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Armored Long RNA Controls or Standards for Branched DNA Assay for Detection of Human Immunodeficiency Virus Type 1▽

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The branched DNA (bDNA) assay is a reliable method for quantifying the RNA of human immunodeficiency virus type 1 (HIV-1). The positive controls and standards for this assay for the detection of HIV-1 consist of naked RNA, which is susceptible to degradation by RNase. Armored RNA is a good candidate for an RNase-resistant positive control or standard. However, its use has been limited by the maximal length of the exogenous RNA packaged into virus-like particles by routine armored RNA technology. In the present study, we produced armored long RNA (armored L-RNA) controls or standards (AR-HIV-pol-3034b) for a bDNA assay of HIV-1 by increasing the amount and affinity of the pac sites (the pac site is a specific 19-nucleotide stem-loop region located at the 5′ terminus of the MS2 bacteriophage replicase gene) by a one-plasmid double-expression system. AR-HIV-pol-3034b was completely resistant to DNase and RNase, was stable in normal human EDTA-preserved plasma at 4°C for at least 6 months, and produced reproducible, linear results in the Versant HIV-1 RNA 3.0 assay. In conclusion, AR-HIV-pol-3034b could act as a positive control or standard in a bDNA assay for the detection of HIV-1. In addition, the one-plasmid double-expression system can be used as a better platform than the one-plasmid expression system and the two-plasmid coexpression system for expressing armored L-RNA.

Human immunodeficiency virus (HIV) infection represents one of the most serious challenges to global public health, since more than 60 million people have been infected with HIV and 25 million have already died of AIDS worldwide (3, 22). Accurate determination of HIV type 1 (HIV-1) RNA levels is important for understanding the natural history of HIV infection, predicting the disease progression to AIDS, and determining the efficacy of antiretroviral therapies for a given patient (1, 15, 16).

The branched DNA (bDNA) assay provides a reliable method for quantifying HIV-1 RNA in human plasma. Its lower limit of quantification is 50 copies of HIV-1 RNA/ml. The bDNA assay directly measures HIV-1 RNA by boosting the reporter signal and thus avoids the errors inherent in the extraction and replication of target sequences. This assay is based on the hybridization of HIV-1 RNA to oligonucleotide probes complementary to the most conserved region (about 2.7 kb) of the HIV-1 *pol* gene and yields a reproducible quantification of HIV-1 RNA that is not affected by the sequence variability of HIV-1 subtypes (8, 12, 17, 28).

The bDNA assay for HIV-1 depends on the use of RNA synthesized by in vitro transcription for its positive controls and standards. A major disadvantage of using naked RNA is that it is susceptible to degradation by RNase. Thus, there is a need for RNase-resistant RNA controls and standards.

Armored RNA is a kind of noninfectious recombinant virus-like particle (VLP) containing target exogenous RNA. It is the most suitable candidate for a positive control or standard for the quantification of an RNA virus, because it is RNase resistant, stable, noninfectious, inexpensive, and easily extracted by conventional methods (2, 4, 6, 31).

A preferred strategy for producing armored RNA is to package the exogenous RNA into the MS2 coat protein by the self-assembly mechanism of MS2. The MS2 self-assembly mechanism is initiated by the highly specific interaction between the coat protein and a single stem-loop structure of 19 bases (pac site) in the MS2 RNA genome located at the 5′ end of the viral replicase gene, containing the Shine-Dalgarno sequence and the start codon of the replicase gene (11).

Theoretically, at least 1,900 bases of the exogenous RNA sequence might be packaged into the coat protein shell by routine armored RNA technology; however, the packaging efficiency decreases quickly as the size of the RNA increases beyond 500 bases. To date, the size of the largest RNA packaged has been 1,200 bases; this was accomplished by utilizing one wild-type pac site (6). However, the controls or standards for a bDNA assay for HIV-1 are approximately 2.7 kb long. Consequently, it is not possible to produce armored RNA controls or standards for this assay using routine armored RNA technology (8).

The pac site plays an extremely important role in the packaging of armored RNA. It has been confirmed that the affinity between the pac site and the coat protein increases significantly when the uridine at position −5 in the pac site is replaced with cytosine (C variant) (5, 9, 10, 14, 23, 24, 26, 27, 30, 35). It has also been shown that increasing the number of pac sites generates a higher affinity between the coat protein and the exogenous RNA (21, 34). We have demonstrated that 1,981-base chimeric RNA can be successfully packaged into the MS2 coat protein by utilizing a one-plasmid expression system with two C-variant pac sites (32). The 2,248-base armored RNA was

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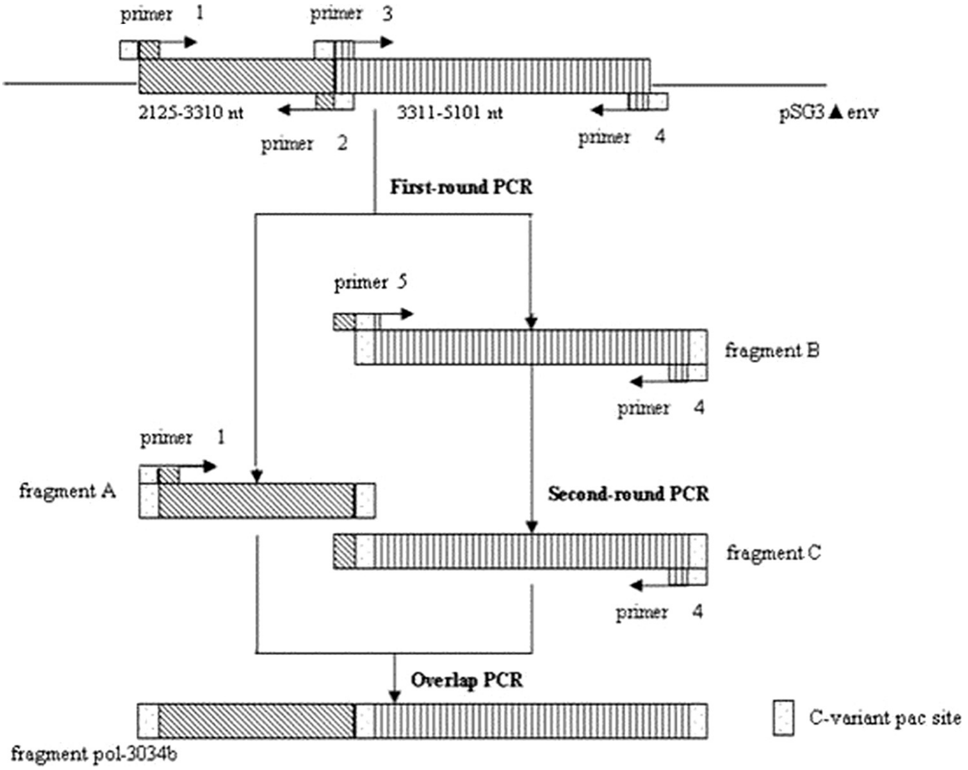

FIG. 1. Method for construction of the exogenous chimeric fragment pol-3034b. During the first-round PCR, two parts (fragments A and B; nucleotides 2125 to 3310, with 1,186 bp, and nucleotides 3311 to 5101, with 1,791 bp, respectively) of the nearly entire HIV *pol* sequence were amplified from the template plasmid pSG3▲env (kindly provided by the National Center for AIDS/STD Control and Prevention, China CDC; containing the entire HIV RNA genome; GenBank accession no. AB221005) using primers 1 and 2 and primers 3 and 4, respectively. In the second-round PCR, fragment C was obtained from fragment B by using primers 5 and 4 to prepare for overlapping extension PCR. Then the 3,034-base exogenous chimeric sequence (pol-3034b) was obtained by overlapping extension PCR using primers 1 and 4 from templates (fragments A and C).

also successfully expressed by a two-plasmid coexpression system with one C-variant pac site (33).

This paper reports the development of armored long RNA (armored L-RNA) controls or standards for the bDNA assay for HIV-1 (AR-HIV-pol-3034b; 3,034 bases). This was accomplished by increasing the number and affinity of pac sites using a one-plasmid double-expression system, in which the cDNA sequence encoding the MS2 coat protein and maturase was cloned into one cloning site of vector pACYCDuet-1 and the HIV *pol* coding sequence (encompassing the target hybridization region for the Versant HIV-1 RNA 3.0 assay) with three C-variant pac sites was cloned into another cloning site of plasmid pACYCDuet-1.

MATERIALS AND METHODS

Construction of the recombinant plasmid pACYC-MS2. The cDNA sequence encoding the MS2 maturase and coat protein was amplified from the pMS_{27} plasmid (kindly provided by D. S. Peabody) by PCR using the following primers: MS2-S (5′-CGGGATCCTGGCTATCGCTGTAGGTAGCC-3′) and MS2-A (5′-AAGGAAAAAAGCGGCCGCTGGCCGGCGTCTATTAGTAG-3′). (The underlined sequences are BamHI and NotI restriction enzyme sites, respectively.) The 1.7-kb amplified DNA fragment was gel purified, digested with BamHI and NotI, and then inserted into one cloning site of the pACYCDuet-1 vector (p15A-type replication origin; Novagen) to generate the recombinant plasmid pACYC-MS2. The inserted DNA was verified by sequencing. The sequencing result was identified using BLAST in the NCBI database.

Construction of pACYC-MS2-pol-3034b. The exogenous chimeric fragment pol-3034b is the cDNA sequence containing the nearly full length HIV *pol* gene (2,977 bases) with three C-variant pac sites inserted at the front, middle, and

TABLE 1. Primers for PCR amplification

Primer name	Primer sequence[a]
Primer 1	5′-TTGGCCGGCC**ACATGAGGAT*C*ACCCATGT**GAATTTCCTTCAGAGCAGACCAGAG-3′
Primer 2	5′-**ACATGGGTGATCCTCATGT**AGCTGTCTTTTTCTGGCAGCACTATAG-3′
Primer 3	5′-**ACATGAGGAT*C*ACCCATGT**GGACTGTCAATGACATACAGAAG-3′
Primer 4	5′-CCTTAATTAA**ACATGGGTGATCCTCATGT**AATCCTCATCCTGTCTACTTGCCAC-3′
Primer 5	5′-CTGCCAGAAAAAGACAGCT**ACATGAGGAT*C*ACCCATGT**GGACTG-3′

[a] The underlined sequences are FseI and PacI restriction enzyme sites, respectively; the sequence in boldface is a C-variant pac site.

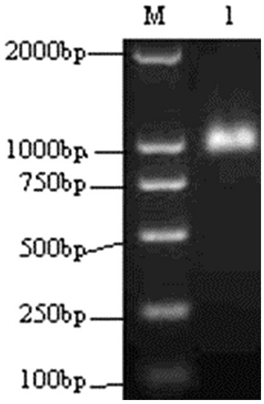

FIG. 2. Results of electrophoresis of AR-HIV-pol-3034b after purification by gel exclusion chromatography (1% agarose gel). Lane M, DNA marker; lane 1, AR-HIV-pol-3034b.

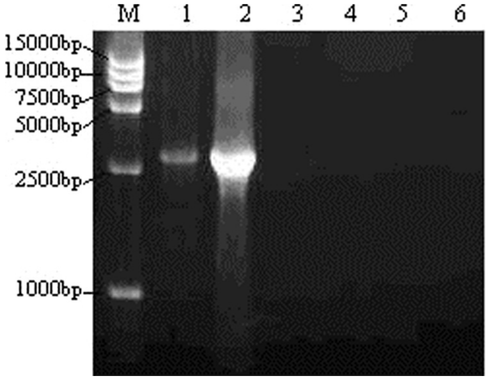

FIG. 3. Results of RT-PCR and PCR with 1% agarose gel analysis. Lane 1, RT-PCR of RNA extracted from AR-HIV-pol-3034b; lane 2, pSG3▲env, the positive control; lanes 3 to 6, the four negative controls (H_2O, H_2O after extraction and RT, RNA extracted from VLPs without RT, and VLPs without extraction and RT).

rear, respectively. This fragment was obtained by overlapping extension PCR as shown in Fig. 1. The primers used in this method are shown in Table 1.

The overlapping extension PCR product pol-3034b was inserted into the other cloning site of the plasmid pACYC-MS2 vector to generate the recombinant plasmid pACYC-MS2-pol-3034b. The recombinant plasmid pACYC-MS2-pol-3034b was verified by sequencing.

Production and purification of armored L-RNA. The recombinant plasmid pACYC-MS2-pol-3034b was transformed into the competent *Escherichia coli* strain BL21(DE3). The armored L-RNA (AR-HIV-pol-3034b) was expressed as described previously (19). Then the cells were harvested by centrifugation and lysed by ultrasonic disruption (Branson Sonifier 350). Twenty milliliters of supernatant was incubated with 1,000 U of RNase A and 200 U of DNase I at 37°C for 40 min in order to eliminate the *E. coli* genome RNA (19). AR-HIV-pol-3034b was further purified by Sephacryl S-200 gel exclusion chromatography (BioLogic DuoFlow chromatography system) and stored at 4°C. After staining with ethidium bromide, 5 μl of the collected fractions was analyzed by agarose gel electrophoresis (1%).

Reverse transcription-PCR (RT-PCR) identification of the length of the packaged RNA. RNA was extracted from purified AR-HIV-pol-3034b using a QIAamp viral RNA minikit (Qiagen, Germany), according to the manufacturer's instructions.

Reverse transcription (RT) reactions were performed in a total volume of 20 μl, containing 5 μl of the RNA extracted from purified AR-HIV-pol-3034b, 4 μl of 5× AMW buffer, 2 μl of the deoxynucleoside triphosphate mixture (10 mM each), 1 μl of 10 mM downstream primer C-3, 0.5 μl of 40-U/μl RNase inhibitor (TaKaRa, Japan), and 0.5 μl of 5-U/μl avian myeloblastosis virus reverse transcriptase (Promega). The mixture was incubated at room temperature for 10 min and then at 42°C for 60 min. It was then cooled at 4°C.

In order to verify whether the full length of the HIV *pol* sequence was packaged into the VLPs, PCR was performed in a 50-μl reaction volume, containing 5 μl of the cDNA obtained from the RT reaction, 5 μl of 10× Pyrobest Buffer II, 1 μl of the deoxynucleoside triphosphate mixture (10 mM each), 1 μl of the 10 mM primers C-1 and C-3, and 1 μl of 5-U/μl Pyrobest DNA polymerase (TaKaRa, Japan), at 94°C for 5 min; 35 cycles of 45 s at 95°C, 30 s at 56°C, and 180 s at 72°C; and 10 min at 72°C.

Several controls, including a positive control (pSG3▲env) and four negative controls (H_2O, H_2O after extraction and RT, RNA extracted from AR-HIV-pol-3034b without RT, and AR-HIV-pol-3034b without extraction and RT), were tested simultaneously.

The PCR products (5 μl) were analyzed by electrophoresis on an agarose gel (1%) containing ethidium bromide. PCR products were then purified and ligated with the pGEM-T Easy plasmids (Promega Corporation) for verification by sequencing.

Incubation with purified nucleases. AR-HIV-pol-3034b, pACYC-MS2-pol-3034b, and RNA isolated from AR-HIV-pol-3034b, each at 0.06 mg/ml, were each incubated with RNase A (5 U/μl) and DNase I (0.1 U/μl) at 37°C for 60 min. After digestion, the samples were stained with ethidium bromide and analyzed by agarose gel electrophoresis (1%) (18, 19, 31).

Stability of AR-HIV-pol-3034b in plasma. AR-HIV-pol-3034b was examined for its stability in EDTA-preserved human plasma. Initially, purified AR-HIV-pol-3034b was quantified, in duplicate, by the Versant HIV-1 RNA 3.0 assay (bDNA), according to the manufacturer's instructions. The quantified AR-HIV-pol-3034b was diluted with normal human EDTA-preserved plasma to yield 500 and 150,000 copies/ml. For each stability study, a single batch was separated into aliquots in individual-time-point samples of 1.0 ml, the volume required for the Versant HIV-1 RNA 3.0 assay. The samples were then incubated at 4°C, 37°C, and room temperature. The AR-HIV-pol-3034b samples were removed at each time point and stored at −80°C until the completion of the experiment. All of the samples were quantified, in duplicate, using the Versant HIV-1 RNA 3.0 assay (18, 31).

The AR-HIV-pol-3034b samples (500 and 150,000 copies/ml) were frozen at −20°C and thawed to room temperature five times. They were quantified, in duplicate, by the Versant HIV-1 RNA 3.0 assay (18, 31).

Performance of AR-HIV-pol-3034b positive controls in a clinical assay. To assess the performance of AR-HIV-pol-3034b as high- and low-positive controls in a clinical assay, the quantified AR-HIV-pol-3034b was diluted with normal human EDTA-preserved plasma to yield 500 and 150,000 copies/ml and was stored at 4°C in aliquots of 1.0 ml. AR-HIV-pol-3034b samples with high and low concentrations were assayed alongside patient samples and the Versant HIV-1 RNA 3.0 assay kit's high- and low-positive controls in regular clinical runs for the determination of HIV loads. Both the AR-HIV-pol-3034b positive controls and the Versant HIV-1 RNA 3.0 assay kit's positive controls were assayed (18, 31).

Linear analysis of AR-HIV-pol-3034b in the bDNA assay for HIV-1. AR-HIV-pol-3034b was diluted in normal human EDTA-preserved plasma in serial 10-fold dilutions (5×10^5, 5×10^4, 5×10^3, 5×10^2, and 50 copies/ml) and was quantified using the Versant HIV-1 RNA 3.0 assay. Triplicate samples and negative controls (normal human EDTA-preserved plasma) for each dilution were evaluated during the same assay run, and the quantification values were averaged (31).

Performance of AR-HIV-pol-3034b standards in a clinical assay. To assess the performance of AR-HIV-pol-3034b as standards in a clinical assay, the quantified AR-HIV-pol-3034b was diluted with normal human EDTA-preserved plasma to yield six samples with different concentrations, equivalent to the six standards provided with the kit. Then the absolute HIV copy numbers of 46 clinical samples were compared by using the commercial RNA standards provided with the kit versus the AR-HIV-pol-3034b standards.

RESULTS

Production and purification of AR-HIV-pol-3034b. After being expressed in *E. coli* BL21(DE3) and digested by DNase I and RNase A at 37°C for 40 min, AR-HIV-pol-3034b was purified by Sephacryl S-200 gel exclusion chromatography (data not shown). The purified AR-HIV-pol-3034b was electrophoresed, and a single band of approximately 1.0 kb could be seen by agarose gel analysis (1%) (Fig. 2), indicating that AR-HIV-pol-3034b was expressed and purified successfully.

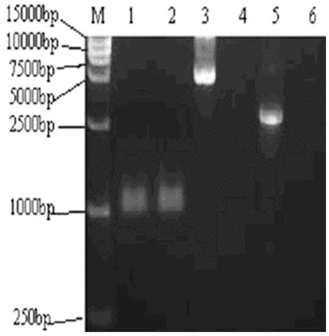

FIG. 4. Resistance of purified AR-HIV-pol-3034b particles to nucleases. Lanes 1, 3, and 5, AR-HIV-pol-3034b, pACYC-MS2-pol-3034b, and RNA isolated from AR-HIV-pol-3034b, respectively, before incubation in RNase A and DNase I. Lanes 2, 4, and 6, AR-HIV-pol-3034b, pACYC-MS2-pol-3034b, and RNA isolated from AR-HIV-pol-3034b, respectively, after incubation in RNase A and DNase I at 37°C for 60 min. AR-HIV-pol-3034b was completely resistant to DNase and RNase treatment; however, naked DNA and RNA were both degraded rapidly.

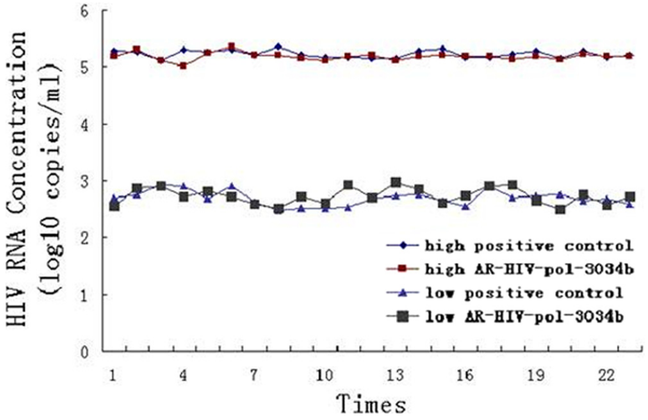

FIG. 6. Comparison of the AR-HIV-pol-3034b positive controls and the Versant HIV-1 RNA 3.0 assay controls in a clinical setting. The CVs for the AR-HIV-pol-3034b positive controls and the Versant HIV-1 RNA 3.0 assay controls were comparable. The high- and low-positive AR-HIV-pol-3034b controls both performed reliably compared with the corresponding controls provided with the kit.

Analysis of the length of the packaged RNA. The RT-PCR amplification product of the RNA extracted from AR-HIV-pol-3034b was full length (3,034 bp) (Fig. 3). This result indicated that AR-HIV-pol-3034b was expressed successfully by the one-plasmid double-expression system with three C-variant pac sites.

Durability of AR-HIV-pol-3034b. AR-HIV-pol-3034b was completely resistant to DNase and RNase treatment under conditions under which naked DNA and RNA are both degraded rapidly (Fig. 4).

Stability of AR-HIV-pol-3034b in plasma. The high-copy-number VLP-HIV-pol-3034b in normal human EDTA-preserved plasma was stable at 4°C for at least 6 months and at 37°C and room temperature for 3 months. The mean concentration for the high-copy-number samples at 4°C was 144,544 copies per ml (5.16 $\log_{10}$; range, 120,226 to 162,181 copies per ml), and the coefficient of variation (CV) was 11.4%. The low-copy-number VLP-HIV-pol-3034b in normal human EDTA-preserved plasma was stable at 4°C for at least 6 months and at 37°C and room temperature for 11 weeks. The mean concentration for the low-copy-number samples at 4°C was 505 copies per ml (2.70 $\log_{10}$; range, 278 to 825 copies per ml), and the CV was 27.5% (Fig. 5).

In addition, samples of AR-HIV-pol-3034b at high and low concentrations were all stable after being frozen and thawed five times.

AR-HIV-pol-3034b as positive controls in a clinical assay. The mean concentration for high-positive AR-HIV-pol-3034b controls was 151,324 copies/ml (5.18 $\log_{10}$), with a range of 101,238 to 219,962 copies/ml and a CV of 25.8%. For the high-positive controls provided with the kit, the mean concentration was 166,676 copies/ml (5.22 $\log_{10}$), with a range of 125,379 to 226,781 copies/ml and a CV of 26.0%. The mean concentration for low-positive AR-HIV-pol-3034b controls was 559 copies/ml (2.75 $\log_{10}$), with a range of 306 to 933 copies/ml and a CV of 33.6%. For the low-positive controls provided with the kit, the mean concentration was 508 copies/ml (2.71 $\log_{10}$), with a range of 294 to 843 copies/ml and a CV of 32.3%. The CVs for AR-HIV-pol-3034b positive controls and the Versant HIV-1 RNA 3.0 assay controls were comparable. The high- and low-positive AR-HIV-pol-3034b

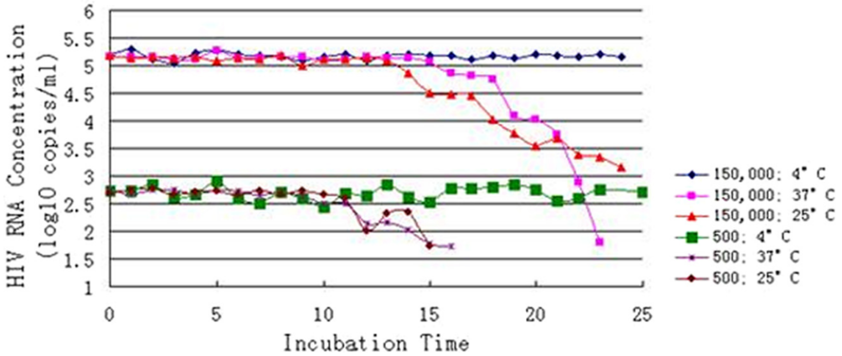

FIG. 5. Study of the stability of AR-HIV-pol-3034b. AR-HIV-pol-3034b samples of different concentrations in normal human EDTA-preserved plasma were stable at 4°C for at least 6 months. The key identifies high- and low-copy-number samples by their concentrations (150,000 and 500 copies/ml, respectively).

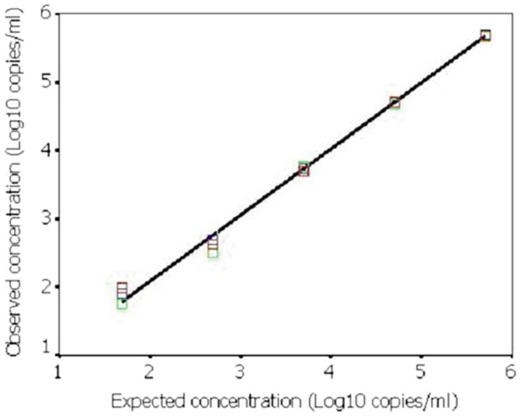

FIG. 7. Linearity of AR-HIV-pol-3034b in the Versant HIV-1 RNA 3.0 assay. AR-HIV-pol-3034b samples were diluted in serial 10-fold dilutions throughout the range of the HIV bDNA test. Tenfold dilutions of AR-HIV-pol-3034b produced linear results ($y = 0.14 + 0.97x$; $r^2 = 0.997$). The three differently colored symbols represent three observed concentrations for each expected concentration.

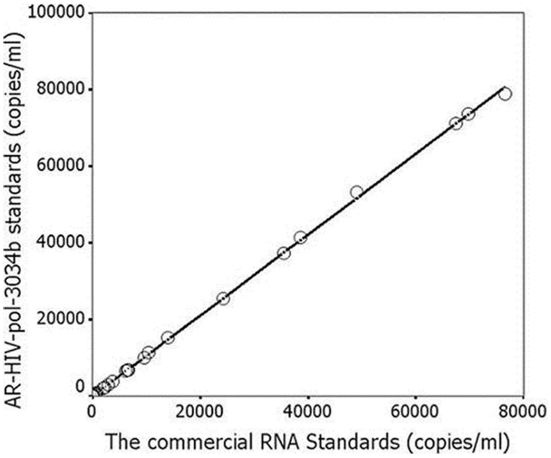

FIG. 8. Correlation of the AR-HIV-pol-3034b standards with the commercial RNA standards provided with the kit, determined by processing 46 clinical samples with each set of standards separately. The relationship between the standards is defined by the equation $y = 15.40 + 1.05x$ ($r^2 = 0.984$).

controls all performed reliably compared with the corresponding controls provided with the kit (Fig. 6).

Linear analysis of AR-HIV-pol-3034b in the bDNA assay for HIV-1. The relationship between the observed concentration and the expected concentration can be defined by the equation $y = 0.14 + 0.97x$. An r^2 value of 0.997 was calculated by linear regression analysis (Fig. 7). The linear results show that AR-HIV-pol-3034b is capable of functioning in the Versant HIV-1 RNA 3.0 assay for the clinical quantification of HIV loads.

AR-HIV-pol-3034b as standards in a clinical assay. To validate AR-HIV-pol-3034b standards in a clinical assay, viral loads in 46 clinical samples were detected. The viral loads measured with the two sets of standards showed a high correlation ($r^2 = 0.984$) (Fig. 8). The results showed that AR-HIV-pol-3034b is capable of functioning as standards in clinical assay.

DISCUSSION

In the present study, long RNA sequences (3,034 bases) could be encapsulated into VLPs using a one-plasmid double-expression system by increasing the number and affinity of the pac sites. The phenomenon that the packaging capacity could be enhanced by increasing the affinity of the pac site (a wild-type pac site replaced by a C-variant pac site) has not been illustrated clearly. It is proposed that the packaging of the genome or exogenous RNA by the MS2 coat protein is triggered by the sequence-specific binding of a coat protein dimer to the pac site (25, 29, 30, 36). Therefore, the affinity between them has a crucial influence on the packaging capacity. The replacement of uridine at position −5 of the wild-type pac site with cytosine gives rise to the formation of extra hydrogen bonds that will significantly improve the stability of the initiation complexes for assembly, thus further increasing the packaging capacity (7, 9, 30). In addition, once the assembly initiation complex is formed, the large single-stranded RNA molecule will be folded into compact and ordered secondary and tertiary structures appropriate for packaging. At the same time, the coat protein dimers will be switched into an allosteric conformation, from a largely symmetrical structure to an asymmetric structure, and subsequently will cooperatively bind to the RNA-protein complex by a nonspecific interaction with the pac site (13, 25, 29, 30, 36). Therefore, the enhanced packaging efficiency induced by increasing the number of pac sites may be due to one or more of the following mechanisms. (i) The initiation complex is able to form more quickly and to be more stable with the increase in the number of pac sites (7, 30), thus triggering packaging more efficiently. (ii) The initiation complex induces the conformational change of the RNA and coat protein dimers, so the increase in the number of pac sites can facilitate the continuation of the packaging with high efficiency and at a high rate (29, 30). (iii) The presence of a second pac site presumably makes the two coat protein dimers bind to the RNA in a cooperative manner, resulting in higher affinity and lower sensitivities to pH, ionic strength, and temperature than those with only a single pac site (21, 34).

In addition, another innovation in this study is that the one-plasmid double-expression system was applied to the expression of armored L-RNA for the first time, and the results revealed that this expression system is more favorable than other expression systems. The maximum theoretical length of the exogenous RNA packaged into the MS2 coat protein by the one-plasmid expression system is only about 1.9 kb, because the 1.7-kb MS2 gene encoding the maturase, the coat protein, and the pac site was also packaged simultaneously (20). On the other hand, the two-plasmid coexpression system has the disadvantage of lower expression efficiency, because the coat protein gene and the exogenous RNA constructed in two distinct plasmids may not be expressed at an optimal ratio, which is particularly important for efficient and specific packaging (21, 29). The one-plasmid double-expression system could compensate for these deficiencies, thus, it can act as the optimal expression system for the packaging of longer RNA.

In this study, AR-HIV-pol-3034b encompassed the target hybridization region for the Versant HIV-1 RNA 3.0 assay. It was shown to be completely resistant to DNase and RNase, to be stable in normal human EDTA-preserved plasma at 4°C for at least 6 months, and to produce reproducible, linear results in the Versant HIV-1 RNA 3.0 assay. AR-HIV-pol-3034b is homogeneous and noninfectious. All of these results demonstrated that AR-HIV-pol-3034b can be used as a positive control or standard for the Versant HIV-1 RNA 3.0 assay (bDNA).

In addition, 3,034-base exogenous RNA was successfully encapsulated into VLPs by using the one-plasmid double-expression system in conjunction with an increase in the number and affinity of the pac sites. It is possible that other armored L-RNA positive controls or calibrators can be produced by this method to meet other needs in clinical testing, such as the detection of more varieties of viral genomes simultaneously or comparison of the data from different clinical laboratories.

In conclusion, the one-plasmid double-expression system can be used as a better expression platform than the one-plasmid expression system and the two-plasmid coexpression system for armored L-RNA. Additionally, AR-HIV-pol-3034b, which exhibits DNase and RNase resistance, stability, and high levels of linearity and reproducibility, can be used as a positive control or standard in the Versant HIV-1 RNA 3.0 assay (bDNA) for HIV.

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