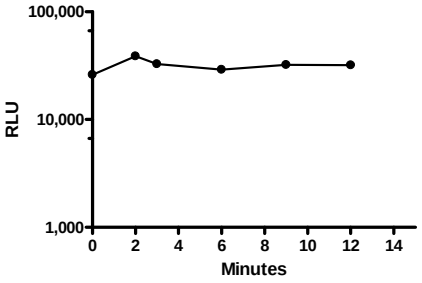

Figure 26. Results of timed incubations of the PerkinElmer Britelite luciferase reaction kit.

Next, the two kits were compared for measurements of TCID50 as described in SOP HVTN02-A0010 using two Env-pseudotyped viruses (SF162.LS and QH0692.42). Nearly equivalent results were obtained with the two reaction kits **(Fig. 27)**.

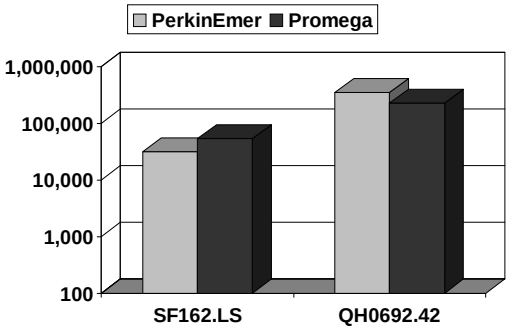

Figure 27. Results of TCID50 assays using either the PerkinElmer Britelite luciferase reaction kit or Promega Bright-Glo luciferase reaction kit.

Finally, the two kits were compared for meaurements of neutralization. Six monoclonal antibodies were assayed against pseudovirus SF162.LS using duplicate plates and SOP HVTN02-A0009. One plate was processed using the PerkinElmer Britelite kit, whereas the second plate was processed using the Promega Bright-Glo kit. As seen in **Fig. 28**, the neutralization curves were superimposed, indicating that equivalent results are obtained with both reaction kits.

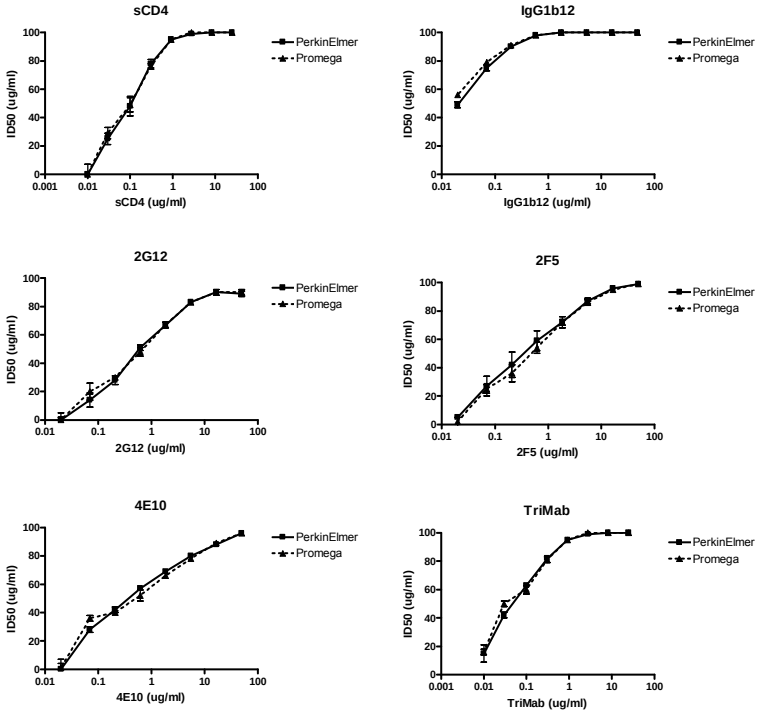

Figure 28A. Comparison of Britelite and Bright-Glo kits for measurements of neutralization of SF162.LS in TZM-bl cells.

In 2007 we were notified by PerkinElmer that the Britelite™ Luciferase substrate reagent will no longer be manufactured and therefore we evaluated their replacement Britelite™ Plus Reporter Gene Assay System (PerkinElmer Cat. # 6016769). We first determined the optimal length of time of incubation by measuring the infectivity of a wide range of dilutions of Env-pseudotyped virus HIV-SC422661 (ID#960) in a TCID50 assay format in TZM-bl cells. Luciferase reactions were performed every 3 minutes starting 1 minute after substrate addition and ending after 16 minutes. The highest RLU reading was obtained after 1 minutes of incubation and RLU loss was minimal after 16 minutes (Fig. 28B, top panel).

Next we compared the Britelite™ Plus to the Britelite™ (PerkinElmer Cat.# 6016979) currently in use. We measured the infectivity of a wide range of dilutions of Env-pseudotyped virus HIV-SC422661 (ID#960) in a TCID50 assay format in TZM-bl cells and applied each kit to parallel plates after two days of incubation. Results were identical between the two kits (Fig. 28B, bottom panel). We conclude that the two kits are equivalent for measuring HIV-1 Env-pseudotyped virus infection in TZM-bl cells.

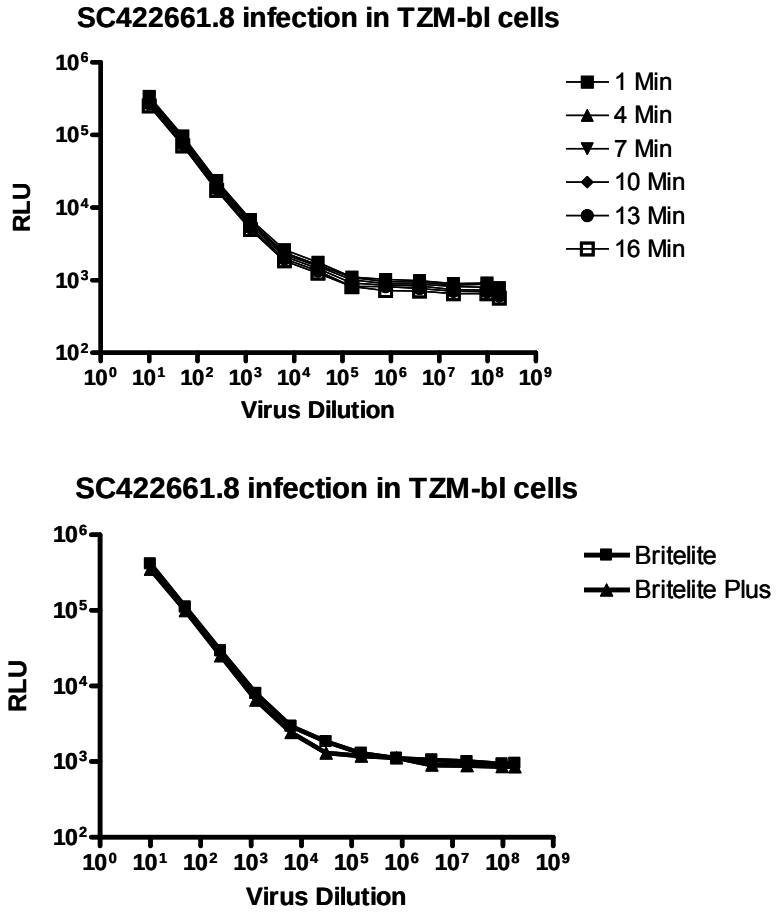

Figure 28B. Comparison of Britelite and Britelite Plus kits for measurements of neutralization of SC42661.8 in TZM-bl cells.

3.6.8 Use of multiple luminometers

Three different luminometers are used in the Duke HVTN Endpoint Neutralizing Antibody Assay Laboratory. All luminometers are the same make and model. Uniformity was assessed comparing intra- to inter-luminometer variation. Three MAb reagents (IgG1b12, 4E10, TriMab) were assayed against pseudovirus SF162.LS in TZM-bl cells (SOP HVTN02-A0009). Nine plates were set-up for each MAb. The RLU for three plates for each MAb was measured in each of the three luminometers. As shown in **Table 19**, all luminometers performed equivalently.

Table 19. Comparison of intra- versus inter-luminometer performance for measuring neutralizing antibodies in the TZM-bl assay.

			ID50 (μg/ml)		
Serial #	**Room**	**Test**	**IgG1b12**	**4E10**	**TriMab**
4205576	215 SORF	1	<0.02	0.54	0.03
		2	<0.02	0.62	0.03
		3	<0.02	0.71	0.03
		Mean (±SD)	***<0.02 ± 0.0***	***0.58 ± 0.06***	***0.03 ± 0.0***
4205754	216 SORF	1	<0.02	0.66	0.03
		2	<0.02	0.82	0.03
		3	<0.02	0.59	0.03
		Mean (±SD)	***<0.02 ± 0.0***	***0.74 ± 0.11***	***0.03 ± 0.0***
4205142	229 SORF	1	<0.02	0.71	0.03
		2	<0.02	0.66	0.03
		3	<0.02	0.60	0.03
		Mean (±SD)	***<0.02 ± 0.0***	***0.69 ± 0.04***	***0.03 ± 0.0***

3.6.9 Validation of a duplicate assay well format

The TZM-bl assay was originally optimized and validated to test each dilution of test sample in triplicate wells. A duplicate well assay format would increase throughput, conserve sample and reduce costs. Before adopting the duplicate format, it was necessary to determine how the neutralization curves, standard deviations and ID50 values compared to the triplicate format. In this experiment, three operators assayed 5 positive serologic reagents (sCD4, IgG1b12, 2G12, 2F5 and TriMab) against 2 strains of Env-pseudotyped HIV-1 (SF162.LS and QH0692.42) in TZM-bl cells (SOP HVTN02-A0009). As shown in **Fig. 29A and B**, nearly equivalent neutralization curves were obtained in the two formats. All ID50 values agreed within 3-fold per operator **(Table 20)** and no difference was seen in the range of standard deviations (range was 0 - 8% at ID50 values ≥50%) **(Table 21)**. Neutralization curves were linear over an approximate range of 20 - 85% reductions in RLU **(Fig. 29)**.

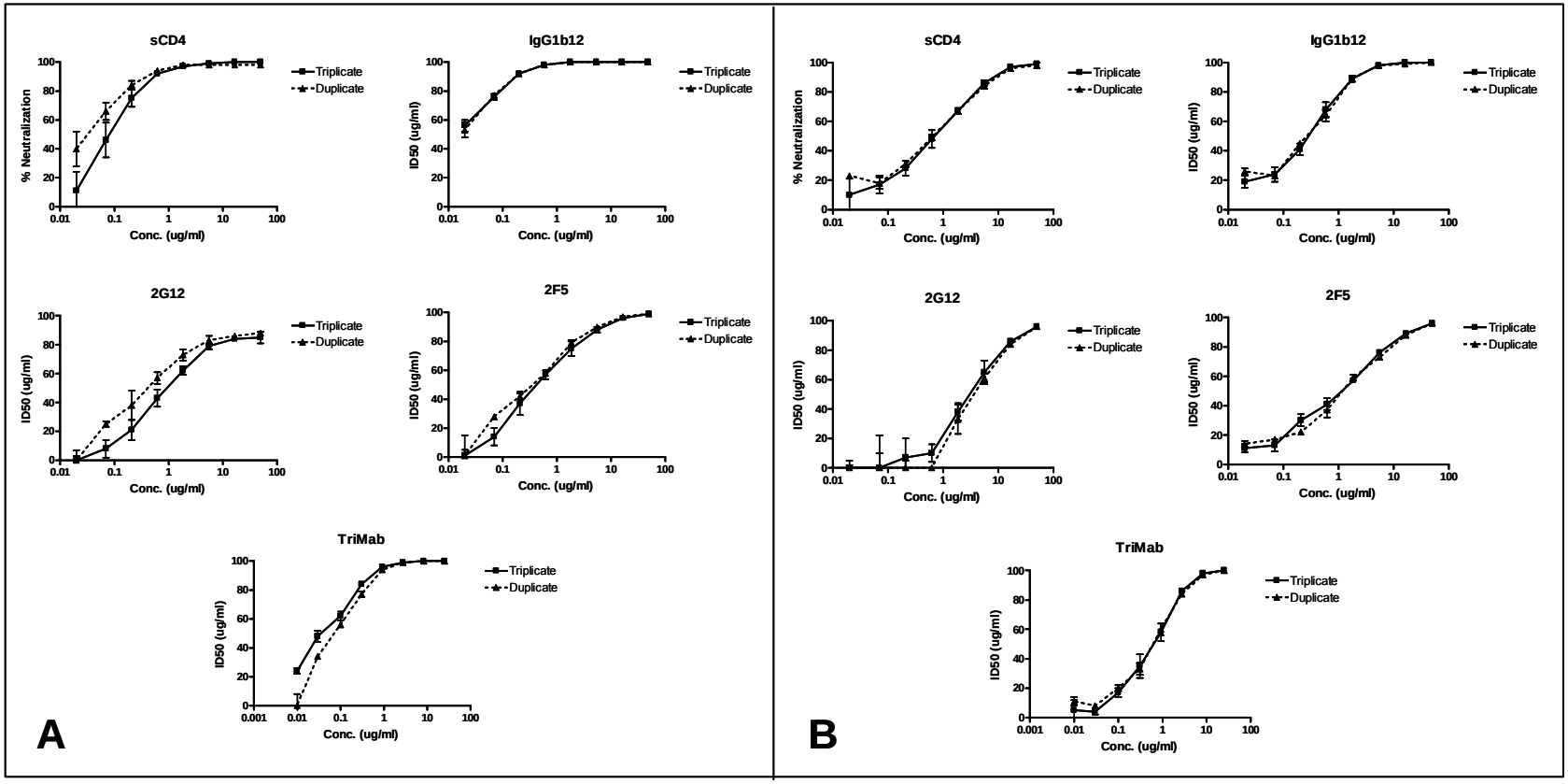

Figure 29. Neutralization assays performed with Env pseudotyped viruses SF162.LS (A) and QH0692.42 (B) using either a duplicate or triplicate well format.

Table 20. Comparison of ID50 values in the duplicate versus triplicate assay well format.

	ID50 in TZM-bl cells:[1] SF162.LS					
	B. Sokolik-Wolak		H. Gao		D. Nickischer	
Sample	Dup	Trip	Dup	Trip	Dup	Trip
sCD4	0.03	0.08	0.06	0.06	0.05	0.05
IgG1b12	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
2G12	0.42	0.93	0.39	0.94	0.38	1.17
2F5	0.35	0.43	0.63	0.39	0.18	0.18
TriMab	0.08	0.04	0.07	0.04	0.03	0.03
	ID50 in TZM-bl cells:[1] QH0692.42					
	B. Sokolik-Wolak		H. Gao		D. Nickischer	
Sample	Dup	Trip	Dup	Trip	Dup	Trip
sCD4	0.67	0.68	0.91	0.76	0.40	0.42
IgG1b12	0.27	0.28	0.34	0.30	0.19	0.19
2G12	3.66	2.77	2.70	3.04	2.93	2.93
2F5	0.66	0.75	1.19	1.17	0.28	0.33
TriMab	0.36	0.39	0.62	0.64	0.24	0.29

[1]*Values are the concentration at which relative luminescence units (RLU) were reduced 50% compared to virus control wells.*

Table 21. Comparison of standard deviations in the duplicate versus triplicate assay well format.

	Range in SD (at values ≥ID50):[1] SF162.LS					
	B. Sokolik-Wolak		H. Gao		D. Nickischer	
Sample	Dup	Trip	Dup	Trip	Dup	Trip
sCD4	0-6	0-6	0-1	0-3	0-1	0-4
IgG1b12	0-5	0-4	0-2	0-3	0-1	0-2
2G12	0-4	1-4	1-3	0-5	0-3	2-4
2F5	0-2	1-5	0-3	0-2	0-8	0-3
TriMab	0-2	0-3	0-2	0-3	0-8	0-2
	Range in SD (at values ≥ID50):[1] QH0692.42					
	B. Sokolik-Wolak		H. Gao		D. Nickischer	
Sample	Dup	Trip	Dup	Trip	Dup	Trip
sCD4	1-2	1-2	0-4	0-3	0-2	0-1
IgG1b12	0-4	0-5	0-1	0-3	0-1	0-5
2G12	1-3	1-4	0-1	0-8	0-4	0-1
2F5	0-3	1-3	0-2	0-1	0-2	0-3
TriMab	0-8	0-3	0-4	0-6	0-4	0-3

[1]*Values are the concentration at which relative luminescence units (RLU) were reduced 50% compared to virus control wells.*

3.6.10 Influence of the backbone vector on pseudovirus yield and neutralization phenotype.

The production of Env-pseudotyped viruses by transfection requires the use of an Env-minus backbone vector. It is desirable to use a vector that generates high titers of infectious pseudovirions. Moreover, it is important that the pseudoviruses maintain a neutralization phenotype resembling the uncloned parent. The standard backbone vector used in our laboratory is SG3Δenv. We have shown that the neutralization phenotype of pseudovirions made with this backbone resemble the uncloned parental virus (see section 2.9). Little is know about the influence that different backbone vectors might have on either the efficiency of pseudovirus production or the neutralization phenotype of the pseudovirions. We obtained a second backbone vector, Q23-17Δenv (Dr. Julie Overbaugh) and asked whether this backbone would be more efficient for transfection while maintaining a genuine neutralization phenotype. In this experiment, both backbone vectors were used to produce stocks of 5 different strains of Env-pseudotyped HIV-1 by transfection in 293T cells (SOP HVTN02-A0010). The neutralization phenotype of each pseudovirion stock was compared by using IgG1b12, 2F5, 4E10, TriMab and sCD4 as assayed according to SOP HVTN02-A0009.

As shown in **Figure 30**, only minor differences were seen in p24 Gag antigen synthesis, where p24 production was consistently higher (~30% higher) with the SG3Δenv backbone **(Fig. 30, left)**. Infectivity was equivalent in 3 cases (5768.4, WITO.33 and PVO.4) and nearly equivalent in the remaining 2 cases **(Fig. 30, right)**. Despite these similarities in pseudovirion production, neutralization-sensitivity was often diminished using the Q23-17Δenv backbone **(Fig. 31)**. This effect was <3-fold in most cases; however, **Table 22** shows that neutralization-sensitivity was reduced >7-fold in 4 cases. The possibility the a particular backbone vector can influence the neutralization phenotype of Env-pseudotyped strains of HIV-1 is scientifically interesting and suggests that a single backbone should be used for standardized assessments of a neutralizing antibody response. This is a preliminary observation that requires further investigation for confirmation.

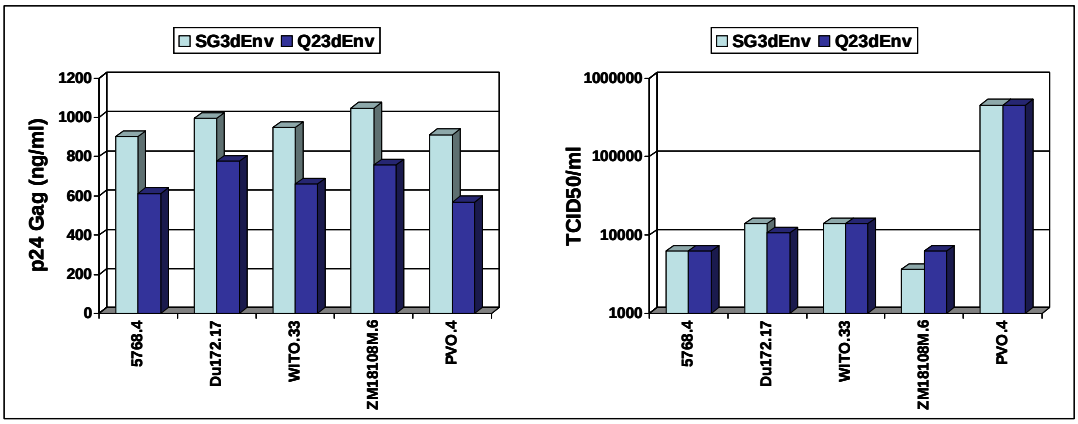

Figure 30. Effect of the backbone vector on the yield of pseudovirus in transfected 293T cells.

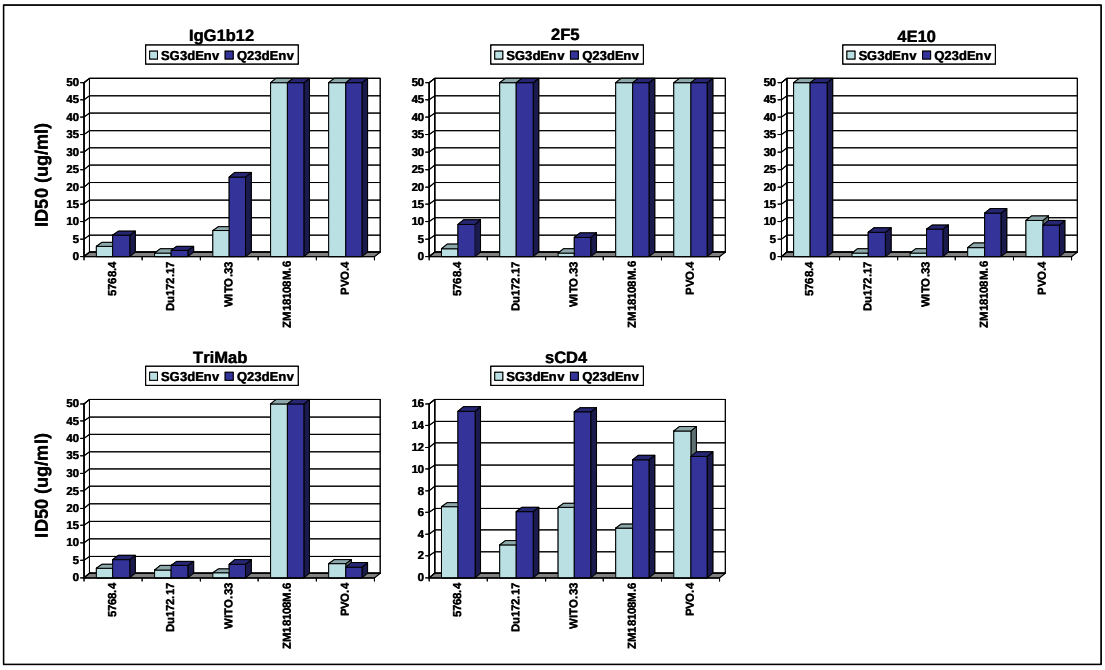

Figure 31. Effect of the backbone vector on neutralization phenotype.

Table 22. Fold-differences in the neutralization sensitivity of viruses made with either the SG3Δenv or Q23-17Δenv backbone.

VIRUS			Differential in ID50[1]				
VIRUS NAME	HARVEST DATE	DUKE ID#	IgG 1B12	2F5	4E10	TriMab	sCD4
5768.4	7-21-05	896	+2.1	+4.0	0 (R)	+1.9	+2.3
DU172.17	7-21-05	900	+1.7	0 (R)	+7.9	+1.5	+2.0
WITO4160.33	7-21-05	904	+3.1	+9.2	+10.0	+2.5	+2.4
ZM18108M.6	7-21-05	908	0 (R)	0 (R)	+9.4	0 (R)	+2.4
PVO.4	7-21-05	914	0 (R)	0 (R)	-1.1	+1.3	-1.2

[1]*Positive values, greater sensitivity with SG3Δenv. Negative values, greater sensitivity with Q23-17Δenv. R, Env was resistant in both backbones. IgG1B12 [3-3-05] Scripps; 2F5 [6-16-04] Polymun lot#hmAk C2F5 T580703-A; 4E10 [2-24-05] Polymun lot# T53/C4E10/1002A; TriMab [10-26-04]; sCD4 Progenics Lot#50.*

Because all SHIV viral genomes are SIV except for Env, we characterized the neutralization phenotype of a SHIV (SHIV-89.6P.18) using an additional set of backbone plasmids that were derived from SIV (GAB2, 7312A and TAN1 910, obtained from Dr. Beatrice Hahn) by comparing them to backbone plasmids pSG3Δenv, Q23-17Δenv and NL4-3LucR-E. Env-pseudotyped viruses containing the complete gp160 of SHIV-89.6P clone 18 were prepared with each backbone plasmid by transfection in 293T/17 cells using our standard protocol. Each backbone generated infectious Env-pseudotyped virus. When tested in the TZM-bl neutralization assay, all six pseudovirus preparations exhibited a similar neutralization phenotype

with IgG1b12, 2G12, 2F5, 4E10 and TriMab **(Table 23)**. These results are additional evidence that different backbone plasmids have little effect on the neutralization phenotype of Env-pseudotyped viruses.

Table 23. Neutralization phenotype of Env-pseudotyped viruses made with different Env-minus backbone plasmids.

			ID50 in TZM-bl cells (μg/ml)				
Env pseudovirus	**Backbone**	**TCID50**	**IgG1b12**	**2G12**	**2F5**	**4E10**	**TriMab**
SHIV-89.6P.18	SG3Δenv	14,867	>50	2.56	2.32	3.39	1.73
SHIV-89.6P.18	NL4-3LucR-E	10,687	>50	1.85	1.66	4.19	1.58
SHIV-89.6P.18	Q23-17Δenv	13,975	>50	1.77	2.47	5.01	2.18
SHIV-89.6P.18	GAB2	91,376	>50	0.54	0.47	2.69	1.30
SHIV-89.6P.18	7312A	2,795	>50	2.00	2.61	6.95	1.57
SHIV-89.6P.18	TAN1 910	13,975	>50	0.47	1.34	4.19	1.01
Mean			**>50**	**1.53**	**1.81**	**4.40**	**1.57**

We also compare the neutralization phenotype of a SHIV gp160 (SF162P3) made with our standard HIV-1 backbone plasmid (pSG3Δenv) and an SIVmac239Δenv backbone plasmid. The results shown in Table 23.1 revealed very little difference in neutralization phenotype with a variety of neutralizing antibody specificities. TriMab (a mixture of IgG1b12, 2G12 and 2F5) was the only exception.

Table 23.1. Neutralization phenotype of SHIV-SF162P3 Env-pseudotyped virus made with HIV-1 compared to SIV Env-minus backbone plasmids.

	ID50 (μg/ml) in TZM-bl cells[1]	
Reagent	**SHIV-SF162P3.5/SG3Δenv backbone**	**SHIV-SF162P3.5/239Δenv backbone**
sCD4	2.7	3.8
IgG1b12	0.6	1.7
2G12	2.7	1.9
2F5	0.4	1.1
4E10	5.4	12.7
TriMab	10.5	1.3
HIVIG	914	856

3.6.11 Effect of different 293T cells on infectious pseudovirus yield and neutralization phenotype.

Our assays prior to December, 2005 utilized a stock of 293T cells for transfection that were not adequately traceable. We therefore obtained 293T/17 cells from the American Type Culture Collection (ATCC) for routine use. For validation purposes, neutralization assays were performed to assess the neutralization phenotype of Env-pseudotyped viruses made in each cell type. In each case, Env-pseudotyped viruses were prepared by co-transfection with SG3Δenv according to our standard protocol. Our results showed that infectious yields and neutralization phenotypes were nearly identical for each cell type **(Table 24)**.

Table 24. Equivalency of Env-pseudotyped viruses made in 293T/17 cells (ATCC) compared to 293T cells with an unspecified history.

			ID50 in TZM-bl cells (µg/ml)				
Env pseudovirus	**Cells**	**TCID50**	**IgG1b12**	**2F5**	**4E10**	**TriMab**	**sCD4**
Du172.17	293T	2,795	1.32	>25	0.39	2.54	1.56
	293T/17 ATCC	2,795	1.09	>25	0.38	3.98	2.22
	293T	13,975	0.87	>25	0.42	1.75	1.08
	293T/17 ATCC	13,975	0.99	>25	0.44	2.45	1.63
Mean			**1.07**	**>25**	**0.41**	**2.68**	**1.62**
SS1196.1	293T	13,975	0.67	13.37	0.73	1.72	0.89
	293T/17 ATCC	13,975	1.01	13.38	0.29	2.01	0.59
	293T	13,975	0.72	14.68	1.23	1.88	1.01
	293T/17 ATCC	31,250	1.12	16.78	1.22	2.02	1.08
Mean			**0.88**	**14.55**	**0.87**	**1.91**	**0.89**
CAAN5342.A2	293T	91,376	>25	5.11	4.08	14.41	13.71
	293T/17 ATCC	69,877	>25	6.11	5.71	16.19	13.69
Mean			**>25**	**5.61**	**4.90**	**15.30**	**13.70**

3.6.12 Effect of different Env:backbone plasmid DNA ratios on pseudovirus neutralization phenotype.

During the course of our studies we have observed that different Env plasmids exhibit wide variation in the yield of infectious Env-pseudotyped virus after transfection. It follows that different expression levels of Env might affect the number and quality of Env glycoprotein spikes on the pseudotyped viruses. This in turn might affect neutralization. To examine this possibility, we prepared four strains of Env-pseudotyped viruses using different ratios and amounts of plasmid DNAs and examined the neutralization phenotype of each pseudovirus preparation with a panel of reagents. In each case, pseudoviruses were prepared in 293T/17 cells using the pSG3Δenv backbone plasmid and the FuGENE-6 transfection kit. Neutralization phenotypes were characterized with sCD4, three human monoclonal antibodies (IgG1b12, 2F5, 4E10) and six HIV-1-positive plasma samples (BB8, BB12, BB28, BB55, BB70, BB106). As shown in **Table 25**, no significant difference in neutralization phenotype was observed over a wide range of Env plasmid : backbone plasmid DNA ratios.

Table 25. Effect of plasmid DNA ratio on the neutralization phenotype of Env-pseudotyped viruses.

	ID50 in TZM-bl cells (μg/ml for sCD4 and MAbs, dilution for plasma samples)									
	SF162.LS	**SF162.LS**	**SF162.LS**	**SF162.LS**	**6535.3**	**6535.3**	**H077.31**	**H077.31**	**TV1.21**	**TV1.21**
Env DNA (μg)/ backbone DNA (μg)	16/4	6.6	4/16	4/0.5	4/8	4/2	4/8	8/2	4/8	8/2
Ratio	4:1	1:1	1:4	8:1	1:2	2:1	1:2	4:1	1:2	4:1
SAMPLE										
sCD4	0.3	0.6	0.4	0.5	0.9	1.4	7.5	8.9	0.3	0.3
IgG1b12	0.1	0.2	0.2	0.1	4.4	9.8	>50	>50	>50	>50
2F5	2.1	2.5	1.5	4.5	0.8	2.6	0.2	0.4	13.0	18.8
4E10	5.7	4.5	3.4	9.9	0.2	0.7	0.6	1.1	2.0	2.8
BB8	3,419	1,896	2,649	2,378	63	39	<20	<20	85	87
BB12	535	452	406	322	224	222	68	49	32	30
BB28	511	476	381	324	94	76	227	174	117	115
BB55	4,695	2,132	2,807	1,414	591	416	32	48	284	342
BB70	7,308	3,365	5,008	2,514	202	127	301	167	454	419
BB106	380	355	330	223	78	102	<20	<20	87	95

3.6.13 Evaluation of the need for indinavir for single round infection with replication-competent viruses.

Our earlier experiments with indinavir (section 2.8) indicated that very little replication of uncloned PBMC viruses occurs in TZM-bl cells over a 72-hour period of time. This suggests that indinavir is not needed in a 48-hour assay to limit replication to a single cycle. In an effort to determine whether indinavir could be excluded, neutralization assays were performed with an H9-grown stock of HIV-1 MN and with a PBMC-grown stock of SHIV-89.6P in the presence and absence of indinavir in the validated 48-hour assay format. Nearly identical results were obtained under both conditions **(Table 26)**. We conclude that indinavir is not needed in a 48-hour assay with replication-competent strains of virus.

Table 26. Equivalent neutralization results are obtained in the presence and absence of indinavir when replication competent viruses are used in a 48-hour assay.

	ID50 in TZM-bl cells (μg/ml)			
	HIV-1 MN		SHIV-89.6P	
Reagent	with indinavir	without indinavir	with indinavir	without indinavir
sCD4	0.04	0.06	1.59	0.79
IgG1b12	0.30	0.34	>25	>25
2F5	0.39	0.48	15.03	13.47
4E10	0.25	0.17	8.14	18.14
TriMab	0.03	<0.01	9.30	7.59

3.6.14 Stability of Env-pseudotyped viruses after temporary storage at -20°C.

Our Env-pseudotyped viruses are typically stored in -80°C freezers. In the event that a freezer malfunctions, the pseudoviruses may experience a temporary increase in freezing temperature. Our -80° freezers are connected to an emergency alarm system that notifies workers when the temperature increases to -60°C. We respond immediately by transferring the contents to a -80°C back-up freezer until repairs are made. To determine the effect of these minor, temporary increases in temperature on pseudovirion stability, we conducted a test whereby separate vials of four pseudoviruses were transferred to a -20°C freezer and stored there for 24 hours. These conditions were meant to be in excess of those that arise when a -80°C freezer malfunctions. Pseudovirion stability after this 24 hr period of incubation at -20°C was determined in infectivity assays and compared to the infectivity of virus stored continuously at -80°C. As shown in **Figure 32**, this temporary storage at -20°C had no obvious effects on pseudovirion infectivity.

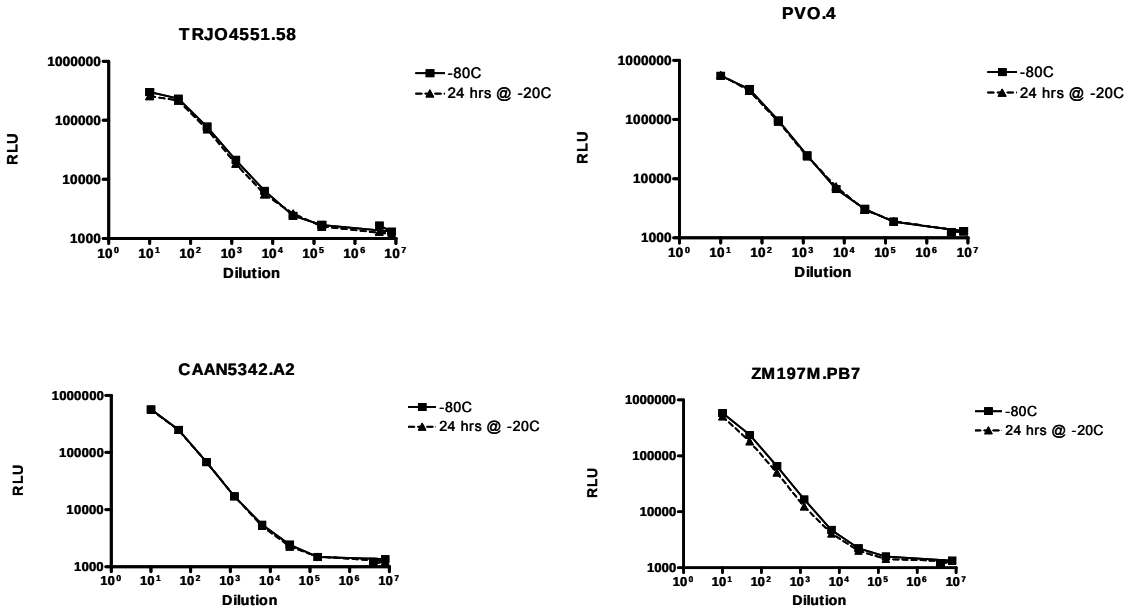

Figure 32. Effect of temporary -20°C storage on pseudovirion infectivity.

3.6.15 Qualification of sCD4, Mabs and HIVIG for competency and proficiency testing: stability after multiple freeze-thaw cycles.

It is often necessary to ship reagents to outside laboratories for competency and proficiency testing. For greatest stability, shipments on dry ice are often preferred. This requires that thawed samples be aliquoted and frozen prior to shipment. They must then be thawed at the recipient laboratrory. In some cases the recipeint laboratory may choose to re-freeze unused portions for future work. We therefore sought to determine the stability of several key serologic reagents after multiple freeze-thaw cycles. sCD4, IgG1b12, 2G12, 2F5, 4E10, TriMab and HIVIG were assayed after 0, 1, 2, 3 and 4 freeze-thaw cycles. We prepared IgG1b12, 2G12, 2F5, 4E10 and sCD4 in 0.5 ml volumes at 0.5 mg/ml in PBS. We also prepare a vial of HIVIG (0.5 ml undiluted) and TriMab (0.5 ml at 0.5 mg/ml). One hundred microliters were removed and stored in the refrigerator. The remaining amounts were stored at -80°C. Each week the frozen reagents were thawed, 100 µl were placed in separate tubes in the refrigerator, and the original vials re-frozen at -80°C. At the end of 4 weeks, the final amounts in the freezer were thawed. Each aliquot was assayed against three Env-pseudoyped viruses in the TZM-bl assay. As shown in Table 27, all reagents were highly stable throughout all freeze-thaw cycles.

Table 27. Neutralizing activity of sCD4, IgG1b12, 2G12, 2F5, 4E10, triMab and HIVIG after multiple freeze-thaw cycles.

		ID50 (µg/ml) in TZM-bl cells		
Reagent	**Freeze-thaw cycle**	**SF162.LS**	**QH0692.42**	**WITO.33**
sCD4	0	0.12	3.1	11.0
	1	0.11	3.2	12.4
	2	0.07	3.2	8.7
	3	0.11	3.5	15.5
	4	0.10	2.5	8.9
IgG1b12	0	0.05	2.1	16.6
	1	0.04	2.2	15.8
	2	0.03	2.1	17.6
	3	0.04	1.8	16.4
	4	0.05	1.5	16.1
2G12	0	2.1	11.0	2.3
	1	1.3	9.1	1.6
	2	1.1	8.9	1.5
	3	1.9	9.1	2.1
	4	0.7	6.5	2.2
2F5	0	1.1	4.4	0.9
	1	0.9	4.7	0.6
	2	0.7	2.9	0.7
	3	1.9	4.8	1.4
	4	1.6	2.7	1.7
4E10	0	3.0	8.5	0.7
	1	2.5	6.4	0.5
	2	3.9	9.6	0.6
	3	4.6	9.3	0.9
	4	4.8	6.4	1.2
TriMab	0	0.20	2.1	1.5
	1	0.13	1.9	0.8
	2	0.20	2.9	1.0
	3	0.45	3.3	2.2
	4	0.30	3.1	2.0
HIVIG	0	3.3	1398	823
	1	2.1	1514	784
	2	1.4	1270	701
	3	2.1	1620	977
	4	2.0	1105	696

3.6.16 Presence and impact of replication-competent virus (RCV) in Env-pseudotyped virus preparations.

The backbone vector we most commonly use for pseudovirus production, pSG3Δenv, is a full-length HIV-1 subtype B genome in plasmid pTZ19U that contains a four nucleotide insertion that inactivates the *env* gene. Because most of *env* is preserved, potential exists for homologous recombination between this backbone plasmid and a functional *env* plasmid during either transfection or infection that could produce replication-competent virus (RCV). The presence of RCV could compromise the neutralization assay by representing an unintended viral target for neutralization. In addition, RCV could allow the virus to multiply and kill cells after prolonged incubation (e.g., in a 3 day assay). Thus, we tested ~ 90 separate stocks of Env-pseudotyped viruses made with the SG3Δenv backbone for the presence of RCV. In addition, we tested a subset of these Env-pseudotyped viruses made with either an HIV-1 subtype A backbone plasmid (Q23-17Δenv) or a different HIV-1 subtype B backbone plasmid (NL4-3) . PHA-PBMC were inoculated with undiluted Env-pseudovirus. PBMC were washed after one day to remove the virus inoculum and the medium was replaced. Fresh PHA-PBMC were added on day 7. Cultures were incubated for 12-14 days with regular changing of the medium every 3 days. Culture fluids from days 4, 7, 10 and the final day of incubation were tested for the presence of p24 Gag antigen. Culture fluids on the final 2 days of incubation were further tested for infectious virus by transferring 50 µl to 96 well plates containing 100 µl of growth medium and 100 µl of TZM-bl cells (this transfer was done before collecting culture fluid for p24 assay). Luciferase activity was measured after 2 days of incubation with the culture fluids. Samples were considered RCV positive if elevated levels of p24 Gag antigen and RLU were detected.

Results of three separate experiments are shown in Tables 28-30 below. As can be seen, 16/60 (27%) of subtype B Envs made with the SG3Δenv backbone plasmid tested positive for RCV. Multiple preparations of the subtype B virus SF162.LS were positive when made with this backbone and when made with the subtype A backbone (Q23-17Δenv) but a stock made with the NL4-3 backbone was negative. In addition, among four different preparations of subtype B virus QH0692.42 made with the SG3Δenv backbone (IDs 1308, 817, 1799, 1767), only two tested positive for RCV. Among three different preparations of subtype B virus THRO4156.18 made with SG3Δenv backbone, one was positive 2/2 times whereas the other two were negative in a single test each. Thus, certain Envs appeared to be more problematic than others. Notably, subtype A, C, BC and AG viruses made with the subtype B backbone (SG3Δenv) were rarely RCV positive (1/48 cases). However, 3/8 subtype A viruses made with the subtype A backbone (Q23-17Δenv) tested positive. These results indicate that the potential for RCV is greater when using a backbone plasmid that is clade-matched to the Env clone.

We next tested whether RCV impacted the outcome of neutralization assays. Any impact would depend on where the RCV arose (i.e., either during transfection of after infection) and how much RCV was present. Any RCV those arose after infection of TZM-bl cells would be of little concern for an assay that measures entry inhibition. On the other hand, if recombination occurred during transfection, the resulting virus would be a mixed population containing the intended Env and a recombinant Env containing elements of the backbone plasmid. In this case, levels of RCV that are sufficient to register in the luciferase reaction would complicate the assay by measuring neutralization of the RCV rather than solely the intended virus.

For our test we selected two separate stocks of SF162.LS and one stock of QH0692.42 that scored among the highest values in our RCV test. These stocks of RCV-positive Env-pseudotyped viruses were assayed in parallel with a stock of each Env-pseudotyped virus that tested negative for RCV. All Env-pseudotyped viruses were made with the SG3Δenv backbone except for the RCV-negative stock of SF162.LS, which was made with the NL4-3 backbone. As shown in Table 31, equivalent neutralization results were obtained with a wide variety of antibodies regardless of RCV status. These results indicate that RCV has no measurable effect on the TZM-bl neutralization assay.

Table 28. Multiple HIV-1 subtypes made with the subtype B backbone SG3Δenv.

Virus Name	Stock ID	Backbone	Clade	p24 Day 4	p24 Day 7	p24 Day 10	p24 Day 14	RLU in TZM-bl (d10 PBMC sups)	RLU in TZM-bl (d12 PBMC sups)
SF162.LS	**1630**	**SG3ΔEnv**	**B**	**15.2**	**343.5**	**122.2**	**70.4**	**103669**	**221969**
SS1196.1	1344	SG3ΔEnv	B	1.5	1.2	0.1	0.1	2311	2892
BZ167.12	**1121**	**SG3ΔEnv**	**B**	**1.9**	**8.6**	**19.9**	**21.3**	**22187**	**16897**
Bal.26	723	SG3ΔEnv	B	4.3	4.0	0.2	0.1	4242	5586
6101.1	**736**	**SG3ΔEnv**	**B**	**4.2**	**3.4**	**0.5**	**2.2**	**9256**	**33183**
6535.3	1645	SG3ΔEnv	B	0.4	0.3	0.1	0.1	1773	1600
QH0692.42	**1308**	**SG3ΔEnv**	**B**	**11.1**	**18.1**	**42.2**	**91.9**	**112494**	**187048**
PVO.4	1313	SG3ΔEnv	B	4.2	3.7	0.2	0.1	3705	4891
AC10.0.29	674	SG3ΔEnv	B	2.6	2.4	0.2	0.1	2491	2422
THRO4156.18	**1316**	**SG3ΔEnv**	**B**	**9.1**	**23.6**	**45.6**	**58.3**	**114443**	**94297**
SHIV-SF162P3.5	**873**	**SG3ΔEnv**	**B**	**1.6**	**2.1**	**4.4**	**8.1**	**73568**	**109604**
H022.7	770	SG3ΔEnv	B	3.5	3.2	0.1	**3.3**	2102	**9824**
H030.7 (T2)	1459	SG3ΔEnv	B	2.2	2.2	0.1	0.1	1796	1808
H035.18	783	SG3ΔEnv	B	2.3	2.2	0.1	0.1	1889	1483
H079.2 (T1)	1640	SG3ΔEnv	B	0.3	0.3	0.1	0.1	1924	1238
H077.31 (T1	1434	SG3ΔEnv	B	2.3	2.0	0.1	0.1	1708	1341
Du151.2	550	SG3ΔEnv	C	5.0	4.5	0.2	0.1	1801	1522
Du156.12	753	SG3ΔEnv	C	1.3	1.0	0.1	0.1	4387	3334
Du172.17	1292	SG3ΔEnv	C	0.9	0.8	0.1	0.1	2082	1821
Du422.1	1339	SG3ΔEnv	C	1.6	1.8	0.1	0.1	1787	1099
ZM197M.PB7	732	SG3ΔEnv	C	13.1	7.6	0.2	0.1	1733	1413
CAP45.2.00.G3	1571	SG3ΔEnv	C	2.8	2.2	0.1	0.1	1770	1603
ZM106F.PB9	823	SG3ΔEnv	C	5.3	5.0	0.2	0.1	1810	1566
HIV-00836-2.5	1196	SG3ΔEnv	C	0.7	0.8	0.1	0.1	1904	1303
HIV-0013095-2.11	1361	SG3ΔEnv	C	0.8	0.6	0.1	0.1	572	1554
HIV-16845-2.22	1365	SG3ΔEnv	C	1.8	1.2	0.1	0.1	561	1594
HIV-25710-2.43	1304	SG3ΔEnv	C	2.1	1.4	0.1	0.1	712	1631
HIV-25925-2.22	1202	SG3ΔEnv	C	3.3	2.4	0.2	0.1	614	1759
CH038.12	1382	SG3ΔEnv	BC	1.3	1.4	0.3	0.4	8243	8835
CH070.1	1490	SG3ΔEnv	BC	2.3	2.1	0.1	0.1	1948	1675
CH110.2	938	SG3ΔEnv	BC	2.9	2.7	0.2	0.1	1922	1644
CH181.12	945	SG3ΔEnv	BC	2.6	2.4	0.1	0.1	1823	1705
CH119.10	946	SG3ΔEnv	BC	1.7	1.5	0.1	0.1	1907	1657
CH115.12	937	SG3ΔEnv	BC	0.3	0.3	0.1	0.1	1871	2036
Q23.17	1658	SG3ΔEnv	A	6.4	5.4	0.2	0.1	553	1558
Q769.d22	1521	SG3ΔEnv	A	4.4	3.2	0.1	0.1	461	1271
Q842.d12	1606	SG3ΔEnv	A	1.2	0.7	0.1	0.1	570	1278
Q168.a2	654	SG3ΔEnv	A	2.5	1.7	0.1	0.1	729	1317
T257-31	1053	SG3ΔEnv	AG	0.2	0.1	0.1	0.1	1732	1519
271-11	1228	SG3ΔEnv	AG	2.1	1.4	0.1	0.1	1791	1675
263-8	1176	SG3ΔEnv	AG	1.8	1.3	0.1	0.1	1811	1758
T251-18	1119	SG3ΔEnv	AG	6.6	5.4	0.2	0.1	1781	1736
T255-34	1137	SG3ΔEnv	AG	0.9	0.6	0.1	0.1	1926	2243
235-47	1220	SG3ΔEnv	AG	2.3	1.7	0.1	0.1	1752	1773
T266-60	1093	SG3ΔEnv	AG	1.0	0.8	0.1	0.1	1642	1412
T280-5	1097	SG3ΔEnv	AG	1.1	0.8	0.1	0.1	1753	1584
252-7	1222	SG3ΔEnv	AG	3.2	2.6	0.2	0.1	1902	1942
SIVmac239CS.23	735	SG3ΔEnv	SIV	9.7	14.9	0.4	0.227	4020	3400

RCV-positive Env-pseudotyped viruses are shown in boldface type.

Table 29. Multiple HIV-1 subtypes made with either the subtype B backbones SG3Δenv or NL4-3.

Virus Name	Stock ID	Backbone	Clade	p24 Day 4	p24 Day 7	p24 Day 10	p24 Day 12	RLU in TZM-bl (d10 PBMC sups)	RLU in TZM-bl (d12 PBMC sups)
SF162.LS	**1630**	**SG3ΔEnv**	B	**3.9**	**29.4**	**182.9**	**256.7**	**125384**	**142390**
SF162.LS	1424	NL4-3	B	0.8	0.4	0.3	0.5	3109	2108
SF162.LS	**1781**	**SG3ΔEnv**	**B**	**14.0**	**64.7**	**266.1**	**343.3**	**225205**	**132423**
BZ167.12	1121	SG3ΔEnv	B	1.6	1.0	0.4	0.3	4200	2146
6535.3 ID	1645	SG3ΔEnv	B	0.3	0.1	0.1	0.1	3117	2057
QH0692.42	**817**	**SG3ΔEnv**	**B**	**2.2**	**6.0**	**142.5**	**198.0**	**153092**	**92427**
QH0692.42	1308	SG3ΔEnv	B	4.1	2.2	0.7	0.8	3090	1940
QH0692.42	**1799**	**SG3ΔEnv**	**B**	**3.5**	**2.1**	**2.0**	**3.1**	**7708**	**10815**
PVO.4	1313	SG3ΔEnv	B	1.3	0.8	0.3	0.3	3207	2030
AC10.0.29	1722	SG3ΔEnv	B	1.4	0.9	0.3	0.4	2963	2062
CAAN5342.A2	734	SG3ΔEnv	B	1.6	1.1	0.4	0.5	3057	2118
REJO4541.67	636	SG3ΔEnv	B	23.2	1.0	0.2	0.3	3072	2000
RHPA4259.7	839	SG3ΔEnv	B	1.1	0.6	0.2	0.3	3041	2028
SC422661.8	576	SG3ΔEnv	B	2.5	1.3	0.4	0.5	2956	1923
THRO4156.18	758	SG3ΔEnv	B	2.0	1.1	0.4	0.5	5251	1878
THRO4156.18	**1316**	**SG3ΔEnv**	**B**	**2.6**	**1.5**	**19.1**	**39.4**	**5978**	**80029**
TRJO4551.58	762	SG3ΔEnv	B	1.0	0.6	0.2	0.3	2985	1763
TRO.11	772	SG3ΔEnv	B	0.9	0.6	0.2	0.3	3021	2080
WITO4160.33	1103	SG3ΔEnv	B	0.5	0.3	0.2	0.2	4765	1984
SHIV-SF162P3.5	873	SG3ΔEnv	B	1.0	0.4	0.2	0.2	3283	2153
SHIV-SF162P3.5	880	SG3ΔEnv	B	1.5	0.8	0.3	0.3	3080	2082
62357.14.D3.4589	**1686**	**SG3ΔEnv**	**B**	**4.0**	**3.3**	**7.2**	**7.5**	**56901**	**119276**
6240.08.TA5.4622	1688	SG3ΔEnv	B	1.5	1.0	0.4	0.3	3216	2118
6244.13.B5.4576	**1682**	**SG3ΔEnv**	**B**	**1.9**	**3.7**	**41.4**	**61.1**	**95284**	**110315**
700010040.C9.4520	1862	SG3ΔEnv	B	2.8	2.2	0.4	0.4	2975	2069
9021-14.B2.4571	1684	SG3ΔEnv	B	5.4	3.4	0.9	0.8	2831	2034
BB-1006-11.C3.1601	1601	SG3ΔEnv	B	0.6	0.4	0.2	0.2	3058	2081
BB-1012-11.TC21	1672	SG3ΔEnv	B	0.9	0.7	0.3	0.2	3023	1952
BB-1054-07.TC4.1499	**1668**	**SG3ΔEnv**	**B**	**1.3**	**3.3**	**20.5**	**32.0**	**195872**	**98849**
BB-1056-10.TA11.1826	1670	SG3ΔEnv	B	0.8	0.5	0.3	0.2	3530	2107
PRB926-04.A9.4237	**1866**	**SG3ΔEnv**	**B**	**2.1**	**3.3**	**29.3**	**27.0**	**81144**	**119652**
SC05.8C11.2344	1872	SG3ΔEnv	B	1.8	1.2	0.4	0.3	3138	1988
WEAU-d15.410.787	1696	SG3ΔEnv	B	3.4	2.4	0.5	0.5	2912	2095
CAP210.2.00.E8	1033	SG3ΔEnv	C	0.4	0.3	0.1	0.2	3104	1935
CAP45.2.00.G3	1571	SG3ΔEnv	C	0.6	0.3	0.2	0.2	3202	1993
Du156.12	753	SG3ΔEnv	C	0.8	0.5	0.2	0.2	2947	2008
Du172.17	1292	SG3ΔEnv	C	0.7	0.4	0.2	0.2	3217	1985
Du422.1	1339	SG3ΔEnv	C	1.0	0.6	0.3	0.3	2995	2023
ZM109F.PB4	831	SG3ΔEnv	C	0.5	0.4	0.2	0.2	2953	1994
ZM135M.PL10a	1496	SG3ΔEnv	C	0.3	0.2	0.1	0.1	2945	2036
ZM197M.PB7	732	SG3ΔEnv	C	0.6	0.5	0.2	0.2	2921	1973
ZM214M.PL15	889	SG3ΔEnv	C	0.9	0.7	0.3	0.2	2906	2074
ZM233M.PB6	593	SG3ΔEnv	C	0.3	0.2	0.2	0.2	2889	2057
ZM249M.PL1	916	SG3ΔEnv	C	0.5	0.4	0.2	0.2	3063	1941
ZM53M.PB12	**821**	**SG3ΔEnv**	**C**	**1.7**	**1.3**	**10.3**	**15.8**	**7741**	**56045**
Q23.17	1658	SG3ΔEnv	A	1.7	1.3	0.3	0.3	2972	2192
Q259.d2.17	656	SG3ΔEnv	A	2.5	1.8	0.5	0.5	2982	2033
Q461.e2	658	SG3ΔEnv	A	1.8	1.2	0.4	0.4	3002	2054
Q769.d22	1521	SG3ΔEnv	A	0.6	0.5	0.2	0.2	3013	1916
Q842.d12	1606	SG3ΔEnv	A	0.2	0.2	0.1	0.1	3024	1918
Q168.a2	654	SG3ΔEnv	AD	1.1	0.6	0.2	0.2	3084	1889
SIVmac239CS.23	735	SG3ΔEnv	SIV	7.6	4.6	1.5	1.3	3663	2152

RCV-positive Env-pseudotyped viruses are shown in boldface type.

Table 30. Multiple HIV-1 subtypes made with either the SG3Δenv, NL4-3 or Q23-17Δenv backbone.

Virus Name	Stock ID	Backbone	Clade	p24 day 4	p24 day 7	p24 day 10	p24 day 13	RLU in TZM-bl (d7 PBMC sups)	RLU in TZM-bl (d13 PBMC sups)
5768.4	898	Q23-17Δenv	B	3.3	1.2	0.5	1.2	1501	1351
6535.3	651	Q23-17Δenv	B	0.7	0.1	0.1	0.1	1517	1628
AC10.0.29	1704	SG3ΔEnv	B	1.3	0.4	0.2	0.6	1607	1829
CAAN5342.A2	1318	SG3ΔEnv	B	2.5	1.1	0.8	2.5	2248	3349
CAAN5342.A2	993	SG3ΔEnv	B	1.1	0.4	0.2	0.5	1596	1816
SC422661.8	965	SG3ΔEnv	B	3.6	1.9	0.8	1.7	1423	1280
QH0692.42	1996	Q23-17Δenv	B	10.1	5.4	2.9	5.1	1554	1529
QH0692.42	1767	SG3ΔEnv	B	5.6	3.5	2.0	3.3	2033	1989
SF162.LS	**2002**	**Q23-17Δenv**	**B**	**4.8**	**11.7**	**29.9**	**57.1**	**322232**	**125593**
SF162.LS	**2000**	**Q23-17Δenv**	**B**	**2.0**	**4.2**	**25.2**	**56.5**	**129383**	**121712**
SF162.LS	1998	Q23-17Δenv	B	3.3	1.2	0.7	0.5	1861	1894
SF162.LS	**1446**	**SG3ΔEnv**	**B**	**3.8**	**17.4**	**19.9**	**63.2**	**7425**	**81844**
SF162.LS	**1719**	**SG3ΔEnv**	**B**	**1.7**	**4.5**	**38.4**	**132.2**	**65235**	**197350**
SF162.LS	1565	NL4-3	B	0.6	0.1	0.1	0.2	1685	1066
REJO4541.67	791	SG3ΔEnv	B	4.3	1.9	1.3	2.1	1632	1761
RHPA.7	562	SG3ΔEnv	B	1.8	0.7	0.4	0.7	1691	1664
THRO4156.18	564	SG3ΔEnv	B	2.6	1.0	0.6	1.1	1579	1737
TRJO4551.58	1789	SG3ΔEnv	B	0.2	0.1	0.1	0.1	1690	1890
TRO.11	1787	SG3ΔEnv	B	0.8	0.2	0.2	0.2	1684	1797
WITO4160.33	906	Q23-17Δenv	B	3.1	1.1	0.8	1.4	1641	1826
PVO.4	914	Q23-17Δenv	B	6.3	2.4	1.4	2.4	1765	1795
SHIV89.6P.18	921	NL4-3	B	0.3	0.1	0.1	0.1	1811	1847
SHIV89.6P.18	794	NL4-3	B	0.2	0.01	0.1	0.1	1679	1849
SHIV89.6P.18	796	Q23-17Δenv	B	4.7	1.5	0.9	1.5	1725	1932
DU172.17	903	Q23-17Δenv	C	0.2	0.1	0.1	0.000	1664	1747
ZM233M.PB6	910	Q23-17Δenv	C	2.1	0.8	0.6	1.2	1528	1296
97ZA012.12	678	Q23-17Δenv	C	2.4	0.7	0.4	0.8	1568	1817
Q842.d12	1988	Q23-17Δenv	A	6.3	3.5	1.6	3.1	1781	1596
Q769.d22	1986	Q23-17Δenv	A	12.7	7.8	4.3	8.2	3049	**31689**
Q461.e2	1984	Q23-17Δenv	A	11.7	7.2	4.1	6.6	4423	2615
Q168.a2	1980	Q23-17Δenv	A	5.0	2.1	1.2	2.2	2535	2061
Q259.d2.17	1982	Q23-17Δenv	A	7.3	3.5	1.9	3.6	2076	1859
Q23.17	**1990**	**Q23-17Δenv**	**A**	**7.7**	**39.7**	**47.2**	**123.0**	**1232***	**141010**
Q23.17	**1992**	**Q23-17Δenv**	**A**	**5.9**	**33.3**	**42.1**	**115.7**	**34848**	**123238**
Q23.17	**1994**	**Q23-17Δenv**	**A**	**5.6**	**32.6**	**55.9**	**157.2**	**19136**	**35888**

RCV-positive Env-pseudotyped viruses are shown in boldface type.

*Extensive cell-killing.

Table 31. Validation of SF162.LS and QH0692.42 PV with either high or undetectable RCV.

	ID50 in TZM-bl cells[1]				
Sample	**SF162.LS ID#1630 RCV-pos**	**SF162.LS ID#1781 RCV-pos**	**SF162.LS ID#1424 RCV-neg**	**QH0692.42 ID#817 RCV-pos**	**QH0692.42 ID#1308 RCV-neg**
sCD4	0.2	0.2	0.2	1.1	1.0
IgG1b12	0.1	0.1	0.1	0.9	1.0
2G12	0.8	0.8	1.0	2.2	2.6
2F5	2.2	4.0	3.5	1.0	1.0
4E10	5.3	8.3	7.5	1.4	1.0
TriMab	0.2	0.3	0.2	0.6	0.8
Zepto 01648	3,561	3,655	3,375	103	114
Zepto 01652	1,292	728	1,017	82	89
Zepto 01686	5,484	5,012	4,396	111	104
HIV-011	7,403	7,273	5,808	211	157
HIV-012	2,304	1,652	2,402	<20	<20
HIV-013	840	627	338	157	94
HIV-014	12,256	11,777	7,971	528	412
HIV-015	9,273	8,817	7,446	<20	<20
HIV-016	7,234	7,561	4,650	132	138
HIV-017	3,356	3,069	2,807	153	154
HIV-018	1,652	1,215	1,774	106	100
HIV-019	550	417	376	26	27
HIV-020	7,348	4,594	4,532	53	40

[1]*ID50 values for sCD4 and mAbs are in μg/ml. ID50 values for the HIV-1-positive plasma samples are dilution.*

3.6.18 Lack of an endotoxin artifact in the TZM-bl assay.

Recently, several antibodies have been shown to exhibit much greater neutralization potency in the PBMC assay than in the TZM-bl assay, (Binley et al., J. Virol. 78:13232-13252, 2004; Brown et al., J. Virol. 81:2087-2091, 2007; Choudhry et al., Virology 363:79-90, 2007; Polonis et al., Virology 375:315-320, 2008; Zhang et al., J. Virol. 82:6869-6879, 2008), raising concern that the TZM-bl assay falsely underestimates the neutralizing activity of some reagents and creating uncertainty as to which assay provides a more meaningful assessment of antibody-mediated virus neutralization. We tested whether much of this discrepancy in assay performance may be an artifact of endotoxin contamination that is only detected in the PBMC assay. Bacterial lipopolysaccharide (LPS) has been shown to potently inhibit both R5-tropic and X4-tropic HIV-1 in $CD4^+$ monocyte-derived macrophages (MDM) and in lymphocytes by down-regulating receptors and by the release of soluble inhibitory factors that include the β-chemokines MIP-1α, MIP-1β, RANTES (Verani et al., J. Exp Med. 185:805-816, 1997; Verani et al., J. Immunol. 168:6388-6395).

In Figure 33 we confirmed that LPS inhibits HIV-1 in PBMC and we show that LPS has no antiviral effect in TZM-bl cells. Low concentrations of LPS (0.1-10 EU/ml) can reduce HIV-1 infection in the PBMC assay by ≥80%. Though LPS did not neutralize HIV-1 in the TZM-bl assay, supernatant from PBMCs that were incubated overnight with LPS did (Fig. 34). This suggests that HIV-1 neutralization does not occur via

direct virus-LPS interactions. Rather, LPS appears to act on PBMC to stimulate the production of soluble HIV-1-inhibitory factors. To investigate this possibility further, we tested LPS-conditioned PBMC medium for the presence of β-chemokines. As shown in Figure 35, high levels of MIP-1α and MIP-1β and low levels of RANTES were detected at LPS concentrations as low as 0.3 EU/ml. Moreover, a substantial portion of this antiviral activity of LPS could be blocked by an anti-MIP-1α antibody and, to a lesser extent, by anti-MIP-1β antibody (Fig. 36), suggesting that these two β-chemokines contribute to the antiviral effects of LPS. As further evidence that β-chemokines are mediators of the anti-HIV-1 effects of LPS, we show in Table 32 that RANTES and the LD78β isoform of MIP-1α have potent antiviral activity against R5 strains of HIV-1. Notably, the R5X4 virus WEAU was not neutralized by any of the β-chemokines, suggesting that there remain some unidentified soluble inhibitory factors that are released by endotoxin stimulation.

Finally, we tested whether LPS contamination affects the neutralizing activity of IgG1b12, 2G12, 2F5, 4E10 and HIVIG in PBMC and in TZM-bl cells. Aliquots of the mAbs and HIVIG were intentionally spiked with a relatively large amount (30,000 EU/ml) of LPS and tested in both assays along side with the corresponding non-contaminated samples. Samples were assayed against the Env-pseudotyped virus QH0692.42 in the TZM-bl assay and against the IMC-rLuc virus Bal in the PBMC assay. LPS contamination increased the potency of the samples by ~100-fold in the PBMC assay but had no measureable effect in the TZM-bl assay (Fig. 37).

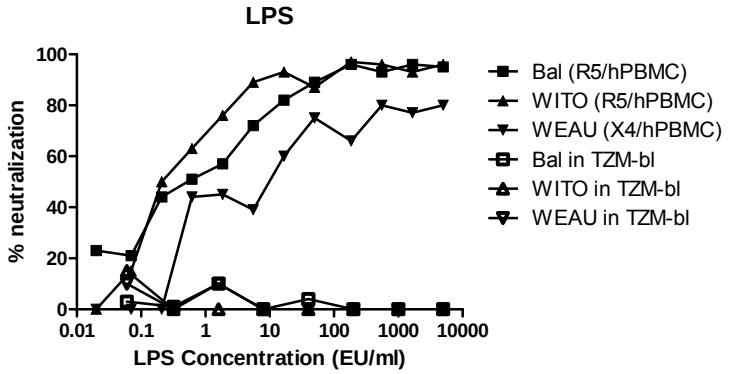

Figure 33. LPS inhibits HIV-1 in PBMC but not in TZM-bl cells*. Two R5 viruses and one R5/X4 virus were assayed in the presence of multiple concentrations of LPS.*

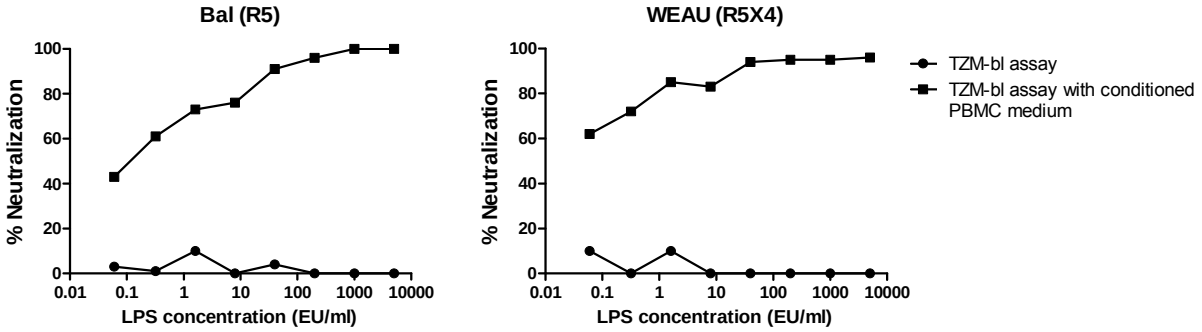

Figure 34. Endotoxin activity is related to the release of soluble HIV-1 inhibitory factors*. PHA-stimulated PBMC were incubated overnight in the presence of multiple concentrations of LPS. Conditioned culture fluids were transferred to corresponding 96-well plates and TZM-bl cells and virus were added. As a control, LPS was assayed directly in TZM-bl cells. Neutralization was assessed by Luc activity two days later.*

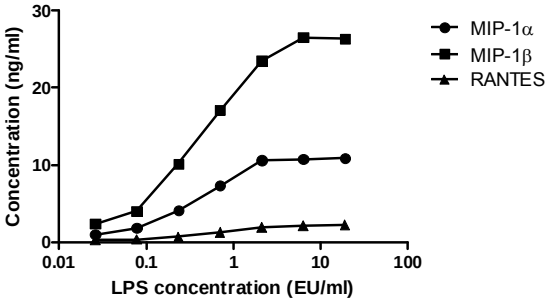

Figure 35. Overnight exposure of PBMC to LPS induces the production of β-chemokines MIP-1α, MIP-1β and RANTES.

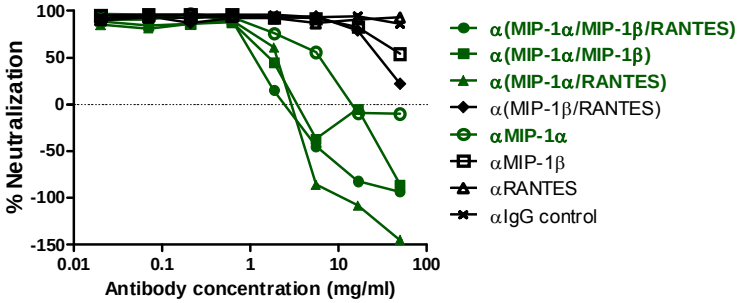

Figure 36. Antibodies against β-chemokines, especially MIP-1α, blocked the neutralizing activity of 5,000 EU LPS/ml against HIV-1 Bal in a PBMC assay.

Table 32. β-Chemokines inhibit R5 HIV-1 in PBMC.

	IC80 (ng/ml)						
Chemokine	Bal	WITO	SF162	CH40	CH58	CH77	WEAU
MIP-1α LD78α	400	120	>1250	126	>1250	>1250	>1250
MIP-1α LD78β	13	10	43	7	18	109	>1250
MIP-1β	>1250	1160	>1250	615	>1250	>1250	>1250
RANTES	66	36	171	37	75	>1250	>1250

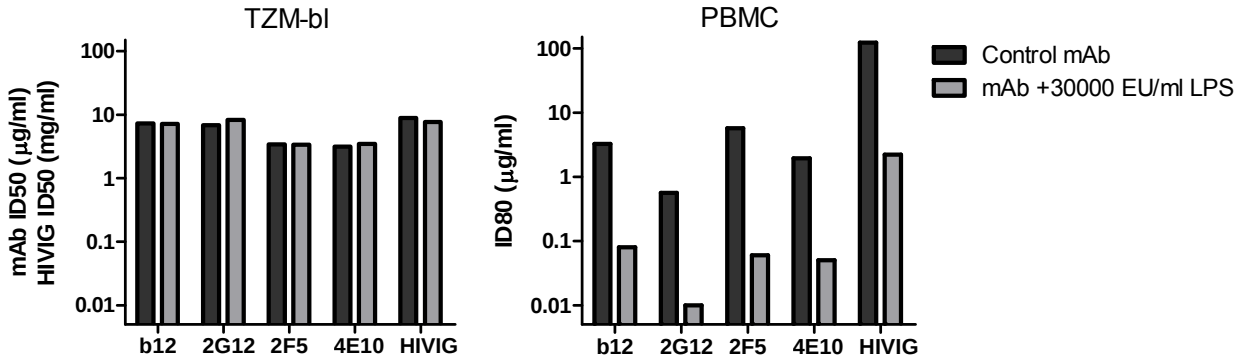

***Figure 37. LPS has no effect on neutralizing antibodies in the TZM-bl assay**. Left panel: QH0692.42 assayed in TZM-bl cells. Right panel: Bal.LucR.T2A.ecto assayed in PBMC. Assays were performed with mAbs and HIVIG containing or lacking LPS.*

3.6.17 Consequences of murine leukemia virus in TZM-bl cells.

A recent report demonstrated the presence of murine leukemia virus (MLV) in the TZM-bl cell line (Takeuchi et al., J. Virol. 82:12585-12588, 2008). MLV appears to have been introduced during the transduction procedures that were used to express CD4 and CCR5 in the progenitor HeLa cell line (Dr. David Kabat, personal communication). Thus, Dr. Kabat has confirmed that the $CD4^+$, $CCR5^+$ cell line, JC.53, was contaminated with replication-competent MLV prior to receipt of the cells by Dr. John Kappes, who subsequently introduced the Tat-regulated Luc and β-gal reporter genes to create the TZM-bl cell line. Dr. Kabat has documented very low levels of MLV and shown that MLV has no effect on HIV-1 infection in TZM-bl cells (personal communication).

In order to determine whether MLV affects the measurement of HIV-1 neutralizing antibodies in TZM-bl cells, we obtained a $CD4^+$, $CCR5^+$ HeLa cell line (JC.48) from Dr. Kabat that was confirmed by him to be MLV-free by Western blot analysis and by an inability to transfer of MLV infection to NIH/3T3 cells (TZM-bl cells were positive in both tests). The MLV-free JC.48 cells express CCR5 at somewhat lower levels than TZM-bl cells (~5-fold lower), which may be expected to reduce their susceptibility to HIV-1 infection and may also be expected improve their sensitivity for detecting neutralizing antibodies (Choudhry et al., 2006). We quantified infectivity in both cell line and as expected, infectivity was reduced in JC.48 cells but was still adequate for neutralization assays (Fig. 38). Using this information, we performed neutralization assays with adjusted virus doses such that their relative infectivity was equal. As seen in Figure 39, assay results in the two cell lines were equivalent for 3/3 viruses assayed with a wide range of neutralizing reagents (all results were within 2-fold). These results indicate that the presence of MLV in TZM-bl cells has no measurable effect on the performance of HIV-1 neutralization assays.

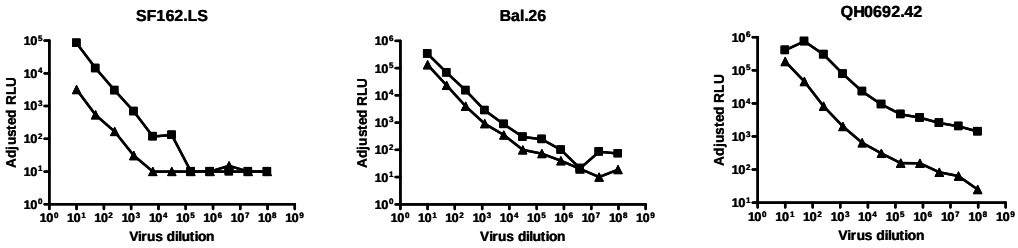

Figure 38. HIV-1 infectivity in TZM-bl compared to JC.48 cells. *Squares, TZM-bl cells; Triangles, JC.48 cells.*

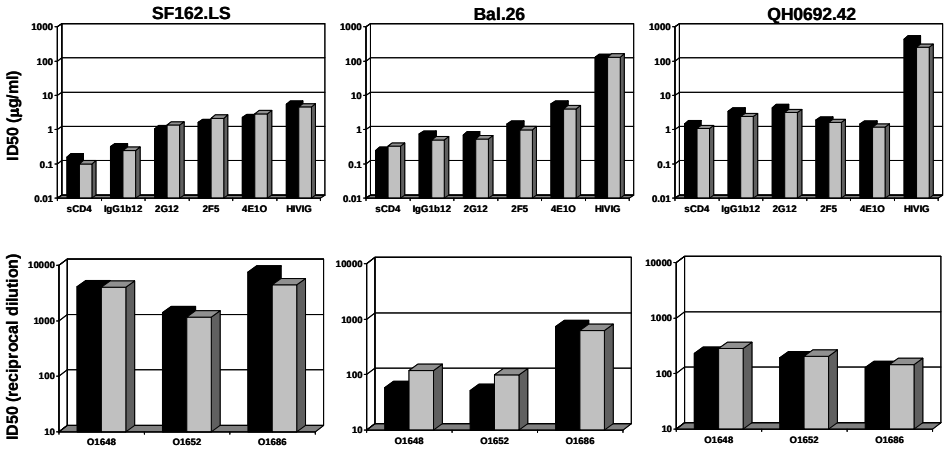

Figure 39. Assay performance in TZM-bl compared to JC.48 cells using adjusted virus doses. *Input virus dose was adjusted to be equivalent for two viruses. Black bars, TZM-bl cells; Grey bars, JC.48 cells. Top panel: sCD4, Mabs and HIVIG (purified IgG from pooled HIV-1-positive plasmas). Bottom pane: Individual HIV-1 positive plasma samples.*

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