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RNase-Resistant Virus-Like Particles Containing Long Chimeric RNA Sequences Produced by Two-Plasmid Coexpression System▽

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ABSTRACT

RNase-resistant, noninfectious virus-like particles containing exogenous RNA sequences (armored RNA) are good candidates as RNA controls and standards in RNA virus detection. However, the length of RNA packaged in the virus-like particles with high efficiency is usually less than 500 bases. In this study, we describe a method for producing armored L-RNA. Armored L-RNA is a complex of MS2 bacteriophage coat protein and RNA produced in *Escherichia coli* by the induction of a two-plasmid coexpression system in which the coat protein and maturase are expressed from one plasmid and the target RNA sequence with modified MS2 stem-loop (pac site) is transcribed from another plasmid. A 3V armored L-RNA of 2,248 bases containing six gene fragments—hepatitis C virus, severe acute respiratory syndrome coronavirus (SARS-CoV1, SARS-CoV2, and SARS-CoV3), avian influenza virus matrix gene (M300), and H5N1 avian influenza virus (HA300)—was successfully expressed by the two-plasmid coexpression system and was demonstrated to have all of the characteristics of armored RNA. We evaluated the 3V armored L-RNA as a calibrator for multiple virus assays. We used the WHO International Standard for HCV RNA (NIBSC 96/790) to calibrate the chimeric armored L-RNA, which was diluted by 10-fold serial dilutions to obtain samples containing 10^6 to 10^2 copies. In conclusion, the approach we used for armored L-RNA preparation is practical and could reduce the labor and cost of quality control in multiplex RNA virus assays. Furthermore, we can assign the chimeric armored RNA with an international unit for quantitative detection.

Armored RNA is a complex of MS2 bacteriophage coat protein and RNA produced in *Escherichia coli* by the induction of an expression plasmid that encodes the bacteriophage sequence consisting of the maturase, the coat protein, the pac site, and an exogenous RNA sequence. This method produces recombinant virus-like particles that are noninfectious and contain predefined RNA (2-6, 8, 11, 12, 15, 16, 28). These armored RNAs are RNase resistant by virtue of their encapsulation within an MS2 coat protein, and they have been widely used as controls, standards, or calibrators for the detection of

hepatitis C virus (HCV) (11, 12, 28), human immune-deficiency virus (11, 15), severe acute respiratory syndrome coronavirus (SARS-CoV) (3, 11), enterovirus (2, 5), avian influenza virus 5 (4), and West Nile virus (6) using reverse transcription-PCR (RT-PCR), real-time RT-PCR, and branched DNA assays (2-6, 11, 12, 15, 28).

The MS2 bacteriophage consists of 180 U of the bacteriophage coat protein that encapsulates the bacteriophage genome (25). The MS2 phage RNA genome comprises a single plus-sense strand encoding 3,569 nucleotides. The genes are organized from the 5′ end as follows: the maturase or A protein, the bacteriophage coat protein, a 75-amino-acid lysis protein, and a replicase subunit. Packaging of the RNA genome by coat protein is initiated by high-specificity binding to a unique site on the RNA, a single stem-loop structure, containing the initiation codon of the gene for the viral replicase. The armored RNA contains approximately 1.7 kb of bacteriophage RNA sequence encoding the maturase, the coat protein, and the pac site. The wild-type MS2 bacteriophage contains an RNA genome of approximately 3.6 kb. In addition, the extensively folded nature of MS2 RNA (22) may make it particularly suitable for uptake into the confines of a small capsid. Thus, theoretically, at most, 1.9 kb of nonbacteriophage RNA sequence might be encapsulated by this method. Practically, the packaging of 500 bases of RNA has been demonstrated to be very efficient; however, packaging of 1- and 1.5-kb amounts of RNA is inefficient (15). Recently, Huang et al. (11) used armored RNA technology to package a 1,200-nucleotide foreign RNA sequence by deleting some disposable sequences between the multiple cloning site and the transcription terminator; however, to date, there have been no reports of armored RNA with sizes greater than 1,200 bp.

Although most RT-PCR assays do not target RNA sequences longer than 500 bases, there are some advantages if longer target RNA sequences are packaged. For example, the human immunodeficiency virus Quantiplex assay (Chiron Corp.) uses a standard that is approximately 3 kb in length; consequently, it is not possible to produce a single armored RNA standard for this assay using routine armored RNA technology. A further advantage of using armored RNA of several kilobases is that PCR primers for different regions of these genes may be used with a single armored RNA standard. Accordingly, it is not necessary to construct a different armored RNA standard for each PCR primer pair that might be used. With such a standard, different research groups and clinical laboratories could directly compare their quantitative data. In addition, if long RNA sequences were to be packaged, we could produce a single chimeric armored RNA standard and control that could meet the needs of a variety of different viral assays designed to detect different viral genomes. This would in turn simplify and reduce the cost of multivirus detection. Furthermore, if chimeric armored RNA sequences from different RNA viruses contain an HCV 5′ untranslated region (5′UTR), we could easily assign the chimeric armored RNA with an international unit for quantitative detection since an international standard for HCV RNA is available from the National Institute for Biological Standards and Controls (NIBSC) (20, 21).

In this article, we describe a method for packaging a long (>2,000 bp) RNA sequence, which is referred to as armored L-RNA technology. We sought to determine whether the bacteriophage sequences encoding the maturase and the coat protein could be replaced with nonbacteriophage RNA sequences, thereby enabling long-fragment RNA sequences to be packaged. In order to achieve this, we took advantage of a two-plasmid coexpression system in which the maturase and coat protein were expressed from one plasmid [pET-28(b)] and the target RNA containing a modified stem-loop (pac site) of MS2 was produced by a second plasmid (pACYCDuet-1). The pac site was located in the middle of the target sequence. In the present study, we used a C-5 variant of the wild-type stem-loop in which uridine had been substituted by cytosine. The replacement of the wild-type uridine with a cytosine at position −5 significantly increases the affinity of the RNA for the coat protein dimer (9, 19, 27, 29). The affinity increase has been estimated to be 6-fold (26) or even as high as 50-fold (13). The stronger

binding of the C-5 variant compared to the wild-type sequence has been suggested to be due to an interaction involving the donation of a hydrogen by the amino group of the cytosine or the corresponding hydroxyl group of the uracil enol tautamer (24).

MATERIALS AND METHODS

Construction of pET-MC. MS2 maturase and coat protein genes were amplified by using primers S-MC and A-MC (Table 1) from the pMS_{27} plasmid (kindly provided by D. S. Peabody) containing nucleotides (nt) 81 to 1749 of the MS2 bacteriophage gene (GenBank accession no. V00642). Sense and reverse primers contained BamHI and HindIII restriction sites (underlined), respectively. These primers correspond to nt 81 to 101 and nt 1721 to 1741 of the MS2 phage genome sequence. The 1.7-kb PCR-amplified DNA fragments were gel purified, digested with BamHI and HindIII, and then ligated to a linearized pET28b (Novagen) vector to generate the recombinant plasmid pET-MC. This plasmid was transformed into competent *E. coli* DH5α cells according to the manufacturer's instructions. The pET-MC plasmids in positive clones that could replicate in LB agar in the presence of 100 μg of kanamycin ml^{-1} were isolated by using a Takara MiniBEST plasmid purification kit (TaKaRa). The DNA insert was sequenced with vector-specific primers using an automated fluorescent DNA sequencer (model 3730XL; Applied Biosystems). The resulting sequences were identified by a search of the NCBI databases for homologous sequences using BLAST.

TABLE 1.

Primers used for PCR amplification

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[a]BamHI, HindIII, FseI, and PacI restriction sites are indicated by underscoring; a C vairiant is indicated in boldface type.

Construction of pACYC-3V. An exogenous chimeric sequence 2,248 bp in length comprising the following sequences was inserted into a pACYCDuet-1 plasmid (p15A-type replication origin; Novagen): M-300 (nt 17~373, 357 bp from avian influenza virus matrix gene; GenBank accession no. DQ864720), SARS-CoV1 (nt 15224 to 15618, 395 bp from SARS-CoV; GenBank accession no. AY864806), SARS-CoV2 (nt 18038 to 18340, 303 bp from SARS-CoV; GenBank accession no. AY864806), SARS-CoV3 (nt 328110 to 28692, 583 bp from SARS-CoV; GenBank accession no. AY864806), a pac site (19 bp), HCV (nt 18 to 310, 293 bp from HCV 5′UTR; GenBank accession no. AF139594), and HA300 (nt 295 to 611, 317 bp from H5N1 avian influenza virus; GenBank accession no. DQ864720). The target sequence included the forward and reverse primer sites, flanking regions, and probe-binding sites previously published or described. A 19-mer pac site was placed between SARS-CoV3 and HCV (Fig. 1). We spliced the six target DNA sequences using overlapping extensions (10). During the first-round PCR, these six small fragments were amplified as follows. SARS-CoV1 was amplified from a pBSSR-V6 plasmid (kindly provided by the Chinese Academy of Medical Sciences and Peking Union Medical College, Institute of Basic Medical Sciences), containing nt 13785 to 16051 of the SARS-CoV gene, using the primers S-SARS1 and LAP-SARS1. SARS-CoV2 was amplified from a pNCCL-SARS plasmid (constructed by our laboratory), containing nt 18038 to 18340 of the SARS-CoV gene, using the primers LAP-SARS2 and A-SARS2. SARS-CoV3 was amplified from a pBSSR7-8 plasmid (kindly provided by the Chinese Academy of Medical Sciences and Peking Union Medical College, Institute of Basic Medical Sciences), containing nt 27730 to 29212 of the

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SARS-CoV gene, using the primers LAP-SARS3 and A-SARS3. The HCV fragment was amplified from a pNCCL-HCV plasmid (constructed by our laboratory), containing nt 18 to 310 of the HCV gene, using the primers S-HCV and HCV-LAP1(underlined for 19mer pac site). HA300 was amplified from a pNCCL-H5N1 plasmid (constructed by our laboratory) using the primers HA300LAP and A-HA300. M300 was amplified from a pNCCL-H5N1 plasmid (constructed by our laboratory) using the primers M300-S′ and M+SLAP1. The PCR products from the first-round amplifications were gel purified and used, together with outside primers, in the overlap extension PCR. In the second-round PCR, amplified SARS-CoV1 plus SARS-CoV2 and HCV plus HA300 were amplified using the primers pairs S-SARS1-LAP-SARS2^{+} and FIVELAP2-OverlapA′, respectively. The PCR products from the second round were gel purified. The third-round PCR amplified SARS-CoV1 plus SARS-CoV2 plus SARS-CoV3 using the primers M+SLAP2 and FIVELAP1. The PCR products from the third round were gel purified. The fourth-round PCR amplified SARS-CoV1 plus SARS-CoV2 plus SARS-CoV3 plus HCV plus HA300 using primers M+SLAP2 and OverlapA′. The fifth-round PCR amplified M300 plus SARS-CoV1 plus SARS-CoV2 plus SARS-CoV3 plus HCV plus HA300 using the primers M300-S′ and OverlapA′. Sense and reverse primers contained FseI and PacI restriction sites (underlined in Table 1), respectively. The fifth-round PCR products were gel purified. The purified fragments were cloned into a pGEM-T Easy vector (Promega) and then excised from the resulting recombinant plasmid with the FseI and PacI restriction enzymes. Simultaneously, the pACYCDuet-1 plasmid was digested with FseI and PacI, and the resulting fragments were ligated into the linearized pACYCDuet-1 plasmid to produce a new donor plasmid (pACYC-3V). pACYC-3V plasmids in positive clones, which could replicate on LB agar in the presence of 100 μg of chloramphenicol ml^{-1}, were confirmed by PCR and sequencing.

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FIG. 1.

Armored L-RNA packaging system. Two expressing vectors were constructed, in which the maturase and the coat protein were expressed from one plasmid [pET-28(b)] and the pac site and the six-target chimeric RNA sequence were produced from the second plasmid (pACYCDuet-1). The pac site was located between SARS3 and HCV. 3V armored L-RNA was produced by inducing and expressing the two-plasmid system.

Construction of pET-MS2-3V. In order to compare armored RNA particles and armored L-RNA particles, we constructed pET-MS2-3V according to routine armored RNA technology (15). The DNAs encoding the maturase; the coat protein; the pac site; and the exogenous chimeric sequence (1,900 bp) containing SARS-CoV1, SARS-CoV2, SARS-CoV3, HCV, and HA300 were cloned downstream of the inducible T7 promoter of pET28b.

Expression and purification of virus-like particles. Both pET-MC and pACYC-3V plasmids were cotransformed into *E. coli* strain BL21(DE3). The 3V armored L-RNA was expressed as described previously (16). The cells were harvested by centrifugation and then washed three times with phosphate-buffered saline. The cells were pelleted and then resuspended in 20 ml of sonication buffer (5 mM $MgSO_4$, 0.1 M NaCl, 50 mM Tris [pH 8.0]). The cells were sonicated (Branson Sonifier 350) by using a small sonication probe operating at 50% duty cycle (unit 5 power) for five pulses. The sonicate was placed on ice for 1 min, and then the sonication step was repeated another five times. The sonicate was centrifuged in order to pellet the cell debris. A total of 20 ml of supernatant was then incubated with 1,000 U of *E. coli* RNase 1 and 200 U of bovine pancreatic DNase 1 at 37°C for 40 min in order to eliminate *E. coli* RNA and DNA. After nuclease treatment, 5 μl of supernatant was

electrophoresed on an agarose gel in TAE buffer and stained with ethidium bromide to assay for armored L-RNA. A CsCl gradient was then performed as the standard method (16). In order to compare the densities of 3V armored L-RNA and 3V armored RNA particles, the 3V armored RNA from pET-MS2-3V was expressed as described previously (16). Each RNA was loaded on separate gradients. After ultracentrifugation, the ultracentrifugation tube was stabilized in an upright position. An 18-gauge needle was slowly inserted into the bottom of the tube, and 0.5-ml fractions were collected and weighed in order to determine the density of the CsCl. The optical density of each fraction at 260 nm was measured in order to quantify the 3V armored L-RNA and 3V armored RNA particles. A 5-μl portion of each fraction was electrophoresed on an agarose gel in TAE buffer and stained with ethidium bromide in order to determine the fractions containing armored L-RNA and those containing armored RNA. The fractions were then pooled and dialyzed against sonication buffer in order to remove CsCl. The dialysate was collected and stored at 4°C.

RNA extraction. RNA was extracted from 140 μl of the purified armored L-RNA by using a QIAamp viral RNA minikit (Qiagen) according to the manufacturer's instructions. The extracted RNA was eluted in 60 μl of diethyl pyrocarbonate-treated H_2O and then used as a template for RT-PCR and RNA electrophoresis.

Identification of armored L-RNA by RT-PCR. RT of the 3V armored L-RNA was carried out by using the Overlap-A downstream primer; this primer corresponds to nt 581 to 605 of the H5N1 gene. PCR was carried out with the primers M300RT-S and HA300RT-A in order to amplify the full length of the 3V L-RNA. RT-PCR was conducted in separate (two-step) reactions by using an Eppendorf PCR system Autorisierter thermal cycler (Eppendorf). For the RT step, each reaction mixture (20 μl) contained 4 μl of first-strand buffer (Invitrogen), 1 μl of 10 mM deoxynucleoside triphosphates, 1 μl of RNaseOUT, 1 μl of 0.1 mM dithiothreitol, 1 μl of reverse primer (Overlap-A), 1 μl of SuperScript III reverse transcriptase (Invitrogen), 5 μl of RNA, and sterile distilled water to 20 μl. The reaction mixture was incubated initially at 55°C for 50 min and then at 70°C for 15 min. An aliquot (5 μl) of the resulting cDNA was amplified by PCR using a 25-μl mixture that contained 1× PCR buffer (Promega), 1.5 mM $MgCl_2$, 300 μM deoxynucleoside triphosphate, 1.5 μM concentrations of each primer, and 1.25 U of *Taq* DNA polymerase (Promega). After an initial incubation at 95°C for 3 min, 40 cycles of the following temperature conditions were used: 95°C for 30 s, 56°C for 30 s, and 72°C for 140 s. A final extension at 72°C for 10 min was performed. Several controls, including a negative control with no template, a positive control with DNA from pACYC-3V, and a negative control with supernatant of the virus-like particles without RT, were tested simultaneously. PCR products (5 μl) were analyzed by electrophoresis on agarose gels containing ethidium bromide.

In order to increase the sensitivity of the RT-PCR, a second amplification was performed in a 25-μl reaction mixture containing 5 μl of the amplification product under the same PCR conditions.

The identity of the amplification products was confirmed by agarose gel electrophoresis. The strands of full-length 3V PCR product were cloned into pGEM-T Easy vectors (Promega), and then the recombinant AT clones were sent to Beijing Sunbiotech Co. to be sequenced using T7 and SP6 primers. The sequences obtained were compared to the target sequences.

In order to determine the nature of the RNA packaged into 3V armored RNA, RT of the RNA was carried out with three downstream primers: Overlap-A, HCV-LAP1, and A-SARS3. PCR was carried out with the primers S-SARS1 and HA300RT-A in order to amplify the full length of the 3V RNA and with the primer pairs S-SARS1 and HCV-LAP1, S-SARS1 and A-SARS3, and S-SARS1 and A-SARS2 to amplify the SARS-CoV1+SARS-CoV2+SARS-CoV3+HCV sequence, the SARS-CoV1+SARS-CoV2+SARS-CoV3 sequence, and the SARS-CoV1+SARS-CoV2 sequence, respectively.

Stability of 3V armored L-RNA. The armored L-RNA was examined for stability in newborn calf serum. Initially, the purified 3V armored L-RNA preparation was quantified, in duplicate, with an HCV RNA PCR fluorescence quantitative diagnostic kit (Shanghai Kehua Bio-Engineering Co., Ltd.). The quantified 3V armored L-RNA was serially diluted 10-fold with the newborn calf serum to obtain 10,000 and 1,000,000 copies/ml. For each stability study, a single batch was separated into aliquots in individual time point samples of 100 μl. The samples were then incubated at 4°C, 37°C, or room temperature. 3V armored L-RNA plasma samples were removed at each time point and were stored at −80°C until completion of the experiment. All samples were quantified by using an HCV RNA RT-PCR fluorescence quantitative diagnostic kit (Shanghai Kehua) and LightCycler thermal cycler (Roche). The data were then analyzed by using LightCycler software (Roche).

Calibration of the chimeric armored RNA against an international standard for HCV RNA. Initially, the purified 3V armored L-RNA preparation was quantified, in duplicate, using a HCV RNA PCR fluorescence quantitative diagnostic kit (Shanghai Kehua). The quantified 3V armored L-RNA was diluted with newborn calf serum, and 500-μl aliquots were prepared by 10-fold serial dilution to obtain samples containing 10^6 to 10^2 copies/ml.

In order to calibrate the chimeric armored RNA, the National Reference material for HCV RNA (GBW09151, 2.26×10^3 IU ml^{-1} to 4.22×10^7 IU ml^{-1}) was used; these RNA values were assigned using the WHO HCV International Standard (NIBSC 96/790). This material is HCV gene type I. The reference material was redissolved in 300 μl of diethyl pyrocarbonate-H_2O when used.

Three different commercially available reagent kits were used for the calibration. The HCV RNA PCR-fluorescence quantitative diagnostic kit (Shanghai Kehua) was used for the HCV RNA tests, the SARS virus RNA PCR fluorescence quantitative diagnostic kit (Shenzhen PG Bio-Technology Co., Ltd.) was used for the SARS-Cov2 RNA tests, and the AIV-H5 virus RNA PCR fluorescence quantitative diagnostic kit (Shenzhen PG) was used for the HA300 RNA tests. The tests were carried out using a LightCycler thermal cycler (Roche). First, we used an international standard to calibrate the chimeric armored RNA. We isolated template RNA from the international standard and the diluted chimeric armored RNA. Newborn calf serum was used as a negative control. All RNA templates were assayed in a single run using the HCV RNA PCR fluorescence quantitative diagnostic kit. We then used the calibrated chimeric armored RNA to prepare calibrators of the SARS-CoV2 and HA300 real-time RT-PCR assays. Samples were assayed in three replicates in a 25-μl final volume containing 12.5 μl of extracted RNA and 12.5 μl of the master mix supplied with the respective kits. The thermal cycling conditions used for the three different kits are given in Table 2.

TABLE 2.

Thermal cycle conditions for the three different kits used in real-time RT-PCR assays

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RESULTS

Homogeneity of armored L-RNA. RNA was isolated from purified armored L-RNA particles. The majority of the 3V L-RNA packaged was approximately 2,200 bases in length, as detected by ethidium bromide staining (Fig. 2).

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FIG. 2.

Characterization of the recombinant RNA packaged in armored L-RNA. Recombinant RNA was isolated from 3V armored L-RNA, fractionated in a denaturing 3% agarose gel, stained with ethidium bromide, and detected by using UV fluorescence. Abbreviations: M, RNA marker; 3V, 3V armored L-RNA recombinant RNA.

Density gradient analysis of armored RNA and armored L-RNA particles. Armored RNA and armored L-RNA particles form narrow bands at 1.37 and 1.36 g ml^{-1}, respectively, when sedimented to their buoyant density in CsCl density gradients. This compares favorably with the previously reported value of 1.35 g ml^{-1} (15).

Cloning and sequencing of the RT-PCR products. The size of the RT-PCR amplification products of the RNA extracted from 3V armored L-RNA was full length (2,248 bp), whereas the size of RNA extracted from 3V armored RNA was between 1,000 and 2,000 bp (Fig. 3). The sequencing result demonstrated that the size was 1,200 bp.

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FIG. 3.

Ethidium bromide-stained 1% agarose gel of RT-PCR amplification products of RNA extracted from 3V armored L-RNA and 3V armored RNA. (A) RT-PCR amplification products of RNA extracted from 3V armored L-RNA. Lane 1, negative control with no template; lanes 2 and 3, negative control without RT; lanes 4 and 5, positive control of pACYC-3V plasmid; lanes 6 to 8, RT-PCR of 3V full-length L-RNA. (B) RT-PCR amplification products of RNA extracted from 3V armored RNA: lane 1, positive control of pET-MS2-3V plasmid using the primers S-SARS1 and HA300RT-A; lane 2, RT-PCR of SARS-CoV1 plus SARS-CoV2 plus SARS-CoV3 plus HCV+HA300 using the primers S-SARS1 and HA300RT-A; lane 3, positive control of pET-MS2-3V plasmid using the primers S-SARS1 and HCV-LAP1; lane 4, RT-PCR of SARS-CoV1 plus SARS-CoV2 plus SARS-CoV3 plus HCV using the primers S-SARS1 and HCV-LAP1; lane 5, positive control of pET-MS2-3V plasmid using the primers S-SARS1 and A-SARS3; lane 6, negative control without RT using primers S-SARS1 and A-SARS3; lane 7, RT-PCR of SARS-CoV1 plus SARS-CoV2 plus SARS-CoV3 using the primers S-SARS1 and A-SARS3; lane 8, RT-PCR of SARS-CoV1 plus SARS-CoV2 using the primers S-SARS1 and A-SARS2; lane 9, positive control of pET-MS2-3V plasmid using the primers S-SARS1 and A-SARS2; lane 10, negative control without reverse transcription using the primers S-SARS1 and A-SARS2.

Durability of armored L-RNA. The armored L-RNA was completely resistant to DNase and RNase treatment under conditions in which naked DNA and RNA are both degraded rapidly (data not shown).

Armored L-RNA plasma stability. The 3V armored L-RNA in newborn calf serum incubated at 4, 37, and 25°C was stable over 2 months (Fig. 4).

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FIG. 4.

Stability study of 3V armored L-RNA. 3V armored L-RNA was added to newborn calf serum to a final concentration of 10,000 and 10,000,000 copies/ml. Samples were incubated at 4°C, 37°C, or room temperature for 0, 1, 2, 4, and 8 weeks. Samples were removed at each time point and were stored at ~80°C until the completion of the experiment. From these materials, we isolated template RNA for real-time RT-PCR assays. Water was used as a negative control. All RNA templates were assayed in a single run by using an HCV RNA PCR fluorescence quantitative diagnostic kit (Shanghai Kehua Bio-Engineering Co., Ltd.). Real-time RT-PCR was conducted by using LightCycler technology (Roche). The mean for low-copy samples was 67,226 IU/ml (4.83 $\log_{10}$; range, 50,100 to 79,400 IU/ml [range, 4.70 to 4.92 $\log_{10}$]), and the coefficient of variation was 12.9%. The mean for high-copy samples was 29,060,000 IU/ml (7.45 $\log_{10}$; range, 22,900,000 to 41,700,000 IU/ml [range, 7.36 to 7.62 $\log_{10}$]), and the coefficient of variation was 22%.

Calibration of the chimeric armored RNA. In order to evaluate the 3V armored L-RNA as a calibrator for multiple virus assays, we used the National Reference HCV RNA assigned the HCV International Standard (NIBSC 96/790) to calibrate the serially diluted chimeric armored L-RNA. The concentrations of the chimeric armored L-RNA for the five samples (10^6, 10^5, 10^4, 10^3, and 10^2) were 1.354×10^7 IU ml^{-1}, 5.740×10^5 IU ml^{-1}, 6.580×10^4 IU ml^{-1}, 5.428×10^3 IU ml^{-1}, and 9.613×10^2 IU ml^{-1}, respectively (Fig. 5).

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FIG. 5.

Calibration of the real-time RT-PCR assay for HCV, SARS-CoV2, and HA300 (H5N1). First, the quantified 3V armored L-RNA was diluted with newborn calf serum 10-fold serially to obtain 100, 1,000, 10,000, 100,000, and 1,000,000 copies/ml. We used the National Reference material for HCV RNA (GBW09151, 2.26×10^3 IU ml^{-1} to 4.22×10^7 IU ml^{-1}) to calibrate the serial dilutions of chimeric armored L-RNA and then used the calibrated L-RNA to prepare calibrators for the two real-time RT-PCR assays. From these materials, we isolated RNA template for RT-PCR assays. Newborn calf serum was used as a negative control. Real-time RT-PCR was conducted on a LightCycler thermal cycler (Roche). (A) Log concentration of the international standard for HCV RNA versus the cycle number for the HCV RT-PCR; (B) amplification curve for the HCV RT-PCR assay; (C) amplification curve for the SARS-CoV2 RT-PCR assay; (D) amplification curve for the HA300 (H5N1) RT-PCR assay.

DISCUSSION

The armored L-RNA (2,248 bp) expressed by our two-plasmid coexpression system differs in several respects from the virus-like particles previously described by Pickett and Peabody (17). These authors also used a two-plasmid expression system; their goal was to determine whether the 21-nt Operator (pac site) would confer MS2-specific packageability on nonbacteriophage RNA in vivo. The *E. coli* was induced such that the Operator-*lacZ* hybrid RNA was coexpressed with the MS2 coat protein. The specificity of the Pickett and Peabody bacteriophage packaging system, however, was poor since the host *E. coli* RNA was packaged in preference to the Operator-*lacZ* RNA. In other studies, Pickett and

Peabody modified the packaging of the Operator-*lacZ* RNA by changing the ratios of coat protein to Operator-*lacZ* RNA produced in *E. coli*. By increasing the concentration of the Operator-*lacZ* RNA and decreasing the concentration of the coat protein, these researchers were able to encapsulate mainly the Operator-*lacZ* RNA. These results suggest that the original Pickett and Peabody packaging strategy lacked specificity because they were unable to determine an appropriate ratio of coat protein to Operator-*lacZ* RNA. Furthermore, the size of the *lacZ* RNA purified from these virus-like particles was approximately 500 bases as opposed to the expected full-length 3,000 bases. These authors were, however, unable to determine whether the degradation occurred before or after encapsulation. In their second set of packaging studies, the RNA was not assessed for size by gel electrophoresis. The MS2 bacteriophage sequence of the RNA of Pickett and Peabody's particles consisted only of the coat protein sequence in one recombinant plasmid, which contrasts with the MS2 maturase and coat protein sequences used in our method. The maturase protein is an important component of armored RNA. Its presence in the virus-like particles is required to preserve the integrity of the genomic RNA against RNase digestion (1, 7). In addition, a potential binding site for coat protein has been identified in the MS2 maturase sequence on the basis of structural homology to the translational operator (18). The maturase protein interacts specifically with viral RNA at two sites (23) and may therefore play a facilitating role in packaging. Pasloske et al. (16) used a single plasmid expression system to produce armored RNA. The armored RNA contained approximately 1.7 kb of bacteriophage RNA sequence encoding the maturase, the coat protein, and the pac site. Since the MS2 bacteriophage RNA genome is approximately 3.6 kb, it is likely that the maximal size of target RNA packaged will be approximately 2.0 kb in armored RNA; however, to date, there have been no reports of armored RNA of more than 1,200 bp using the method proposed by Pasloske et al.

In order to arrive at an appropriate ratio of coat protein to the six-target chimeric sequence, we selected the pET28b and pACYCDuet-1 plasmids as expression vectors. These two plasmids are members of different compatibility groups. Therefore, they can be stably maintained together in the same bacterial host. These plasmids contain the same T7 bacteriophage promoter, and they are both low-copy plasmids, having almost equivalent copy numbers. Given this equivalence, the ratio of coat protein from pET28b to the six-target chimeric RNA sequence from pACYCDuet-1 should be appropriate.

Compared to armored RNA of approximately 1,200 bp obtained using the original armored technology, our work indicates that long-fragment (2,248 bp) RNA sequences can be encapsulated by using the two-plasmid coexpression method in conjunction with the C-variant of the wild-type stem-loop. The armored L-RNA particles have all of the characteristics of armored RNA. The technology allows the user to precisely define the control RNA's sequence. The armored L-RNA comprising a 2,248-nt foreign RNA sequence, which includes three SARS-CoV fragments, one HCV fragment, and two H5N1 fragments, can be used as a control or calibrator for SARS-CoV, HCV, and H5N1 qualitative or quantitative detection by RT-PCR. The inclusion of the HCV 5′UTR made it easy to assign an international (IU) value to the SARS-CoV and H5N1 RNAs within the armored L-RNA and avoided complex procedures involved in value assignment of calibrators or standards in situations where their international standard (IS) are not available (20, 21). Moreover, the metrological traceability of nucleic acid measurement of all RNA viruses without IS could be solved by the same model as chimeric armored L-RNA.

From Fig. 5, it can be seen that the highest copy number of chimeric armored L-RNA used was calibrated higher than expected and was not near a 1:1 correlation, a finding that might be explained as follows. First, an error in the first step of dilution could have occurred. Second, it was acceptable that the detection deviation for the samples was in the range of the target value ± 0.27 $\log_{10}$ (14).

In theory, the length of armored L-RNA expressed by the two-plasmid system could be as much as approximately 3.6 kb since the MS2 bacteriophage RNA genome is 3,569 bp in length. The results presented here indicate that at least a 2,248-bp armored L-RNA can be expressed with high efficiency.

We have also successfully expressed an approximate 2,700-bp chimeric armored RNA by the two-plasmid system (data not shown), and the construction of an expression vector for a chimeric armored L-RNA of more than 3,000 bp in length is under way.

Because the pac site is a key point in the interaction between the MS2 coat protein and exogenous RNA, it is believed that the expression efficiency and package capacity could be further enhanced if the number of pac sites were to be increased within the chimeric RNA.

In conclusion, the results presented here demonstrate that the two-plasmid expression system for armored L-RNA (>2,000 bp) is effective. The chimeric armored L-RNA, which exhibits RNase resistance and stability properties similar to armored RNA, can be used as a calibrator in SARS-CoV, H5N1, and HCV RT-PCR assays.

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FOOTNOTES

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