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Clinical Validation of a Novel Diagnostic HIV-2 Total Nucleic Acid Qualitative Assay Using the Abbott m2000 Platform: Implications for Complementary HIV-2 Nucleic Acid Testing for the CDC 4th Generation HIV Diagnostic Testing Algorithm

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Abstract

Background—The 2014 CDC 4th generation HIV screening algorithm includes an orthogonal immunoassay to confirm and discriminate HIV-1 and HIV-2 antibodies. Additional nucleic acid testing (NAT) is recommended to resolve indeterminate or undifferentiated HIV seroreactivity. HIV-2 NAT requires a second-line assay to detect HIV-2 total nucleic acid (TNA) in patients' blood cells, as a third of untreated patients have undetectable plasma HIV-2 RNA.

Objectives—To validate a qualitative HIV-2 TNA assay using peripheral blood mononuclear cells (PBMC) from HIV-2–infected Senegalese study participants.

Study design—We evaluated the assay precision, sensitivity, specificity, and diagnostic performance of an HIV-2 TNA assay. Matched plasma and PBMC samples were collected from 25 HIV-1, 30 HIV-2, 8 HIV-1/-2 dual-seropositive and 25 HIV seronegative individuals. Diagnostic performance was evaluated by comparing the outcome of the TNA assay to the results obtained by the 4th generation HIV screening and confirmatory immunoassays.

Results—All PBMC from 30 HIV-2 seropositive participants tested positive for HIV-2 TNA including 23 patients with undetectable plasma RNA. Of the 30 matched plasma specimens, one

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was HIV non-reactive. Samples from 50 non-HIV-2 infected individuals were confirmed as non-reactive for HIV-2 Ab and negative for HIV-2 TNA. The agreement between HIV-2 TNA and the combined immunoassay results was 98.8% (79/80). Furthermore, HIV-2 TNA was detected in 7 of 8 PBMC specimens from HIV-1/HIV-2 dual-seropositive participants.

Conclusions—Our TNA assay detected HIV-2 DNA/RNA in PBMC from serologically HIV-2 reactive, HIV indeterminate or HIV undifferentiated individuals with undetectable plasma RNA, and is suitable for confirming HIV-2 infection in the HIV testing algorithm.

Keywords

HIV-2; diagnostic; qualitative; total nucleic acid; DNA/RNA; PCR

BACKGROUND

Accurate HIV testing is the cornerstone of HIV/AIDS diagnosis, prevention, care and treatment. In the current United States Centers for Disease Control and Prevention (CDC) 4th generation HIV-1/HIV-2 testing algorithm [1], the initial screening utilizes HIV-1/2 antigen(Ag)/antibody(Ab) combination immunoassays, and resulting reactive samples are further tested using an HIV-1/HIV-2 Ab differentiation immunoassay. Samples determined to be HIV non-reactive or HIV-1 Ab and/or HIV-2 Ab indeterminate are recommended for further testing with FDA-approved HIV-1 nucleic acid tests (NAT). Due to the absence of an FDA-approved HIV-2 NAT, HIV-1/HIV-2 undifferentiated (dually seropositive) and HIV-2 Ab indeterminate samples are not clearly addressed in the algorithm. Although detectable HIV-2 plasma RNA confirms HIV-2 infection, undetectable HIV-2 plasma viral loads (<10 RNA copies/mL) have been reported in 36–46% of antiretroviral (ART)-naïve HIV-2–infected patients [2–4]. A second-line NAT that specifically detects HIV-2 DNA/RNA in PBMC is therefore essential for accurate HIV-2 diagnosis and would fulfill the need for a confirmatory HIV-2 assay in the current CDC testing algorithm.

OBJECTIVES

Our objective was to develop and validate a qualitative HIV-2 total nucleic acid (TNA) assay using the Abbott m2000 platform for clinical and research use.

STUDY DESIGN

Clinical samples

Senegalese study participant data, plasma, and PBMC were collected at the Clinique Des Maladies Infectieuses Ibrahima DIOP Mar, Centre Hospitalier Universitaire de Fann, Universite Cheikh Anta Diop de Dakar, Senegal, as part of ongoing studies investigating HIV-2 and HIV-1/HIV-2 dual infection and antiretroviral therapy. Written informed patient consent was obtained, and the University of Washington (UW) Institutional Review Board and the Senegal Ethics Committee (CNERS) approved all aspects of the study. Whole blood was collected in EDTA vacutainer tubes and PBMC were isolated by Ficoll gradient centrifugation. Plasma and PBMC were saved in Senegal at −80°C, shipped in liquid nitrogen dry shippers or dry ice to Seattle and stored at −80°C at UW. HIV infection status

was determined in Senegal from serum samples using the *Alere Determine*™ HIV-1/-2 rapid test and the ImmunoCombII HIV- 1 & 2 BiSpot assay (ImmunoCombII) (Alere). For genotyping, portions of the HIV-2 *pol* gene were PCR-amplified from PBMC and sequenced, and the data were subjected to phylogenetic analysis [5, 6]. PBMC and plasma from HIV-2 seropositive and HIV-1/-2 dual-positive participants, who at the time of collection were either ART-experienced or ART-naïve, were used for this study.

Paired PBMC and plasma samples from 25 HIV-1-infected and 25 HIV-negative individuals were provided by the UW Center for AIDS Research HIV Specimen Repository Program. All subsequent testing was performed in the CLIA-certified UW Retrovirology Laboratory using good laboratory practice.

Assay controls and standards

A molecular clone (plasmid pROD9) containing the full genome of the HIV-2 group A ROD strain was used to prepare positive controls and standards. The concentration of DNA in the pROD stock was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA) to calculate the copy number of HIV-2 genomes in pROD9 working dilutions. All working dilutions of pROD9 DNA was linearized by digestion with X*ho*I.

The negative control for the assay contained 1.3 million human PBMC prepared from HIV negative donors; one million cells were subject to the assay extraction procedure described below. The positive control contained nominal 180 copies of linear pROD9 DNA/million PBMC. Each serial dilution of pROD9 DNA at nominal 46, 23, 12, 6 or 3, copies were added to one million PBMC to evaluate assay sensitivity. The probit analysis function of Minitab 17 (Minitab statistical software, PA) was used to determine the limit of detection (LOD) and 95% confidence interval of the HIV-2 TNA assay.

To evaluate the potential for anticoagulant interference, venous blood was drawn from an HIV negative individual into vacutainers containing EDTA, sodium citrate, heparin, or acid citrate dextrose (ACD). PBMC were isolated from each blood sample by Ficoll gradient centrifugation. Aliquots of 1.3 million cells were then pelleted by centrifugation for assay testing.

HIV-2 TNA assay

For each sample, a 1.3-million PBMC pellet was mixed with 50μL Abbott proteinase K (>6000 units/mL), and 150μL of bulk lysis buffer (Abbott Molecular, US), and agitated at 56°C for 30 minutes using an Eppendorf Thermomixer at 5000rpm. Then, an additional 1.1-mL of bulk lysis buffer was added to each cell lysate to yield a final volume of 1.3 mL/sample. One-mL of lysate was processed by the Abbott m2000sp instrument using the Abbott *m*Sample DNA preparation kit and an Abbott proprietary customer-specific DNA/RNA extraction protocol. Both DNA and RNA were extracted and eluted in 210μL of buffer. Approximately 1000 copies of exogenous internal control RNA (IC) comprised of *in vitro* transcripts of a recombinant clone including fruit fly and bacteria genes [2]were dispensed to each sample during the extraction procedure, and subsequently co-amplified with HIV-2 DNA/RNA.

HIV-2 specific PCR primers and a fluorescent probe detecting both HIV-2 groups A and B were used as previously described [2]. The final concentrations of the primers and the probe in the RT-PCR step of this assay were 900nM and 250nM, respectively. Amplification with the same primers and a second HIV-2 probe (5′FAM-TGCTGGAGAGAACCTCCC-BHQPlus, Biosearch Technologies, CA) was applied to samples showing inefficient amplification by the first HIV-2 probe.

PCR fluorescence analysis and result reporting for this assay were designed using the Abbott laboratory-defined application program specific for the qualitative assay. The cut-off cycle number (CN) was determined by examining amplification graphs generated for the aforementioned low-copy pROD9 DNA standards used to evaluate assay sensitivity; the cut-off CN was near the largest HIV-2 CN reported for those standards and the level of assay detection limit. The Delta Cycle (DC) value for each sample was calculated as the cut-off CN minus the reported HIV-2 CN. Therefore, larger DC values corresponded to higher levels of HIV-2 TNA in the cell lysates. Samples yielding DC values between 0 and 1 were interpreted as inconclusive status and were subject to re-test. Samples testing negative were reported as “Not Detected”.

The endogenous genomic DNA control was used by amplifying human ribonuclease P gene (RPP30) to ensure adequate DNA was extracted from the approximately 1 million PBMC used in the assay-specific extraction protocol. Briefly, for the RPP30 qPCR assay, 5μL of extracted eluate was amplified along with human genomic DNA standards at 50ng, 5ng and 0.5ng/reaction (Human Taqman Control Genomic DNA, Thermo Fisher Scientific) using the Applied Biosystems 7900HT fast real-time PCR instrument. The primer/probe sequences and thermocycler settings were adopted from Hindson et al, [7].

HIV assays

The Abbott ARCHITECT HIV Ag/Ab Combo assay (Combo) determined HIV serological status and provided a signal-to-cutoff ratio (S/CO) for each sample. The Bio-Rad Multispot HIV-1/-2 Rapid Test (Multispot) was used to confirm and differentiate HIV-1/HIV-2 Ab. Plasma samples with dual-reactive results were diluted by 10-or 100-fold for further testing [8]. Samples that were Multispot dual-reactive following a 1:100 dilution were reported to be HIV reactive but undifferentiated. The Bio-Rad Geenius™ HIV 1/2 Supplemental Assay (Geenius) was used to retest Multispot HIV-undifferentiated specimens[9]. The UW HIV-2 RNA quantitative assay [2] was used to measure HIV-2 RNA levels in the plasma specimens. The UW HIV-1 TNA assay was a modification of the Abbott RealTi*m*e HIV-1 Qualitative assay [10] and used the aforementioned DNA/RNA extraction protocol.

RESULTS

Assay precision and sensitivity

To evaluate assay precision, a total of 24 replicates of positive and negative controls were tested daily over two days. Four sets of positive and negative controls were tested daily over eight additional days for the PBMC samples. There were no false-positive negative controls (N=56) detected by the HIV-2 TNA assay. The assay mean, total assay standard deviation,

and coefficient of variance for the positive control (N=56) were 5.76 DC, 0.40 DC and 6.97%, respectively.

To determine assay sensitivity, nominal copies of linear pROD9 DNA (46, 23, 12, 6, or 3 copies) in 1 million of PBMC were extracted and amplified. Sixteen replicas were tested at each DNA concentration across five days, as summarized in Table 1. The LOD for the assay was determined to be 26 copies/million cells (95%CI, 18–55 copies/million cells).

Assay specificity

PBMC samples from 25 HIV-negative, 25 HIV-1-infected, and 30 HIV-2-infected individuals were tested with assay positive and negative controls over eight different days. All PBMC from 25 HIV-negative and 25 HIV-1–infected individuals tested negative by the HIV-2 TNA assay. Thirty of thirty (100%; 95%CI lower bound 88.4%) PBMC from Senegalese HIV-2 seropositive participants tested positive; the median DC was 6.36 (IQR 5.01–7.74; range, 1.07–9.36). Twenty-three of the 30 (76.7%) tested HIV-2 seropositive participants had undetectable plasma RNA (Table 2). Twenty-three of the 30 samples were HIV-2 group A, 1 was HIV-2 group B and in 6 samples the HIV-2 group was not available. In addition, the HIV-2 TNA assay detected HIV-2 in PBMC from an additional 15 of 15 (100%) Senegalese HIV-2 seropositive participants whose matching plasma samples were unavailable.

Monitoring assay extraction procedure and potential inhibitors

To determine an acceptable range for RNA extraction performance, we analyzed the IC RNA CN for 64 controls and 80 PBMC samples tested over 8 assay runs. The mean (±SD) of IC CN for PBMC samples and controls prepared from EDTA blood was 32.95 (±0.41). Based on these results, samples with an IC CN >3-SD above the mean (i.e., CN> 34.18) were considered for re-testing.

To account for variation due to cell counting and DNA extraction, the extracted eluates were tested separately using the RPP30 qPCR assay. Analysis of RPP30 cycle number across the threshold (Ct) for the 64 controls and 80 tested samples generated a median of 23.10 and a range from 21.18 to 24.85. The acceptable RPP30 Ct for adequate DNA extraction was no more than 25 as determined by this validation study.

To test for potential interference, PBMC prepared from blood samples containing each of four different anti-coagulants were tested in quadruplicate. IC CN (mean ± SD) were 32.54±0.21, 32.83±0.26, 32.52±0.23, and 32.66±0.35 for PBMC pellets prepared with EDTA, sodium citrate, heparin, and ACD anti-coagulant, respectively, indicating no significant differences among the four groups (p=0.364, one-way ANOVA). The RPP30 Ct values were less than 25 for all sixteen PBMC pellets, indicating no interference on DNA extraction by sodium citrate, heparin, or ACD anti-coagulant.

Diagnostic performance compared to the CDC 4th generation testing algorithm

The assay's diagnostic performance for HIV-2 infection status was evaluated by comparing the outcome of PBMC tested using the HIV-2 TNA assay with the serologic results obtained

from the Combo and Multispot immunoassays using matching plasma samples. All 30 (100%) of the HIV-2 infected plasma samples were HIV reactive by the Combo (median S/CO=183.5; range, 5.89–293.67) and 29 of 30 (96.7%) samples were confirmed to be HIV-2 Ab reactive by the Multispot test. A single HIV-2 plasma sample (H2A32) was reactive by the Combo with an S/CO of 5.89 but was non-reactive by the Multispot test. This participant was previously determined to be HIV-2 infected by the ImmunoCombII assay. The H2A32 plasma sample also had undetectable HIV-2 RNA, while the corresponding PBMC was positive for HIV-2 TNA (DC=4.99) and negative for HIV-1 TNA. In addition, two participants (H2A67 and H2A76), whose plasma samples were weakly positive by the Multispot test, were positive for PBMC HIV-2 TNA (DC=1.07, 8.24, respectively; Table 2).

Plasma from 25 HIV-negative individuals tested non-reactive by the Combo assay. Plasma specimens from 25 HIV-1 infected individuals tested HIV reactive by Combo (median S/CO=525.1; range, 444.00–738.80) and were confirmed by the Multispot test. As mentioned above, the matched PBMC samples from 25 HIV-negative and 25 HIV-1 infected individuals were negative by the HIV-2 TNA assay. Therefore, the agreement between the HIV-2 TNA assay and the combined results of the Combo and Multispot immunoassays was 98.75% (79 of 80), as summarized in Table 3.

HIV-1 and HIV-2 dually seropositive Senegalese

PBMC from eight HIV-1/-2 dually-infected participants were tested by both the HIV-1 and HIV-2 TNA assays (Table 4). PBMC from six participants were positive for both HIV-1 and HIV-2 TNA and two were positive for either HIV-1 or HIV-2 TNA. The HD10 PBMC sample negative for HIV-2 TNA likely had HIV-2 DNA/RNA below the assay LOD. All eight corresponding plasma samples diluted up to 100-fold remained positive for HIV Ab (undifferentiated) by the Multispot assay. When tested by the Geenius assay, undiluted plasma samples from the dually-infected participants were re-confirmed as having undifferentiated (untypable) HIV results.

DISCUSSION

We developed and validated a qualitative HIV-2 TNA diagnostic assay running on the Abbott m2000 platform. For clinical usage, endogenous and exogenous controls were implemented to ensure adequate extraction and amplification of DNA and RNA from PBMC specimens. With regard to diagnostic sensitivity, the HIV-2 TNA assay was able to detect HIV-2 DNA/RNA in PBMC samples when the corresponding HIV-2 seropositive plasma samples were undetectable for HIV-2 RNA. The assay diagnostic performance largely agreed with the serological determination by the Combo and Multispot assays, with the exception of one plasma sample (H2A32) with a relatively low S/CO of 5.68 and undetectable HIV-2 plasma RNA (Table 2). This case demonstrates the utility of the plasma HIV-2 RNA and PBMC HIV-2 TNA assays for plasma specimens that yield indeterminate and undifferentiated HIV-1/-2 Ab results when tested using the CDC 4th generation HIV screening algorithm.

The ability to confirm and distinguish HIV-1, HIV-2 and HIV-1/HIV-2 dual infections has important implications for clinical management and antiretroviral therapy [11–13].

Currently, per the CDC 4th generation HIV diagnostic algorithm, the FDA-approved assays to confirm and differentiate HIV-1 or/and HIV-2 serology are the Multispot [14–16] and the Geenius tests [17, 18]. The Multispot reports nonreactive, HIV-1 reactive, HIV-2 reactive, or HIV reactive (undifferentiated) results. The Geenius cassette contains two HIV-2 peptide bands and 4 HIV-1 peptide or protein bands. The Geenius Reader detects the presence or absence of the bands and provides assay results using a proprietary algorithm[9]. In addition to the aforementioned Multispot interpretations, Geenius reports HIV-2 indeterminate, HIV indeterminate and HIV-2 positive with HIV-1 cross-reactivity results. Several recent studies have reported HIV-2 indeterminate cases using the Geenius test, which led to a recommendation to use HIV-2 NAT to further determine HIV infection status [18–20]; as such, the HIV-2 TNA assay has the potential to address this need.

Several groups used “in-house” quantitative assays or a modified Roche Amplicor test to measure HIV-2 proviral DNA in PBMC [3, 21, 22], ranging from not-detectable up to 4 logs copies/million PBMC. Though many HIV-2 infected participants in these three studies were highlighted for having plasma RNA below assay detection limits—40, 100 or 250 copies/mL, some of these studied samples would have had detectable or quantifiable plasma RNA by more current HIV-2 RNA assays [2, 23, 24]. Lately, Styer et al. [25] has developed a droplet digital PCR assay to detect HIV-2 DNA in the whole blood and blood cell pellets. We demonstrated that our assay has improved sensitivity by detecting HIV-2 in PBMC from 23 HIV-2 infected participants with undetectable plasma RNA, <10 copies/mL, and by amplification of both cell-associated DNA and RNA in PBMC.

In conclusion, the described HIV-2 TNA assay is a sensitive NAT for detecting HIV-2 DNA/RNA in PBMC specimens and is suitable for the diagnosis of HIV-2 for patients with serologically indeterminate or undifferentiated HIV status and undetectable HIV-2 plasma RNA viral load. As such, this validated HIV-2 TNA assay addresses an important deficiency in the current CDC 4th generation HIV testing algorithm.

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Highlights

- A qualitative assay detecting HIV-2 total nucleic acid in patients' PBMCs
- A second-line assay to resolve HIV-2 sero-indeterminate cases
- Able to detect HIV-2 DNA and RNA in PBMC from patients with undetected plasma RNA

Table 1

Limit of detection of the HIV-2 TNA assay

Copies of HIV-2 pROD9 DNA/million cells	Number Tested	Number Detected	Percent Detected (%)
46	16	16	100
23	16	15	93.4
12	16	10	62.5
6	16	9	56.3
3	16	3	18.8

Table 2

Paired plasma and PBMC samples from Senegalese HIV-2 seropositive infected participants

		Plasma Sample			PBMC Sample		
		HIV Ag/Ab Combo[#]	Multispot[^]	HIV-2 RNA	HIV-2 TNA		HIV-1 TNA
Specimen ID	Group	Results (S/CO[+])	Interpretation	Results (c/mL)	Results	DC~	Results
H2A32	A	5.89	HIV NEG	TND[*]	Detected	4.99	Not detected
H2A76	A	22.07	HIV-2 (wk)	TND	Detected	8.24	Not detected
H2A24	A	54.78	HIV-2-after 1:10	32	Detected	8.86	Not detected
H2A67	NT[$]	66.66	HIV-2 (wk)	TND	Detected	1.07	Not detected
H2A72	B	99.79	HIV-2	TND	Detected	7.24	Not detected
H2A25	A	122.55	HIV-2	<10, detected	Detected	5.73	NT[$]
H2A66	NT[$]	123.75	HIV-2	TND	Detected	6.17	Not detected
H2A64	A	139.49	HIV-2-after 1:10	TND	Detected	6.92	Not detected
H2A26	A	142.28	HIV-2	TND	Detected	5.03	NT[$]
H2A53	A	149.89	HIV-2-after 1:100	TND	Detected	6.39	Not detected
H2A84	NT[$]	157.64	HIV-2	TND	Detected	3.00	Not detected
H2A14	A	160.84	HIV-2-after 1:100	n/a	Detected	8.34	Not detected
H2A78	NT[$]	165.91	HIV-2	TND	Detected	6.17	Not detected
H2A3	A	168.28	HIV-2-after 1:100	115	Detected	7.97	Not detected
H2A30	A	179.93	HIV-2-after 1:100	TND	Detected	9.39	Not detected
H2A10	A	186.91	HIV-2	TND	Detected	6.58	Not detected
H2A34	A	189.62	HIV-2	<10, detected	Detected	5.32	Not detected
H2A38	NT[$]	192.39	HIV-2-after 1:100	<10, detected	Detected	5.59	Not detected
H2A74	A	192.51	HIV-2	TND	Detected	9.08	Not detected
H2A62	A	196.98	HIV-2	TND	Detected	4.76	Not detected
H2A57	A	205.59	HIV-2	TND	Detected	6.66	Not detected
H2A77	A	206.43	HIV-2-after 1:10	TND	Detected	8.46	Not detected
H2A22	A	207.58	HIV-2-after 1:10	TND	Detected	5.15	Not detected

		Plasma Sample			PBMC Sample		
		HIV Ag/Ab Combo[#]	Multispot[^]	HIV-2 RNA	HIV-2 TNA		HIV-1 TNA
Specimen ID	Group	Results (S/CO[+])	Interpretation	Results (c/mL)	Results	DC~	Results
H2A55	A	219.28	HIV-2	TND	Detected	8.67	Not detected
H2A6	A	222.62	HIV-2	TND	Detected	6.51	Not detected
H2A33	A	223.00	HIV-2-after 1:100	TND	Detected	6.32	Not detected
H2A20	A	225.10	HIV-2	16	Detected	3.92	Not detected
H2A82	NT[$]	234.44	HIV-2	TND	Detected	8.25	Not detected
H2A9	A	234.58	HIV-2	TND	Detected	4.72	Not detected
H2A31	A	293.67	HIV-2	TND	Detected	4.17	Not detected

[#]Abbott ARCHITECT HIV Ag/Ab Combo assay

[^]Multispot results interpreted without dilution are stated without further description. Samples determined to be HIV-2 seropositive after 1:10 or 1:100 dilutions as stated. Wk, weak positive.

[$]NT, Not tested;

[+]S/CO, Signal-to-cutoff ratio;

[*]TND, Target not detected; ~DC, Delta Cycle.

Table 3

Diagnostic performance of HIV-2 TNA assay

		The matching plasma samples tested by HIV Ag/Ab Combo and Multispot assays	
		HIV-2 Ab reactive	**HIV-2 Ab non-reactive**
PBMC tested by HIV-2 TNA assay	Detected	29	1*
	Not-detected	0	50+

*HIV reactive by Abbott ARCHITECT HIV Ag/Ab Combo assay; HIV nonreactive by Multispot. Participant's baseline HIV testing: HIV reactive by *Alere Determine*™ HIV-1/-2 rapid test and HIV-2 reactive by the ImmunoComb II HIV- 1 & 2 BiSpot assay; negative HIV-2 RNA in plasma.

+The number is the combined result for 25 HIV-negative and 25 HIV-1 infected individuals.

Table 4

Multispot-undifferentiated* of HIV-1/2 dually seropositive participants' paired PBMC and plasma samples

Specimen ID	Plasma sample		PBMC sample	
	HIV-1 RNA copies/mL	HIV-2 RNA copies/mL	HIV-1 TNA	HIV-2 TNA
HD01	TND#	TND	Detected	Detected
HD02	85120	2592	Detected	Detected
HD06	510596	311	Detected	Detected
HD09	TND	TND	Detected	Detected
HD12	TND	TND	Detected	Detected^
H2A036	TND	136	Detected	Detected
HD13	Detected, <40c/ml	28726	Not detected	Detected
HD10	13730	TND	Detected	Not detected

* HIV Ab was not differentiated for the sample at a 1:100 dilution.

Target not detected.

^ Detected by a HIV-2 second probe.