A new quantitative HIV load assay
based on plasma virion reverse transcriptase activity
for the different types, groups and subtypes

Short Title: Plasma RT activity assay for HIV viral load

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Abstract

**Background:** Plasma viral load monitoring is an integral part of the standard of care for HIV-infected patients in industrialized countries. In developing countries, viral load assay is either unaffordable or hindered by on-site maintenance and/or technical problems.

**Objectives:** To evaluate a new and simple quantitative assay for plasma HIV reverse transcriptase (RT) activity; and to compare RT activity-based and RNA-based quantification in plasma samples from patients infected by different subtypes of HIV-1 group M, HIV-1 group O and HIV-2.

**Methods:** The RT-based viral load assay involves separation of the virion-protected-RT and quantification of its activity with an enzyme-immunoassay. Plasma viremia was quantified both by RT activity and by RNA copies in 322 samples from 236 HIV-1-group M-infected patients, including serial samples from 54 patients; 74% of the patients were on antiretroviral therapy. Samples from 49 patients infected by HIV-1-group O or HIV-2 were also tested.

**Results:** RT activity and RNA copies were detected in 70% of plasma samples; respectively 25% and 1% of samples contained detectable RNA copies or RT activity alone. Measured RT activity corresponded to 48%, 96% and 100% of samples with 1.7 to 4.0 Log$_{10}$, 4.1 to 4.8 Log$_{10}$ and 4.9 to 6.7 Log$_{10}$ RNA copies/ml, respectively. The values of the two assays correlated independently of the HIV subtype (p<0.0001) and group/type (p<0.03). Patient follow-up showed similar pattern of viremia with the two assays.

**Conclusion:** Plasma RT activity assay is a simple, cheap and reliable alternative for HIV viral load determination. As such, it could be particularly valuable for diagnosis and treatment monitoring in developing countries.

**Key words:** HIV, reverse transcriptase activity, HIV diagnostic tests, treatment monitoring, HIV diversity, HIV-1 RNA.

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**Introduction**

The management of HIV-infected patients in industrialized countries is chiefly based on human immunodeficiency virus (HIV) ribonucleic acid (viral load) assay and CD4 cell counts. In contrast, monitoring of HIV infection is still an unaffordable part of treatment in developing countries. HIV-1 is found worldwide, while HIV-2 is essentially restricted to west Africa [1, 2]. HIV-1 is comprised of the pandemic group M (major) and the divergent group O (HIV-O, Outlier), the latter being most prevalent in central Africa. Viral load assays designed for use in Africa must take this extreme genetic divergence into account. Ideally, they should also be inexpensive, robust and simple. Here, we evaluated an RT activity assay kit (RetroLoad™) which seems to meet these criteria, by testing panels of plasma from patients infected by HIV-1 group M (B and non B subtypes), HIV-O and HIV-2, in comparison with a viral load assay based on RNA copy numbers. We also investigated the reliability of RT activity assay for patient follow-up.
Methods

Population. The study population included 236 HIV-1 group M-infected patients, from whom 322 plasma samples were collected between 1999 to 2002. Two hundred thirty-four samples from 170 patients contained group M subtype-B viruses and 88 samples from 66 patients contained group M subtype-non B viruses. Fifty-three samples (16%) were from 42 treatment-naive patients, 238 (74%) were from 174 patients receiving antiretroviral therapy, and 31 (10%) were obtained from 28 patients during treatment interruption.

Plasma samples from 49 patients infected by HIV-1 group O or HIV-2 were also included in the study. Three of the 15 HIV-1 group O-infected patients (4 of the 16 samples) were on antiretroviral therapy, and 12 were treatment naive. Sixteen of the 34 HIV-2-infected patients (34 of the 52 samples) were on antiretroviral therapy and 18 were treatment naive.

Sample collection. Acid citrate dextrose- or ethylenediaminetetraacetic (EDTA)-preserved blood samples, collected and transported at ambient temperature, were centrifuged within 6 h of collection; plasma was aliquoted and stored at –80°C.

Isolation of virion-associated reverse transcriptase (RT) and RT activity assay. RT was recovered from plasma and quantified using the RT activity kit (RetroLoad™, Cavidi Tech AB, Uppsala, Sweden), as recommended by the manufacturer [3]. Briefly, 1 ml of plasma was treated to inactivate interfering enzymes; virions were then immobilized on a gel column, and washed to remove other interfering factors (e.g. RT-blocking antibodies and residual antiretrovirals). The gel was then conditioned and intra-viral RT was recovered with detergent-containing lysis buffer.

RT activity in the viral lysates was assayed in 96-well microtiter plates with a polyA template immobilized on the bottom of the wells and a reaction mixture containing the primer (oligodT), the dNTP (Bromo-deoxyuridine triphosphate, BrdUTP) and all other components necessary for RT activity. The RT in the sample was allowed to synthesize a BrdU-DNA strand overnight at 33°C. After washings, the incorporated BrdUTP was quantified by adding an RT product tracer (BrdU binding antibody conjugated to alkaline phosphatase). The alkaline phosphatase activity
of the bound tracer was measured after another wash, using a substrate giving a fluorescent product (umbiliferate). Emitted fluorescence is proportional to the RT activity in the sample. The results are expressed as Log_{10} femtograms (fg) of HIV-1 RT activity/ml of plasma. According to the manufacturer, the detection limit is 1 fg/ml.

**Within- and between-run variations.** Aliquots of the same plasma sample were quantified in duplicate. Within-run variation was measured by processing 10 samples (3.5 to 6.4 Log_{10} RNA copies/ml) and between-run variation was measured on 8 samples (3.9 to 5.8 Log_{10} RNA copies/ml). An in-house pool of plasma from 40 HIV-1 infected patients (5.5 Log_{10} RNA copies/ml) was used as control and was quantified in 7 independent experiments (2 to 7 separate columns per experiment).

**Plasma HIV RNA assay.** HIV RNA load was determined in identical plasma aliquots as those used for RT assay. HIV-1 group M and group O viremia were measured with RT-PCR-based assays according to the manufacturers' instructions. The COBAS AMPLICOR HIV-1 Monitor™ v1.5 test (Roche Diagnostics, Meylan, France) used for HIV-1 group M samples has a dynamic range of 50 to 750 000 RNA copies/ml [4]. The LCx HIV RNA quantitative assay (Abbott, Chicago, IL) used for HIV-1 group O samples has a dynamic range of 50 to 6 000 000 RNA copies/ml [5]. HIV-2 viremia was quantified with a real-time PCR assay based on the Light-Cycler system (Roche Molecular, Indianapolis, Ind); the quantification cut-off is set at 250 RNA copies/ml [6].

**Determination of HIV groups and subtypes.** HIV-1 subtype was determined on samples of serum, plasma or peripheral blood mononuclear cells by using a subtype-specific enzyme immunoassay (SSEIA, [7]), or an envDNA enzyme immunoassay (envDEIA, [8]) or by genotyping the protease and RT genes with the TRUGENE™ HIV-1 genotyping kit (Visible Genetics, Epinay sur orge, France), followed by phylogenetic analysis. HIV-2 and HIV-1 group O were identified as previously described [9, 10].

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**Statistical Analysis.** Spearman correlation coefficients were determined between plasma RT activity (fg/ml) and RNA (copies/ml) levels. Variability between replicate tests was calculated using standard deviation (SD) for Log_{10}-transformed RT activity values. The variability of the average difference between Log_{10}-transformed RNA copy numbers and RT activity was calculated using standard error of the mean (SEM).
Results

RT activity assay shows low within- and between-run variation (Table 1).
Within-run RT activity standard deviations ranged from 0.00 to 0.07 Log_{10}. Between-run RT activity standard errors of the mean ranged from 0.01 to 0.37. Within-run and between-run standard deviations obtained with an in-house pool of plasma from HIV-1-infected patients ranged from 0.02 to 0.15 Log_{10} and 0.08 Log_{10}, respectively. No significant differences were found in RT activity measurement for within-run or between-run replicates.

RT activity allows determination of the majority of samples.
In-house negative reference plasma samples from HIV-seronegative individuals were included in each run and were repeatedly negative for RT activity. Samples from 167 HIV-1-infected patients (229 plasma samples) were positive for RT activity (Table 2). Log_{10}-transformed RT activity values were: \( \leq 1 \) Log_{10} in 52 samples, > 1 to \( \leq 2 \) Log_{10} in 97 samples, > 2 to \( \leq 3 \) Log_{10} in 65 samples and > 3 Log_{10} in 15 samples. The lowest and highest values quantified were 1.4 fg RT activity (0.1 Log_{10}/ml) and 4193 fg RT activity (3.6 Log_{10}/ml), respectively. RT activity was undetectable in 93 samples from 86 patients. Seventy-eight of these samples were from patients receiving antiretroviral therapy, and 15 samples were from 10 treatment-naive patients (3 subtype-B and 7 non B) and from three patients who had discontinued treatment (2 subtype-B and 1 non B).
Sixteen samples from 15 HIV-1 group O-infected patients and 52 samples from 34 HIV-2-infected patients were also assayed for RT activity. Eleven of the 16 HIV-1 group O samples were positive for RT activity (1.5 to 163.4 fg/ml); 10 samples were from treatment-naive patients and one from a patient on antiretroviral therapy. Eighteen of the HIV-2 samples were positive for RT activity; eight samples from patients on antiretroviral therapy contained RT activity ranging from 2.2 to 20.3 fg/ml, and ten samples from treatment-naive patients contained RT activity ranging from 1.2 to 913.9 fg/ml.

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**HIV RNA values**

308 HIV-1 group M plasma samples from 222 patients had RNA values above the detection limit (160 subtype-B and 62 non B) (Table 2). HIV RNA was undetectable in 14 samples from 14 patients (10 subtype-B and 4 non B); two of these samples contained detectable RT activity, albeit with low values (both subtype-B, 1.4 and 3 fg RT activity/ml).

Thirteen samples from HIV-O-infected patients were assayed for HIV RNA. Two samples from a patient on antiretroviral therapy were below the detection limit. The other 11 samples, all from treatment-naive patients, had values from 3.7 to 5.2 Log_{10} RNA copies/ml. Seven samples from HIV-2-infected patients were assayed for HIV RNA and had values ranging from 4.1 to 6.1 Log_{10} copies/ml. Four samples were from treatment-naive patients and three were from patients on antiretroviral therapy.

**Sensitivity of RT assay relative to HIV RNA assay.**

RT activity values were compared with HIV RNA values in HIV-1 group M plasma samples (Table 2): 229 (71%) of the 322 samples studied had measurable RT activity, and 308 (96%) contained measurable RNA. Two hundred twenty-seven samples (70%) were positive for both RT activity and HIV RNA; 81 samples (25%) were positive for RNA only; and 2 samples (1%) were positive for RT activity only. Among the 81 samples positive for RNA but negative for RT activity (74 patients), 53 (47 patients) were subtype-B and 28 (27 patients) were subtype-non B; 84% of these samples were from patients on antiretroviral therapy.

Nine of the 11 HIV-O samples with measurable RNA (3.7 to 5.2 Log_{10} copies/ml) had measurable RT activity, while two samples (3.9 and 4.0 Log_{10} copies/ml) were below the detection limit. All seven HIV-2 samples with measurable RNA (4.1 to 6.1 Log_{10} RNA /ml) had measurable RT activity.

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Sensitivity of RT assay is similar for HIV-1 group M subtype-B and non B in samples with measurable HIV RNA.

227 (74%) of the 308 plasma samples with measurable HIV RNA (1.7 to 6.7 Log_{10} copies/ml) had measurable RT activity (Table 3). The sensitivity of the RT assay was similar for subtype-B and non B HIV-1 (77% and 66%, respectively).

RT activity was measurable in 48% of samples containing 1.7 to 4.0 Log_{10} RNA copies/ml (53% of subtype-B and 37% of non B samples). Most of the samples with non measurable RT activity (86%) were from patients receiving antiretroviral treatment. RT activity was measurable in 96% of samples containing 4.1 to 4.8 Log_{10} RNA copies/ml (95% of subtype-B and 100% of non B samples), and in 100% of samples containing ≥ 4.9 Log_{10} RNA copies/ml (62 subtype-B and 22 non B samples).

The average difference between the Log_{10}-transformed values for RNA copies and RT activity was 2.85 for all samples together and was slightly higher (2.99) for samples containing 4.9 to 6.7 Log_{10} RNA copies/ml.

Only two patients (both subtype-B) had RNA loads above 4.0 Log_{10} copies/ml and non measurable RT activity. The first patient had RNA load stable for 112 weeks (maximum 4.4 Log_{10}, minimum 4.0 Log_{10}), was 30 weeks pregnant and had 57 weeks treatment interruption. The second patient had recently been diagnosed as HIV-1 seropositive; his initial RNA value was 4.8 Log_{10} and highly active antiretroviral treatment was started 10 weeks later. His RNA load fell to 2.0 Log_{10} after 5 weeks on treatment and became undetectable after 26 weeks. RT activity was undetectable in both samples tested, one at diagnosis (4.8 Log_{10} RNA copies/ml) and the other 7 weeks later (4.5 Log_{10} RNA copies/ml).

Taken together, these results show the RT activity can be reliably quantified in samples containing viral loads of at least 4.0 Log_{10} RNA copies/ml.

**Strong correlation between Log_{10}-transformed RT activity and RNA copy numbers.**

We tested the correlation between RT activity and RNA viral load for all HIV-1 group M plasma samples analyzed, including replicates and samples with highly divergent RT activity and RNA load values. Log_{10}-transformed values obtained for the 344 HIV-1 group M samples quantified
for RT activity and RNA copies are compared in Fig.1. A strong correlation was found between
the two quantitative assays (r=0.850, p<0.0001). The correlation was independent of the HIV-1
subtype (p<0.0001 for both subtype-B (251 samples, 170 patients) and subtype-non B (93
samples, 66 patients)). In the subset of HIV-2 and HIV-O samples assayed for HIV RNA, the
correlation between RT activity and RNA values was also significant (p=0.0229 and p=0.0099,
respectively).

Serial samples.
The value of RT activity for patient follow-up was examined relative to RNA assay in three HIV-1
subtype-B-infected patients for whom serial samples were available (Fig. 2). Changes in RT and
RNA values were similar in all three patients. Patients #1 and #3 both had a strong fall in RT
and RNA values following a change in therapy. In patient #1, a switch from ddI+d4T+NVP to
ddI+d4T+EFV induced a fall of 1.7 and 1.1 in Log_{10} RT and RNA values, respectively. In patient
#3 a switch from d4T+APV to d4T+3TC+ABT378 induced a fall of 2.3 and 2.0 in Log_{10} RT and
RNA values, respectively. Viral load increased 15 weeks later in patient #1 (RT = 1.1; RNA =
0.8) and in patient #3 (RT = 1.6; RNA = 1.4). In patient #2, changes in antiretroviral therapy,
including a treatment interruption lasting seven weeks, did not significantly affect viral load
values.

Serial viral load values were studied in a further 51 patients (35 subtype-B and 16 non B). Two
samples were available for 38 patients, three for 10 patients and four for 3 patients. RT and
RNA curves again showed similar changes (data not shown).

Taken together, these results show that the RT activity assay can be used to monitor changes
in viral load on antiretroviral therapy.
Discussion

CD4 counts is an immunological marker for treatment initiation and immune reconstitution and viral loads is the best surrogate marker of HIV dynamic changes over time [11, 12]. There is an urgent need for a simple, cheap alternative to HIV RNA assay for patient follow-up in developing countries. Here, we evaluated the RetroLoad™ enzyme-immunoassay which measures intra-viral RT activity.

A major weakness of RT activity assays is their susceptibility to enzyme-inhibiting antibodies and other interfering molecules [13]. Most HIV-infected patients have antibody-mediated RT activity inhibition [14]. Previously described sensitive RT activity assays were applied either to crude or centrifuged plasma, which still contains interfering substances [13, 15]. In the new assay evaluated here, virions are first separated from plasma by passage through a gel column; this step removes antibodies and other interfering substances (drug metabolites and enzymes). RT, protected inside virions is then released by lysing the viral particles, before being quantified.

Although RT activity can only be systematically quantified with this assay in samples with RNA loads of 10 000 copies/ml, this should not hinder the use of this method for diagnosis or follow-up. In addition, within- and between-run reproducibility is good in our hands.

The main drawback of RNA-based viral load assays are their genomic sequence dependency, which limits their use in non-subtype-B infection. It is noteworthy that the pol gene, which codes for HIV RT, is the most highly conserved gene in retroviruses [16]; RT activity assay should not therefore be affected by the broad sequence diversity of HIV subtypes, groups and types, as borne out by our experimental results and by previous reports [13].

We found a strong correlation (p<0.0001) between viral load values based on RT activity and HIV RNA, both in the entire study population and in subtype-B and non B subpopulations. Although relatively few HIV-2 and HIV-1-group O samples were tested here, the assay used reliably quantified the RT activity of these highly divergent viruses, as values correlated well with RNA-based viral load ( p=0.0229 and p=0.0099, respectively). Larger sample panels must now be tested to determine the respective detection limits.

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RNA-based viral load assays are too costly for widespread use in developing countries. In contrast, the cost of the RT activity assay is less than 15 US$ per sample.

Plasma viral load can also be estimated on the basis of p24 antigen assay [17]. However, p24 antigenemia does not reliably reflect RNA-based viral load [18, 19]. Furthermore, RT activity may be a more reliable index of viral load than p24 for divergent strains of HIV [13, 20]. RT activity assay has been shown to be the most sensitive method, after RNA quantification [13].

It has been suggested that plasma RT activity assay may replace RNA-based quantitative assays in the neonatal setting [21]. The present assay requires about 1 ml of plasma, but perinatally-infected infants have high HIV-1 RNA levels (>5 Log_{10} copies/ml) [22, 23], suggesting that a smaller volume might suffice for neonatal diagnosis. Tests for p24 antigen dissociated from immune complexes are also useful in this setting [24], but may miss HIV-O and HIV-2 infection.

The assay described here has already been evaluated in Uganda [25], and we are currently investigating its practical value in western Africa. Preliminary results are promising.

In summary, our data support the use of this quantitative RT activity assay as an affordable alternative to viral RNA quantification, for both diagnosis and follow-up. This approach may prove particularly useful as antiretroviral therapy becomes more affordable in developing countries.

**Acknowledgements**

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Figure Legends

Fig. 1. Correlation of viral load in HIV-1 group M subtype-B and non B samples based on RT activity and RNA assay (Log\textsubscript{10} values).

RT activity was quantified with the RetroLoad™ assay. RNA copies were quantified with the Monitor™v1.5 test. The respective quantification limits (broken lines) were 1.7 Log\textsubscript{10} RNA copies/ml (50 copies/ml) and 0 Log\textsubscript{10} RT activity/ml (1 fg/ml). Samples below the detection limits were attributed arbitrary values of 10 RNA copies/ml and 0.25 fg RT activity/ml. All data of HIV-1 group M samples are shown and included in the correlation. r, Spearman correlation coefficient.

Fig. 2. History of antiretroviral therapy among three HIV-1 subtype-B-infected patients.

RT-based (○) and RNA-based (▲) viral load values are Log\textsubscript{10}-transformed. RT activity was quantified with the RetroLoad™ kit. The quantification limit (broken line) was 0 Log\textsubscript{10} RT activity/ml (1 fg/ml). Arbitrary value of 0.25 fg/ml was attributed for undetectable level of RT activity. RNA copies were quantified with the Monitor™v1.5 test. Antiretroviral therapy in Patient #1: 1=ddI+d4T+NVP, 2=ddI+d4T+EFV; in patient #2: 1=d4T+EFV, 2=APV, 3=TI, 4=3TC+ABT378, 5=3TC+Kaletra; in patient #3: 1=IDV+3TC+NVP+RTV, 2=d4T+APV, 3=3TC+d4T+ABT378, 4=d4T+Kaletra, 5=d4T-ddI. AZT-Zidovudine; d4T-Stavudine; ddI-Didanosine; 3TC-Lamivudine; ddC-Zalcitabine; ABC-Abacavir; SQV-Saquinavir; RTV-Ritonavir; IDV-Indinavir; NFV-Nelfinavir; APV-Amprenavir; ABT378-Lopinavir; NVP-Nevirapine; EFV-Efavirenz; TI-Treatment Interruption.

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Table 1. Within- and between-run variation of HIV-1 group M RT activity quantification.

<table>
<thead>
<tr>
<th>aVL of sample</th>
<th>Subtype</th>
<th>Mean bRT</th>
<th>SD bRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.53</td>
<td>B</td>
<td>0.76</td>
<td>0.01</td>
</tr>
<tr>
<td>4.26</td>
<td>B</td>
<td>1.74</td>
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<td>4.34</td>
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<td>0.07</td>
</tr>
<tr>
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<tr>
<td>4.56</td>
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<td>1.51</td>
<td>0.01</td>
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<tr>
<td>5.40</td>
<td>B</td>
<td>3.20</td>
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<td>5.69</td>
<td>B</td>
<td>1.51</td>
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</tr>
<tr>
<td>6.40</td>
<td>B</td>
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<table>
<thead>
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<td>3.98</td>
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<td>B</td>
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<td>Non B</td>
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<td>5.36</td>
<td>Non B</td>
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<tr>
<td>5.78</td>
<td>B</td>
<td>3.61</td>
<td>0.01</td>
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aRNA viral load (VL) was quantified with the Monitor™v1.5 test. bReverse transcriptase activity (RT) was quantified with the RetroLoad™ kit. Results are Log10 transformed RNA copies/ml and RT activity (fg/ml). SD, standard deviation.

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Table 2. Plasma viral load determination based on RT activity and RNA copy numbers in HIV-1 group M samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total</th>
<th>^aRT-</th>
<th>^bVL-</th>
<th>^aRT+</th>
<th>^bVL+</th>
<th>^aRT+</th>
<th>^bVL+</th>
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<tbody>
<tr>
<td>Plasma subtype B</td>
<td>234 (170)</td>
<td>8 (8)</td>
<td>53 (47)</td>
<td>171 (123)</td>
<td>2 (2)</td>
<td></td>
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<tr>
<td>Plasma subtype non B</td>
<td>88 (66)</td>
<td>4 (4)</td>
<td>28 (27)</td>
<td>56 (42)</td>
<td>0 (0)</td>
<td></td>
<td></td>
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<tr>
<td>Plasma</td>
<td>322 (236)</td>
<td>12 (12)</td>
<td>81 (74)</td>
<td>227 (165)</td>
<td>2 (2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Reverse transcriptase activity (RT) was quantified with the RetroLoad™ kit. Results below <1 fg RT activity/ml are reported as "RT-.

^b RNA viral load (VL) was quantified with the Monitor™v1.5 test. Results below <50 RNA copies/ml are reported as "VL-".
Table 3. Sensitivity of RT activity assay for HIV-1 group M subtype-B and non B in samples with measurable HIV RNA.

<table>
<thead>
<tr>
<th>VL</th>
<th>Samples Investigated</th>
<th>Samples &lt;sup&gt;b&lt;/sup&gt;RT+</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
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</tr>
<tr>
<td>1.7-4.0</td>
<td>n</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>% positif</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>SEM DLog</td>
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<tr>
<td>4.1-4.8</td>
<td>n</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>% positif</td>
<td>96</td>
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<td></td>
<td>SEM DLog</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>% positif</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SEM DLog</td>
<td>0.06</td>
</tr>
<tr>
<td>1.7-6.7</td>
<td>n</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>% positif</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>SEM DLog</td>
<td>0.03</td>
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</table>

n, number of samples. <sup>a</sup>RNA viral load (VL) was quantified with the Monitor™ v1.5 test. Results ≥50 RNA copies/ml are expressed as Log<sub>10</sub>-transformed values. <sup>b</sup>Reverse transcriptase activity (RT) was quantified with the RetroLoad™ kit. Results ≥1 fg RT activity/ml are reported as "RT+". Mean DLog was calculated as the mean difference between Log<sub>10</sub>-transformed values for RNA (copies/ml) and for RT activity (fg/ml). SEM DLog was calculated using the standard error of the mean (SEM) for the mean DLog.

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Figure 1

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Figure 2

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