Introduction:

We have completed the analysis of your HIV RNA Validation Study. The validation plan was designed to verify the installation of an unmodified FDA-approved HIV RNA assay into your laboratory. The validation plan utilized both VQA HIV RNA controls and clinical samples to verify the manufacturer's claimed performance specifications.

The Viral Load Validation Working Group (VLVWG) was formed to help The VQA develop this plan. The VLVWG was comprised of virologists from the AIDS clinical trial networks who reviewed the data from the validation testing while the plan was in the developmental stages. The VQA Quality Assurance Subcommittee (QASC) reviewed all the validation reports in a blinded manner prior to being sent to the laboratory as a final document.

This report was created to summarize the data generated by the testing laboratory for use in the validation of the installation of a new HIV RNA assay platform. The VQA has highlighted potential problems with the data (i.e. those areas where the data exceeded the designated targets), but the laboratory director will make the ultimate decision as to whether or not the validation results are acceptable. A copy of the final un-blinded report has been sent to the testing laboratory director and the primary network laboratory (PNL) or DAIDS as appropriate.

Note: the intention of this report is to provide data on a laboratory's ability to perform a newly installed HIV RNA assay as defined by the expectations derived for clinical trial testing and the manufacturer's specification. This validation plan is not intended to provide an 'Approval' for switching assays or generating data for clinical trials. Any change in HIV RNA testing that involves clinical trial testing will require explicit approval from the network leadership and protocol teams for which the testing is being done.

Table/Figure Descriptions:

Data generated from VQA HIV-1 RNA copy controls were used to evaluate precision, sensitivity (false negative results), accuracy, and reportable range. Data generated from clinical samples were used to assess correlation, carryover effects and specificity (false positive results).

Table 1: A list of the concentrations and number of replicates of the VQA HIV RNA copy controls as well as the number of seropositive and seronegative specimens tested in this evaluation are provided in Table 1. The concentrations of the VQA controls used were dependent on the sensitivity of the assays being tested.

Tables 2, 3, 4: Precision statistics were evaluated using the $\log_{10}$ transformed HIV RNA results. Intra- and inter-assay components of variation were calculated and combined to generate a total-assay standard deviation (SD). Estimates of the intra-assay, inter-assay, and total-assay standard deviations for each nominal concentration are provided in Tables 2, 3, and 4, respectively. Separate calculations are provided for the two data sets. The total assay SD should not exceed the target of 0.15. This target is used by The VQA program for proficiency testing as well and is based on the premise that clinicians wanted to have the statistical power to detect 5-fold changes in HIV virus load.

Table 5: The percentage of positive results obtained for samples with a nominal concentration above and below the assay lower limit of detection is shown in Table 5. This was based on the manufacturer's definition of a detectable result and a separate calculation was provided for each assay platform. A p-value is provided for each assay to indicate if the detection rate of the sample with a nominal value near the lower detection limit of the assay (e.g. 50 copies/mL) is significantly different (too many false negatives) than what would be expected for a 95% detection rate by chance alone.

Table 6: The percentage of results with an estimated RNA concentration > 50 copies/mL for samples with a nominal concentration of 50 copies/mL is shown in Table 6. A p-value comparing the percentage of samples with results > 50 copies/mL across the two assays is also provided. This concentration was selected because it is used in many clinical trials as a cutoff for virologic failure. The expected percentage of samples that yield a result > 50 copies/mL is 50%.

Table 7: The assay templates were designed such that known negative samples were positioned immediately following known high positive samples tested to see if there was any carryover effect between samples that could affect an unknown result. All of the estimates for the HIV high positive controls should be positive, and all of the estimates for the HIV seronegative samples should be undetectable. Separate calculations are provided for each data set in Table 7.

Tables 8a-c: The results generated for VQA HIV RNA copy controls and seropositive clinical samples were compared across the two platforms. For VQA HIV RNA controls, the $\log_{10}$ transformed nominal value (the expected result based on the serial dilution of a stock virus) was subtracted from the $\log_{10}$ transformed result generated for VQA HIV RNA copy controls on each platform. The $\log_{10}$ transformed results from the seropositive donor samples generated on one assay platform were subtracted from the $\log_{10}$ transformed result obtained on the other assay platform. In both cases, differences that exceeded 0.5 were deemed problematic and are tabulated in Table 8a. Table 8b provides the average and the SD of the $\log_{10}$ differences obtained on HIV seropositive clinical samples and VQA HIV RNA copy controls. If there were clinical samples or VQA HIV RNA copy control results with $\log_{10}$ estimates that differed by more than 0.5, then table 8c will exist and list the donor # or control nominal value, the results obtained on each of the HIV RNA platforms and the $\log_{10}$ difference that was calculated. Table 8c will not exist if there were no problems noted.

Figures 1, 2: The results obtained on seropositive samples tested across the two HIV RNA assay platforms are graphically compared in Figures 1 and 2. The $\log_{10}$ estimates of the comparator assay plotted against the $\log_{10}$ estimates of new assay are shown in figure 1. The correlation (r) for this comparison was provided in the graph together with a p-value. A high correlation with a significant p-value would indicate a good correlation between the results obtained on each platform. The target correlation should be > 0.95.

The difference between the estimates obtained on each platform plotted against the average $\log_{10}$ results obtained on both assay platforms to determine if there were any differences in the data sets that may be concentration dependent are shown in figure 2. If the two assays are, on average, performing the same, then the scatter in the plot would form a horizontal band centered on a horizontal reference line of zero difference. Horizontal scatter above or below the line indicate that the assays differ but that the difference does not vary with RNA concentration. Scatter that is not horizontal would indicate that the differences do vary with RNA concentration. The $\log_{10}$ difference for any given sample should not exceed $0.5\log_{10}$ RNA copies/mL and data that do not form a horizontal band or exceed this target may require additional testing. A correlation (r) and p-value are provided to indicate how data correlate. A low correlation with an insignificant p-value is the target; this indicates the differences are not concentration dependent.

Figures 3, 4: Plots of $\log_{10}$ recovery for the new assay and comparator assay, respectively, using the results generated for VQA HIV RNA copy controls are shown in Figures 3 and 4. Log_{10} recovery was plotted against $\log_{10}$ nominal concentration. Recovery is defined as the ratio of measured to nominal RNA (nominal RNA is the value assigned to the sample by The ***VQA LAB***, ref. 1). Thus, $\log_{10}$ recovery was the difference between $\log_{10}$ measured RNA and $\log_{10}$ nominal RNA value. In the linear range, a $\log_{10}$ recovery plot should form a horizontal band. Departures from that shape indicate loss of linearity. A reference line at zero is included in each plot. The controls selected in this study encompassed the reportable range of the new assay as defined by the manufacturer. For validation purposes, a sample with a nominal concentration of 1,500,000 copies/mL was selected to evaluate the upper limit of detection for the newer real-time PCR assays and a sample with a nominal concentration of 50 copies/mL was used to evaluate the lower detection limit. A correlation (r) and p-value are provided to indicate how the data correlate. The correlation between log recovery and log nominal concentration is provided with the p-value for testing the null hypothesis of zero correlation. A statistically significant correlation indicates that recovery varies with concentration, whereas a correlation that is not statistically significant indicates that no variation in recovery was detected across the range of nominal concentrations tested

References:

1. B. Yen-Lieberman, D. Brambilla, B. Jackson, J. Bremer, R. Coombs, M. Cronin, S. Herman, D. Katzenstein, S. Leung, H.J. Lin, P. Palumbo, S. Rasheed, J. Todd, M. Vahey and P. Reichelderfer. 1996. Evaluation of a quality assurance program for quantitation of Human Immunodeficiency Virus Type 1 RNA in plasma by the AIDS Clinical Trials Group virology laboratories. J. Clin. Microbiol. 34:2695.

Laboratory-Specific Comments:

The two assays used for the validation study performed by VQA laboratory 281 (ZA_Drbn_CAPRISA) was the Standard Roche COBAS AmpliPrep/COBAS Amplicor HIV-1 Monitor Test, v1.5 (stdcam) for the comparator test, and Roche COBAS AmpliPrep/COBAS TaqMan Assay (taqman) for their new test. The reportable range for the stdcam assay is 400 – 1,000,000 copies/mL and is 48 – 10,000,000 copies/mL for the taqman assay.

The results from this validation study were all valid and all the data were included in this analysis. Table 1 lists the samples that were included in the validation.

Precision:
The precision statistics for $\log_{10}$ transformed data are provided in Tables 2, 3 and 4. The total assay SD should not exceed the target of 0.15. This target was exceeded in 1 out of 4 control sets tested for both the taqman and the stdcam assay (0.213 for the 1500000cp/mL control for taqman and 0.167 for the 1500cp/mL control for stdcam). The increased variation noted in the stdcam assay was attributed mainly to inter-assay variation, and the main contribution was intra-assay variation for the taqman assay as noted in tables 2 and 3.

Sensitivity:
Tables 5 and 6 provide the summary of the sensitivity analysis. Table 5 shows that the taqman assay detected 87.5% of samples with nominal values of 50 copies/mL and 75% of the samples with a nominal value of 25 copies/mL. The stdcam assay detected 25% and 0% of the 50 and 25 copies/mL controls, respectively. While this is a statistically significant reduction ($p<0.001$), it is important to note that the Standard Monitor test used in this evaluation has a reported lower detection limit of 400 copies/mL so this would be expected. There was no statistically significant reduction in sensitivity noted in the taqman assay.

Table 6 shows that 87.5% of the samples that were detectable on the taqman yielded a result that was > 50 copies/mL. None of the controls yielded a result > 50 copies/mL on the stdcam assay, presumably because the Standard extraction was utilized.

Correlation:
The results from the correlation analysis are provided in figures 1 and 2. In figure 1, the $\log_{10}$ results obtained for the HIV+ clinical samples using the taqman assay were compared to the results obtained using the stdcam assay. The graph shows a correlation (r) of 0.950 ($p < 0.001$) for the virus load results obtained on clinical samples using the stdcam and taqman assays. The target correlation is > 0.95.

Figure 2 is a plot that compares the difference between the results obtained on the taqman and stdcam kits for HIV+ clinical samples with the average $\log_{10}$ result. The data showed no correlation between the difference in $\log_{10}$ recovery vs. average $\log_{10}$ recovery ($r = 0.264$, $p = 0.166$) which indicates the $\log_{10}$ recovery was not concentration dependent. Table 8a shows that the difference in $\log_{10}$ recovery for 5 (17.2%) out of 29 HIV+ clinical samples and 4 out of 34 VQA HIV RNA controls on the taqman assay did exceed the target of 0.5. The actual virus loads obtained on each of the two kits is presented in table 8c. Table 8b shows the average Log_{10} difference noted in the taqman assay was 0.339 vs. and average $\log_{10}$ difference of 0.066 with the stdcam kit for VQA RNA controls. The average $\log_{10}$ difference in virus load results obtained with abbott and usmon on clinical samples is -0.043. There currently is no established target for this criterion.

Accuracy:
The results of the accuracy analysis are provided in figures 3 and 4. The $\log_{10}$ nominal value for VQA HIV RNA copy controls was subtracted from the $\log_{10}$ result obtained on each of the kits (figure 3 is taqman and figure 4 is stdcam) and plotted against the $\log_{10}$ nominal value in a Bland-Altman plot. Controls used for sensitivity evaluations were not included in these plots. A positive correlation was noted in the taqman ($r = 0.438$, $p = 0.010$) suggesting the difference in recovery was concentration dependent. A positive correlation was also noted in the stdcam assay ($r = 0.718$, $p = 0.001$) indicating the $\log_{10}$ recovery obtained for HIV RNA copy controls was also concentration dependent. The average $\log_{10}$ recovery for the taqman assay is consistently higher in the VQA controls (sodium citrated plasma) than in the clinical donor samples (EDTA plasma) and is probably associated with the anticoagulant used to create VQA copy controls. The $\log_{10}$ difference noted in 4 results for 1,500,000 copies/mL controls did exceed the target of 0.5 and may be related to the shifted recovery noted in VQA controls, but this is the control that also yielded high variation.

Reportable Range:
Figures 3 and 4, and table 5 provide an overview of the performance of this assay for the reportable range of these assays. For taqman, the reportable range is 48 – 10,000,000 copies/mL. For the stdcam assay, the reportable range is 400 – 1,000,000 copies/mL. The detection limit for samples with a nominal value of 50 copies/mL was 100% for the

taqman assay but the lower detection limit was not challenged in this evaluation for the stdcam assay. However, all the results with nominal values within the reportable ranges of the respective assays were detectable. There was no sample tested with a nominal value of 10,000,000 copies/mL, but the control with a nominal value of 1,500,000 copies/mL was detectable and represents the titer of a high titered sample that may be seen in their clinical population. There were no problems noted in either data set.

Carryover and Specificity:
Table 7 provides result for the carryover study. All the HIV seronegative samples yielded an undetectable result, even if the sample was preceded by a sample with a high nominal value of 1,500,000 copies/mL. There were no problems noted in either data set.

Summary:
The data suggest the taqman assay is performing within the expectations of the manufacturer, except for the precision noted for the VQA 1,500,000 copies/mL control and may warrant repeat testing. The data obtained on the taqman were comparable to those obtained on the stdcam, but differences in $\log_{10}$ recovery did exceed the target for 4 VQA HIV RNA copy controls and 5 clinical samples and the laboratory should review the data to determine if the difference could be explained. The differences noted in the controls may be anticoagulant related, but the differences noted in the clinical samples may warrant repeat testing.

The significant trends noted in the correlation studies for the HIV+ donor samples tested on the taqman should be reviewed to determine if the differences noted are clinically relevant.

If you have any questions regarding this report, please contact Suzanne Granger as soon as possible. I can be reached by telephone (617-923-7747, X319) or E-mail at SGranger@neriscience.com.

LAB 281 TAQMAN vs. LAB 281 STANDARD COBAS-AMPLIPREP

TABLE 1: SAMPLES INCLUDED IN THE HIV RNA VALIDATION STUDY

	Number of Samples	
VQA HIV RNA Nominal Concentration (copies/mL)	**281 taqman**	**281 stdcam**
25	4	4
50	8	8
1,500	9	9
15,000	9	9
150,000	9	9
1,500,000	7	7
Unique Clinical Donor Samples: HIV+	39	39
Unique Clinical Donor Samples: HIV-	20	20

TABLE 2: INTRA-ASSAY STANDARD DEVIATION OF LOG10 RNA COPIES/ML AT EACH NOMINAL CONCENTRATION

	NOMINAL CONCENTRATION (Copies/mL)			
Lab-Assay	**1,500**	**15,000**	**150,000**	**1,500,000**
281-taqman	0.117	0.068	0.051	0.184
281-stdcam	0.096	0.087	0.111	0.049

TABLE 3: INTER-ASSAY STANDARD DEVIATION OF LOG10 RNA COPIES/ML AT EACH NOMINAL CONCENTRATION

	NOMINAL CONCENTRATION (Copies/mL)			
Lab-Assay	**1,500**	**15,000**	**150,000**	**1,500,000**
281-taqman	0.000	0.027	0.003	0.107
281-stdcam	0.137	0.000	0.056	0.062

TABLE 4: TOTAL-ASSAY STANDARD DEVIATION OF LOG10 RNA COPIES/ML AT EACH NOMINAL CONCENTRATION

	NOMINAL CONCENTRATION (Copies/mL)			
Lab-Assay	**1,500**	**15,000**	**150,000**	**1,500,000**
281-taqman	0.117	0.073	0.051	0.213
281-stdcam	0.167	0.087	0.125	0.079

TABLE 5: PERCENTAGE OF DETECTABLE RESULTS ACCORDING TO MANUFACTURERS DEFINITION

	NOMINAL CONCENTRATION (Copies/mL)	
Lab-Assay	**25 N = 4**	**50 N = 8**
281-taqman	75%	87.5%[1]
281-stdcam	0%	25%[2]

[1] P-value=0.34
[2] P-value=<.001

TABLE 6: PERCENTAGE OF RESULTS >50 COPIES/ML AT A NOMINAL CONCENTRATION OF 50 COPIES/ML

Lab-Assay	**% > 50 copies/mL[1] N = 8**
281-taqman	87.5%
281-stdcam	0%

[1] P-value=0.001, Fisher's exact test

TABLE 7: ANALYSIS OF CARRYOVER

	NEGATIVE CONTROLS & HIV SERO-NEGATIVE DONORS		1,500,000 COPIES/ML VQA CONTROLS	
Lab-Assay	**# Tested**	**# Negative Results**	**# Tested**	**# Detectable Results**
281-taqman[1]	25	25	7	7
281-stdcam[2]	25	25	7	7

[1] Results indicate that no carryover was detected
[2] Results indicate that no carryover was detected

TABLE 8a: NUMBER OF DIFFERENCES EXCEEDING 0.5 LOG10

Lab-Assay	# Samples Tested	# Samples Exceeding 0.5 Log10 Difference
281-taqman[1]	34	4
281-stdcam[1]	34	0
HIV Seropositive Donors[2]	29	5

[1] Only includes VQA controls in the linear range (1,500 - 1,500,000 copies/mL)
[2] Only includes donors in which a quantitatively detected result was obtained from both assays

TABLE 8b: AVERAGE LOG10 DIFFERENCE

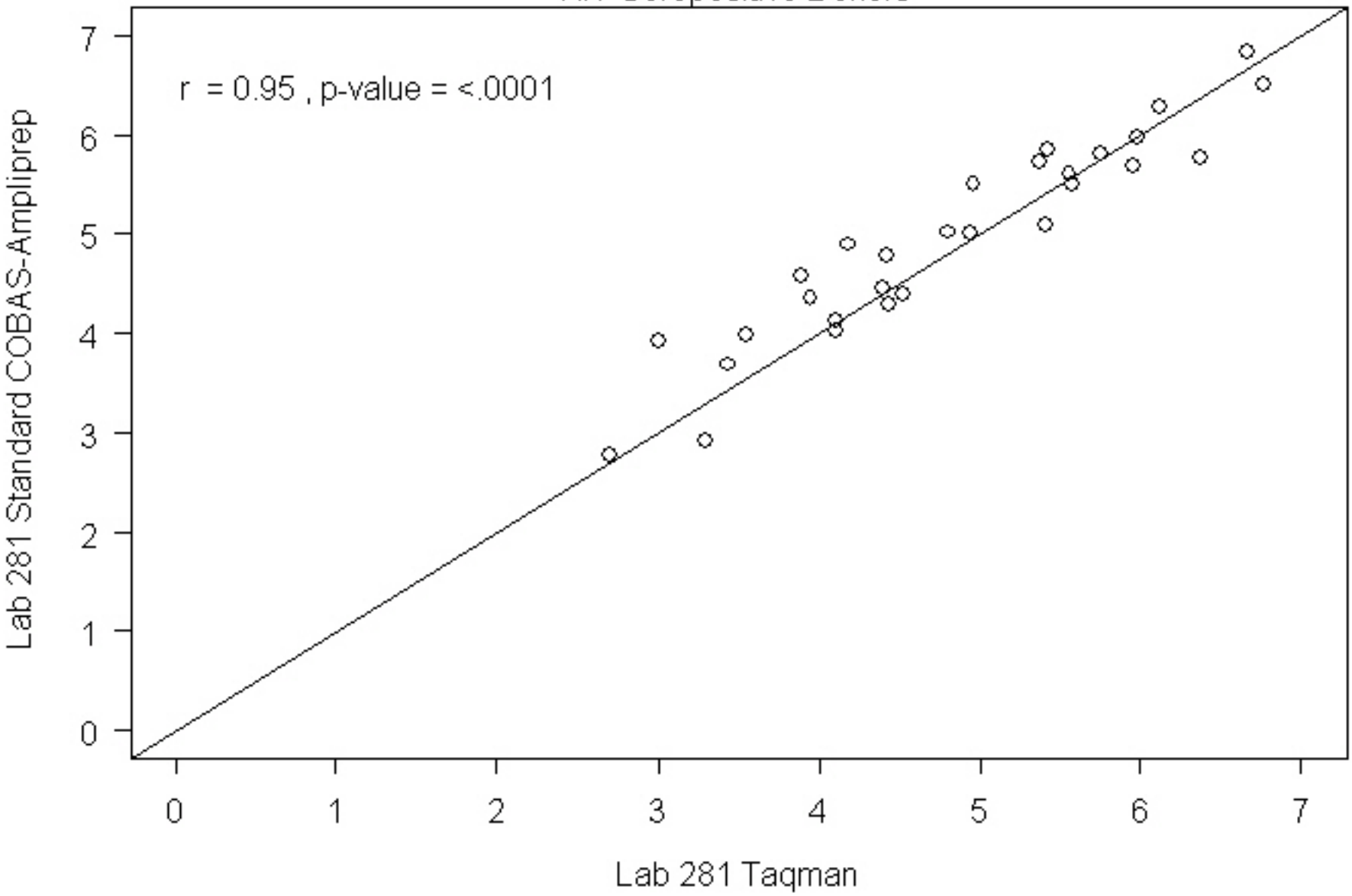
Lab-Assay	# Samples Tested	Average Log10 Difference	Standard Deviation
281-taqman[1]	34	0.339	0.134
281-stdcam[1]	34	0.066	0.162
HIV Seropositive Donors[2]	29	-0.125	0.349

[1] Only includes VQA controls in the linear range (1,500 - 1,500,000 copies/mL)
[2] Only includes donors in which a quantitatively detected result was obtained from both assays

TABLE 8c: LIST OF SAMPLES WITH DIFFERENCES EXCEEDING 0.5 LOG10

Donor #/ VQA Control	taqman result (copies/mL)	stdcam result (copies/mL)	Log10 Difference
Donor #8	15,373	78,500	-0.708
Donor #10	1,017	8,320	-0.913
Donor #12	91,760	312,000	-0.532
Donor #29	7,740	37,900	-0.690
Donor #38	2,339,737	585,000	0.602
1,500,000 copies/mL	4,753,467		0.501
1,500,000 copies/mL	5,365,627		0.554
1,500,000 copies/mL	4,789,190		0.504
1,500,000 copies/mL	9,414,344		0.798

Figure 1
Correlation Evaluation
HIV Seropositive Donors



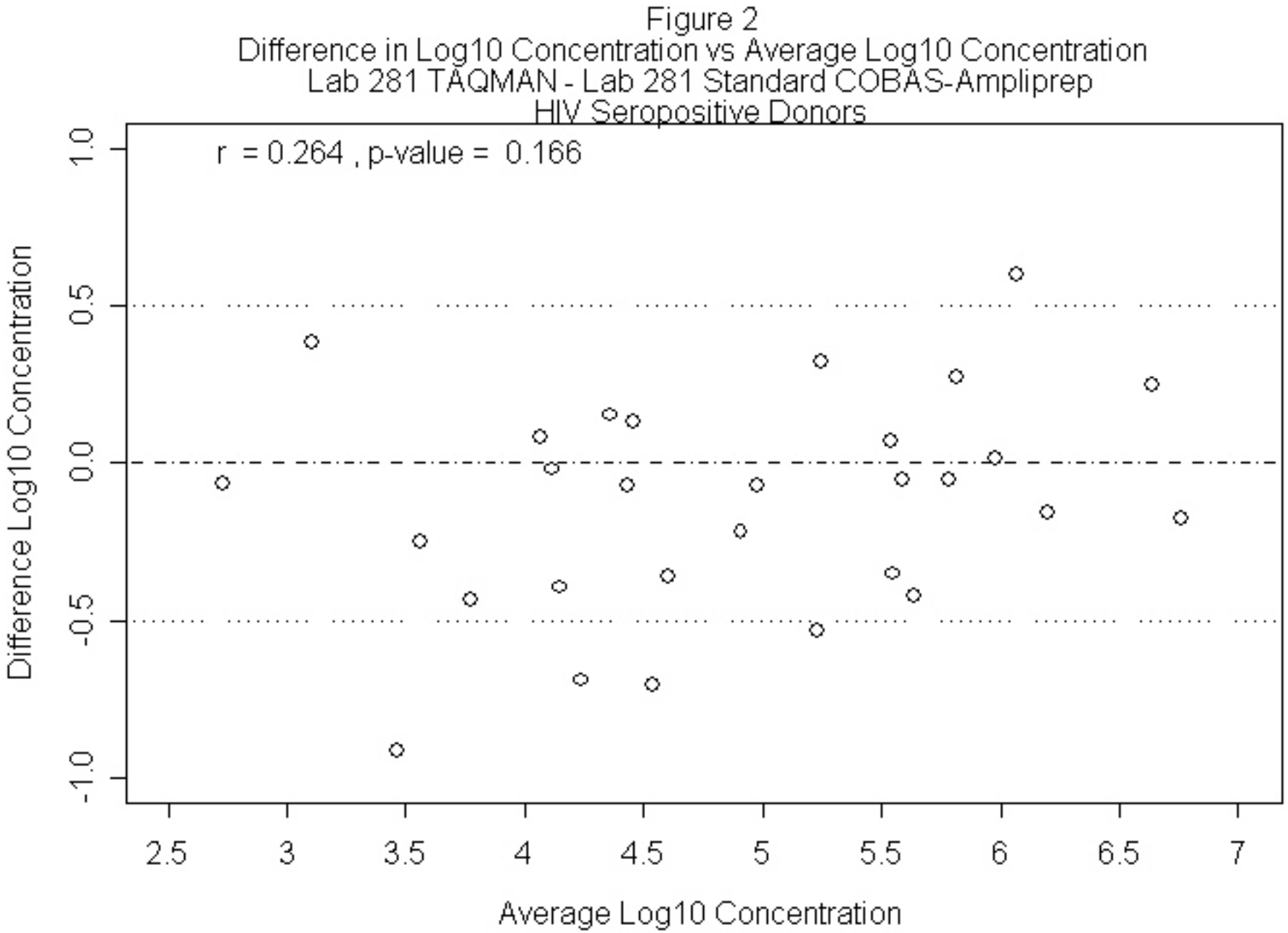


Figure 2
Difference in Log10 Concentration vs Average Log10 Concentration
Lab 281 TAQMAN - Lab 281 Standard COBAS-Ampliprep
HIV Seropositive Donors
r = 0.264 , p-value = 0.166
Difference Log10 Concentration
1.0
0.5
0.0
-0.5
-1.0
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
Average Log10 Concentration

Figure 3
Log10 RNA Recovery by Log10 Nominal Concentration
VQA Controls Lab 281 TAQMAN

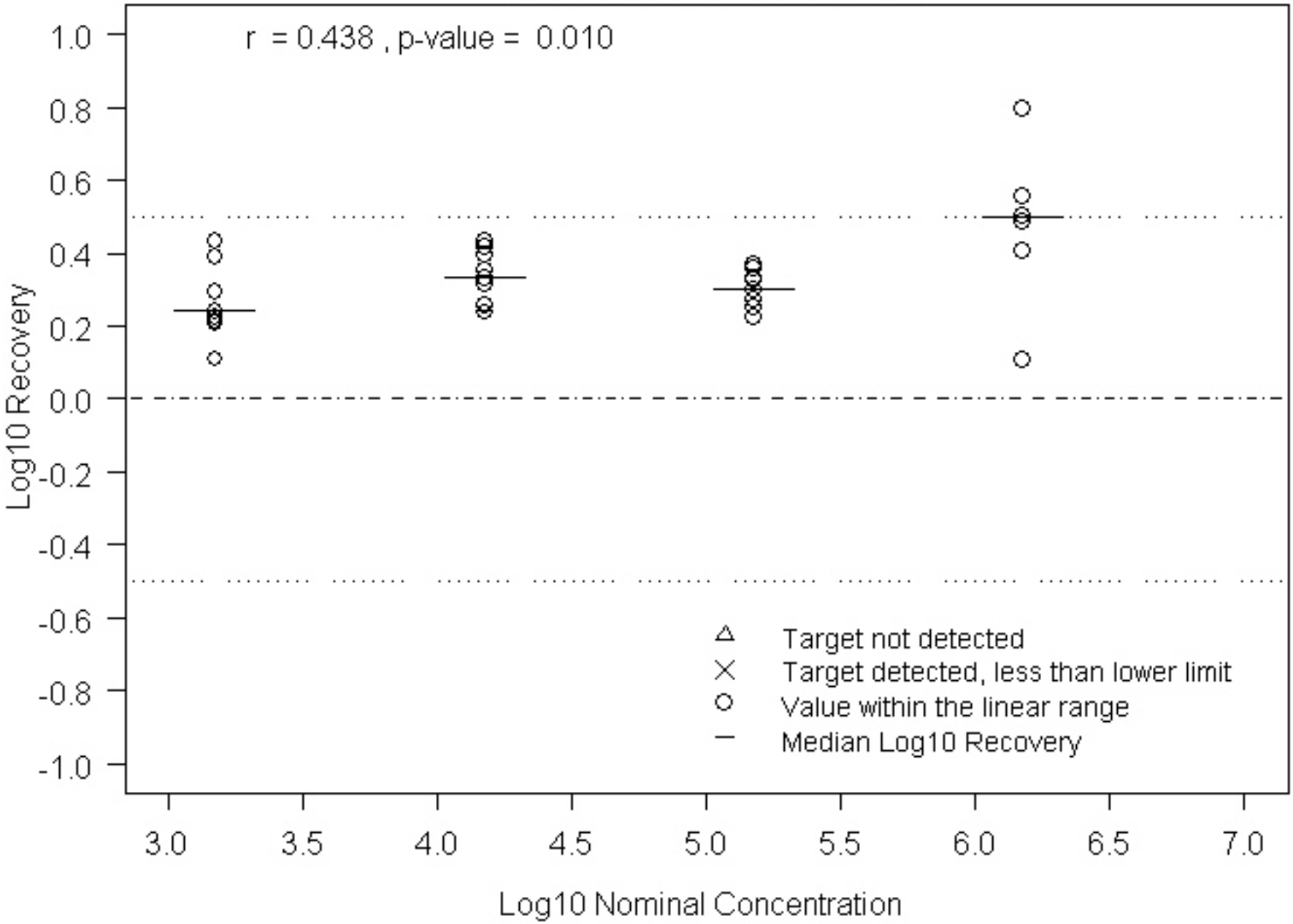


Figure 4
Log10 RNA Recovery by Log10 Nominal Concentration
VQA Controls Lab 281 STANDARD COBAS-AMPLIPREP