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# Construction of Lentivirus-Based Reference Material for RT-PCR Detection of Middle East Respiratory Syndrome Coronavirus and Its Application in External Quality Assessment

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Nucleic acid amplification technology (NAT) has been the most used one for rapid detection of Middle East Respiratory Syndrome coronavirus (MERS-CoV) since MERS-CoV was first detected in 2012. It is important to develop stable and safe reference materials for assessing the quality of NAT kits and performing an external quality assessment (EQA) in different laboratories. In this study, the MERS-CoV RNA fragments including *upE*, *ORF1b*, and *N* were packed within human immunodeficiency virus type 1 (HIV-1)-like particles. The lyophilized virus-like particles (VLPs) were found to be stable at 37 °C or below and safe to be used not only as the control material for PCR detection of MERS-CoV but also as the reference material for EQA. In an EQA organized by Ningbo International Travel Healthcare Center in China, 49 participating institutions achieved 100% agreement in detecting MERS-CoV using various commercial diagnosis kits and different extraction methods. However, different Ct values reported by different sites for the same sample implied that a need exists to standardize the RNA extraction method and/or the PCR detection conditions between the laboratories.



**Keywords:** Middle East Respiratory Syndrome Coronavirus, Virus-Like Particles, External Quality Assessment, Reference Material.

## 1. INTRODUCTION

Since the Middle East Respiratory Syndrome coronavirus (MERS-CoV) was first identified on 22 September 2012,[1] it has caused several outbreaks. In the latest of them, which took place in 2015, 186 South Koreans were infected, and 38 of them died. Due to a lack of effective intervention and treatment, early diagnosis and isolation are the primary methods to control MERS-CoV infection.[2] The method used most commonly and frequently for early detection of MERS-CoV is the real-time reverse transcription polymerase chain reaction (RT-PCR) targeting *upE*,[3] *ORF1a*, or *ORF1b* fragments of MERS-CoV.[4, 5]

Most of the MERS-CoV detection kits utilize plasmids or *in vitro*-transcribed RNA as the positive control for the assay. However, these materials do not offer a control for the extraction procedure. Recombinant bacteriophages such as MS2 could be used to control the efficiency of RNA extraction protocol and the presence of PCR inhibitors in RT-PCR assays.[6–8] Since the maximum load volume of MS2 for foreign genes is about 2 kb, it appears necessary to construct recombinant bacteriophages capable of accommodating different RNA targets of MERS-CoV. Although wild-type virus preparations could be used as controls,[9, 10] their use is limited to laboratories which have access to biosafety level 3 facilities.[11] Furthermore, inactivation of a high-titer virus stock by a single method does not assure safety, and most published procedures that inactivate viruses through a combination of methods may result in disruption of viral RNA, making its suitability as NAT standard uncertain.[12]

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Because camels constitute the natural reservoir of MERS-CoV, it is believed that another MERS-CoV outburst is inevitable. China is one of the countries in closest contact with the Middle East, and a large number of people enter and exit China, especially in harbor cities. As a result, there is a risk that MERS-CoV enters China again. Screening of high-risk populations from MERS-CoV affected regions is therefore essential not only for the control and prevention of MERS-CoV in China natives but also as a part of the global task of fighting the infection. The availability of reference materials for the comparison of the sensitivity of different assays, for the validation of recently developed point-of-care technologies, and for the harmonization of inter- and intra-laboratory results is therefore fundamental for the control of MERS-CoV.

In the present study, we packed the MERS-CoV RNA fragments into human immunodeficiency virus type 1 (HIV-1)-like particles using a lentiviral packaging system. The final product is in the form of pellets which are safe, non-replicating, freeze-fried specimens that can function as the control material for the entire diagnostic procedure from viral RNA extraction to nucleic acid amplification. An external quality assessment (EQA) based on the virus-like MERS-CoV, organized by the Ningbo International Travel Healthcare Center and involving 49 laboratories in China, has shown that the virus-like particles (VLPs) are also suitable for use as the reference material for preparing the sample panel in the EQA.



## 2. MATERIALS AND METHODS

### 2.1. Plasmids Preparation

The nucleotide sequences of MERS-CoV *upE* (27180 bp–27514 bp), *ORF1b* (17700 bp–19000 bp), and *Nseq* (28500 bp–30000 bp) were from the NCBI (GenBank accession number KT029139.1).[13] Fragment *1bN* containing ORF1b and Nseq was synthesized by GenScript Company (Nanjing, China). Fragment *upE* was provided by Professor Zhengli Shi (Wuhan Institute of Virology, Chinese Academy of Sciences, China). These two fragments were sub-cloned into the pCMV-MCS-CopGFP vector (clone sites and primers used are listed in Table I), forming the expression plasmids pCMV-MCS-upE and pCMV-MCS-1bN. Lentiviral vector pCMV-MCS-CopGFP is a commercial lentiviral vector in which the Cytomegalovirus (CMV) major immediate early promoter (MIEP) is upstream of the multi-cloning sites and it contains green fluorescence protein (GFP) as a reporter gene. The constructed plasmids were confirmed by sequencing (Tsingke Co, Wuhan, China) and analyzed by the DNAstar MegAlign software.

**Table I.** The primers used in this study.

| Name | Sequence (5′–3′) |
|---|---|
| MERSVLP-upE-F | CGAATTCCTACATTCCACTGTTT |
| MERSVLP-upE-R | CGTGGATCCCGTTAAACCCACTCGTCAG |
| MERSVLP-1bN-F | CGGAATTCTTTTATTACTGCCAATCC |
| MERSVLP-1bN-R | TCGGATCCAGGTGACAGTCTTTAACAT |

*Note*: Underlining denotes the clone sites.

### 2.2. Expression and Purification of MERS-CoV VLPs

The MERS-CoV VLPs were produced by the third generation lentivirus packaging system, which contains four plasmids: pCMV-MCS-CopGFP, PLP1, PLP2, and VSVG. To block the expression of MERS-CoV proteins, start codons were removed from every fragment. To increase the biosafety of the system, the long terminal repeats in the lentiviral vectors are defective (ΔU3), an internal promoter is missing, and the envelope protein is not expressed in the transfected cells, rendering the HIV-like particles non-infectious. The lentiviral particles were generated by transfection of $5 \times 10^6$ HEK293T-17 (ATCC CRL-11268) cells in a 10 cm dish with a mixture of 18 $\mu$L of lipofectamine 3000 reagent (Promega), 12 $\mu$g of PLP1, 6 $\mu$g of PLP2, 3 $\mu$g of VSVG, 9 $\mu$g of pCMV-MCS-upE or pCMV-MCS-1bNin, and 650 $\mu$L Dulbecco modified essential medium (DMEM, Gibco). After incubation for 5 min at room temperature, the mixture was added drop-wise to 8 mL of DMEM supplemented with 10% fetal calf serum. The cells were then cultivated at 37 °C with 5% $CO_2$ for 72 hours. The supernatant was harvested and filtered using a 0.45 $\mu$M filter (Millipore). The VLPs in the supernatant were purified by ultracentrifugation at 201,400 g at 4 °C on a 20% sucrose cushion in 50 mM sodium phosphate buffer using the SW70 rotor in a Beckman Optima LE-80K Ultracentrifuge. Subsequently, the pellets were washed once with phosphate buffer saline (PBS, pH 7.4) and harvested by ultracentrifugation at 201,400 g at 4 °C for 1 hour. Finally, the MERS-CoV VLPs were resuspended in 100 $\mu$L PBS (pH 7.4) with 5% trehalose dehydrate (Sigma) and 1% Bovine Serum Albumin (BSA) at a concentration of about $10^8$–$10^9$ copies/mL and freeze-dried.

### 2.3. Freeze-Drying Procedure

In separate procedures, the final preparations were aseptically dispensed in 0.1 mL aliquots into 2 mL glass vials. The mixture was pre-frozen at −80 °C for 12 hours, following by lyophilization at −56 °C for 6 hours in a freeze-dryer (ScanVac CoolSave™ 55-9, Germany).

### 2.4. Transmission Electron Microscopy

A 20 $\mu$L aliquot of MERS-CoV VLPs solution in the supernatant after the sucrose-purification was soaked with a Formvar carbon-coated copper grid for 10 minutes. The grids were then removed and washed 3 times in pure water. After that, the grids were stained with 20 $\mu$L of 2% phosphotungstic acid (PTA, pH 6.8) for 30 s, air-dried, and analyzed by transmission electron microscopy (H-7000FA, Hitachi).

### 2.5. Assessment of MERS-CoV VLPs

The number of copies of RNA in the freeze-dried MERSVLPs powder was quantified using droplet digital PCR (ddPCR). The ddPCR was conducted utilizing a QX200™ ddPCR system (Bio-Rad) according to the manufacturer's recommendations. Briefly, the lyophilized powder was reconstituted with 140 $\mu$L water, and RNA was extracted using a QIAamp viral RNA minikit (Qiagen). The obtained RNA was collected with 60 $\mu$L water. Subsequently, 5 $\mu$L of RNA solution were reverse-transcribed in cDNA using the GoScript™ Reverse Transcription system (Promega, USA) with specific primers in accordance with the manufacturer's instructions. Finally, PCR reaction mixture was prepared by mixing 11 $\mu$L of 2× Supermix, 0.25 $\mu$M of each of the probes, 0.9 $\mu$M of each primer, 2 $\mu$L of the cDNA solution, and water for a total volume of 22 $\mu$L. The primers and probes were bought from Liferiver (Shanghai, China). Droplets were generated using the Automated Droplet Generator (Bio-Rad), in which a vacuum was applied to the outlet wells to partition simultaneously the PCR mixtures into nanoliter-sized droplets. The obtained PCR plate was subsequently heat-sealed with pierceable foil using a PX1™ PCR plate sealer (Bio-Rad) and amplified in a conventional thermal cycler (C100 Touch, Bio-Rad). The thermo-cycling parameters were set as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min (temperature ramp 2 °C/s), and final extension at 98 °C for 10 min. After the reactions were completed, the 96-well plate was fixed into a plate holder and placed in a Q200 Droplet Reader (Bio-Rad). The data obtained were analyzed using the provided software package (QuantaSoft, Bio-Rad). Droplet counts below 10,000 droplets were considered unacceptable and, therefore, discarded.

The stability of the lyophilized VLPs powder was tested by storing the powder at 4 °C, room temperature, and 37 °C for one week. After this time, the copies of RNA in the freeze-dried MERS-CoV VLPs powder were checked again using the above procedure.

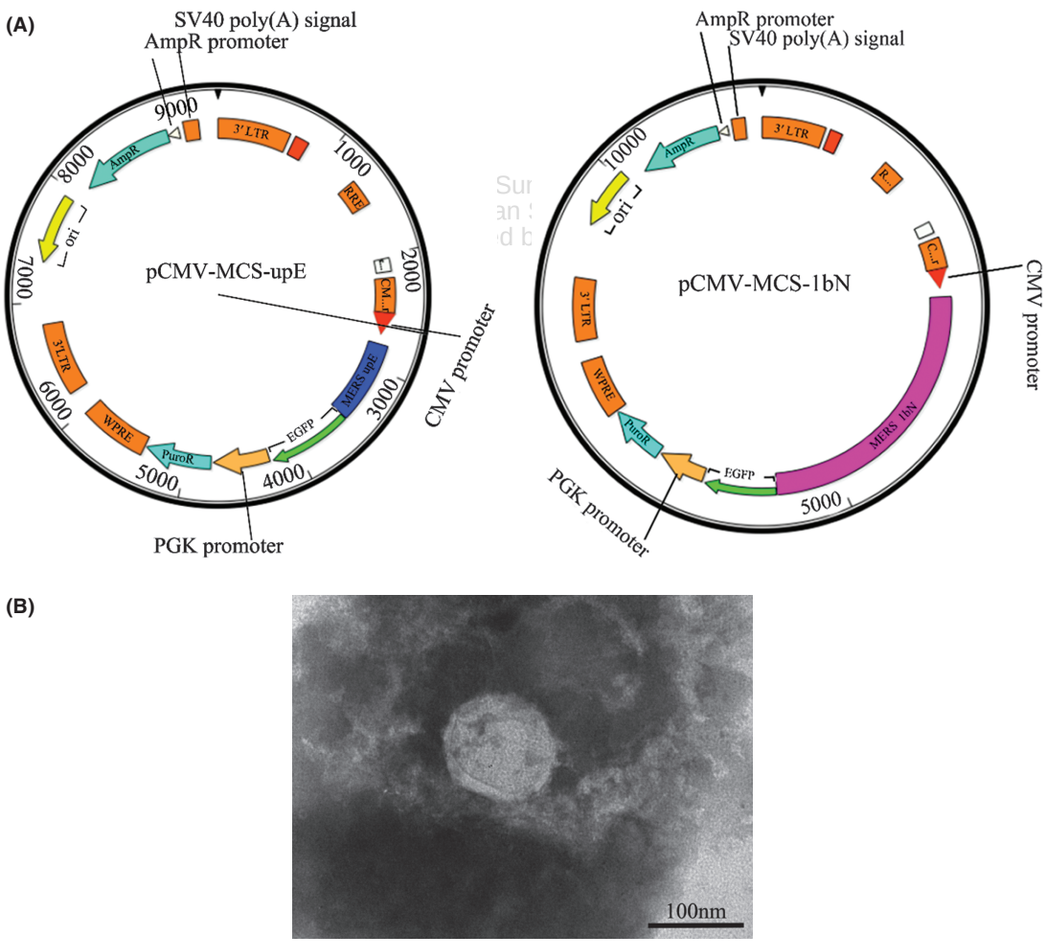


**Figure 1.** (A) MERS-CoV sequences of *upE* (Dark blue) and 1bN (Pink) were subcloned into the lentiviral vectors between the restriction sites *EcoR1* and *BamH1*, respectively. The main elements of the lentiviral vector used for the production of viral RNA and incorporation within the HIV-like particle are indicated. (B) A representative image of MERS-CoV VLPs obtained by negative staining and transmission electron microscopy. The average particle size is 100 nm (average of 6 fields).

**Table II.** DNA content in MERS pseudoviral particles determined by ddPCR.

| Sample | Reverse transcription | Concentration of pseudoviral particles (copies/mL) | Percentage of the total concentration (%) |
|---|---|---|---|
| MERS-upE | + | $1.27 \times 10^{10}$ | 100 |
| MERS-upE | − | $3.6 \times 10^{8}$ | 2.76 |
| MERS-1bN | + | $1.01 \times 10^{10}$ | 100 |
| MERS-1bN | − | $1.05 \times 10^{8}$ | 1.02 |

### 2.6. Sample Panel Preparation and External Quality Assessment (EQA)

A panel consisting of three negative and seven positive samples ranging from $8 \times 10^2$ to $5 \times 10^7$ copies/mL of the VLPs was designed for use in an external quality assessment (EQA) organized by Ningbo International Travel Healthcare Center. Briefly, one bottle of the lyophilized powder was reconstituted using 1 mL of a virus preservation solution (DMEM supplemented with 0.1% BSA, 10% glycerol, and antibiotics), and 10 fold serial dilutions, from $10^2$ to $10^7$ copies/mL, were prepared. A 140 $\mu$L aliquot of each sample was extracted using the QIAamp viral RNA mini kit (Qiagen, US) following the manufacturer's instructions, and eluted in 60 $\mu$L water. Commercial PCR kit (Liferiver, Shanghai, China) was used to test the solutions. At the same time, 100 aliquots of 40 $\mu$L were taken from the solutions with three different concentrations of the MERS-CoV VLPs (Sample SP, $1 \times 10^6$ copies/mL; Sample NP, $5 \times 10^5$ copies/mL, and Sample LP, $1 \times 10^5$ copies/mL). A homogeneity validation test was performed by randomly selecting 10 aliquots for the RT-PCR test. To evaluate the stability of the reconstituted particles, another 4 aliquots of 40 $\mu$L were taken from the SP, NP, and LP solutions of the sample, and stored at −70 °C, −20 °C, 4 °C and 37 °C for 7 days, respectively. The RT-PCR test was performed at 1, 3, and 7 days to determine the stability of the sample during the storage.

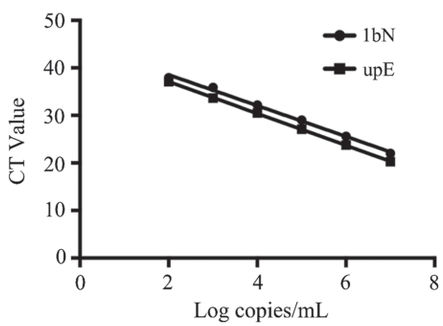


**Figure 2.** Standard curves for qPCR determination of MERS-CoV upE VLPs (squares) and MERS-CoV 1bN VLPs (dots) after RNA extraction by a commercial kit. The extracted RNA was tenfold diluted serially from $10^7$ to $10^2$ copies/$\mu$L. The slope of the MERS-CoV upE VLP standard curve is −3.350 ($R^2 = 0.99981$). The slope of the MERS-CoV 1bN VLPs standard curve is −3.244 ($R^2 = 0.99537$).

**Table III.** Stability of the freeze-dried MERS pseudoviral particles after 7 days of storage at different temperatures.

| Sample | Temperature | | |
|---|---|---|---|
| | −20 °C | 4 °C | 37 °C |
| MERS-upE | 0* | −0.4946 | −0.6204 |
| MERS-1bN | 0 | 0.0679 | −0.6919 |

*Notes*: *The *upE* and ORF 1b genes of two types of pseudoviral particles were detected by ddPCR. After calculating the copy number of virus, the difference of copy number under each temperature and temperature was expressed by delta Log (copies/mL): Log (T °C) −Log (−20 °C).

The EQA was attended by 49 laboratories, most of them under the authority of the Chinese Entry-exit Inspection and Quarantine Bureau and Centers for Disease Control and Prevention of China. A sample panel consisting of different concentrations of the MERS-CoV VLPs was prepared and transported to the laboratories in an ice box. The test results were collected and evaluated within one month.

## 3. RESULTS AND DISCUSSION

### 3.1. Generation of MERS-CoV Virus-Like Particles

Two constructed lentiviral vectors, pCMV-MCS-1bN containing MERS-CoV ORF1b and N, and pCMV-MCS-upE containing *upE*, are illustrated in Figure 1(A). After expression, the pellets obtained were visualized by transmission electron microscopy to confirm that VLPs were produced. As shown in Figure 1(B), the average particle size was 100 nM, similar to that reported previously.[14] The concentrations of MERS-CoV *upE* VLPs determined by the ddPCR assay were $4.701 \pm 1.102 \times 10^8$ copies/mL, while those of MERS-CoV 1bN VLPs were $2.730 \pm 1.483 \times 10^8$ copies/mL. By comparing the copy numbers obtained without reverse transcription (Table II), the plasmid DNA contamination in the VLP solutions was low, from 1% to 2.76% of the total copy numbers obtained with reverse transcription.

### 3.2. Evaluation of MERS-CoV VLPs as the Positive Control Material in NAT

By reconstituting the lyophilized MERS-CoV VLPs with PBS buffer, the resulting solution could be used as

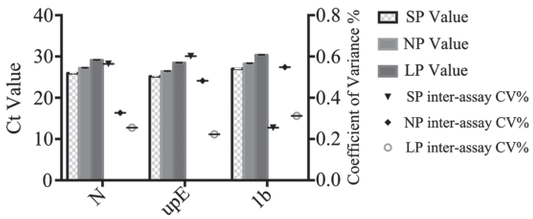


**Figure 3.** Homogeneity of the MERS-CoV VLPs solution prepared by reconstituting the lyophilized powder with the virus protection solution. Sample SP: $1 \times 10^6$ copies/mL, Sample NP: $5 \times 10^5$ copies/mL, and Sample LP: $1 \times 10^5$ copies/mL. The histogram indicates average Ct values of each sample. Dots show the CV of repeated qPCR assays.

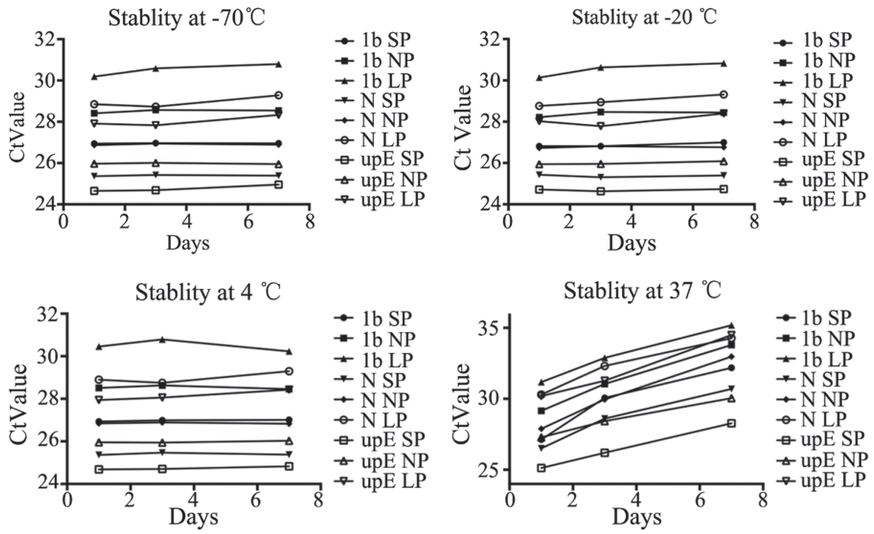


**Figure 4.** Stability of the VLPs solutions after one week of storage at different temperatures from 1 to 7 days. Samples were assessed by quantitative RT-PCR detection of *ORF1b* and *upE*.

the positive control in the NAT assay kits. As shown in Figure 2, good linearity ($R^2 = 0.99537$ for MERS-CoV 1bN VLPs, and $R^2 = 0.99981$ for MERS *upE* VLPs) was obtained using the commercial qPCR kit to test serial dilutions of the VLP ranging from $10^2$ to $10^7$ copies/mL. The lyophilized powder of the MERS-CoV VLPs was quite stable, and no significant differences in the form of ΔLog (copies/mL) were found using the ddPCR after storing at −20 °C, 4 °C, and 37 °C for 7 days (Table III).



**Table IV.** Performance of 49 laboratories participating in the external quality assessment of MERS pseudoviral particles.

| No. | NA extraction kit[a] | NA extraction method | NA detection method[b] | qPCR platform | Sample panel[c] | Agreement with the expected results (%) |
|---|---|---|---|---|---|---|
| 1 | RNAeasy | Magnetic bead | A | ABI 7500 fast | SP/NP/LP/N | 100 |
| 2 | KINGFISHER | Magnetic bead | A | ABI viia7 | SP/LP/N/N | 100 |
| 3 | Un | Un | A | ABI stepone | SP/LP/N/N | 100 |
| 4 | Un | Un | A | Roche LC480 | SP/NP/LP/N | 100 |
| 5 | Daan gene | Magnetic bead | C | Qiagen RGQ | SP/LP/N/N | 100 |
| 6 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 7 | Un | Un | A | Roche LC2.0 | SP/LP/N/N | 100 |
| 8 | QIAamp | Spin column | A | ABI 7300 | SP/NP/N/N | 100 |
| 9 | Un | Un | F | ABI 7500 | SP/NP/LP/N | 100 |
| 10 | QIAamp | Spin column | A | ABI 7500 | SP/NP/LP/N | 100 |
| 11 | PROMEGA | Spin column | A | ABI 7500 | NP/LP/N/N | 100 |
| 12 | Un | Un | A | ABI stepone plus | NP/LP/N/N | 100 |
| 13 | QIAamp | Spin column | A | ABI Qb | NP/LP/N/N | 100 |
| 14 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 15 | QIAamp | Spin column | B | ABI 7500 fast | SP/NP/LP/N | 100 |
| 16 | QIAamp | Spin column | A | ABI stepone plus | NP/LP/N/N | 100 |
| 17 | QIAamp | Spin column | A | Cephid PCR | SP/NP/N/N | 100 |
| 18 | QIA EZ1 | Magnetic bead | D | ABI viia7 | NP/LP/N/N | 100 |
| 19 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 20 | QIAamp | Spin column | A | Roche LC480 | SP/NP/N/N | 100 |
| 21 | ZJ bio-tech | Magnetic bead | A | ABI 7500 | SP/NP/N/N | 100 |
| 22 | QIAamp | Spin column | A | Agilent MX3005P | SP/LP/N/N | 100 |
| 23 | QIAamp | Spin column | A | Qiagen RGQ | SP/NP/LP/N | 100 |
| 24 | Un | Un | A | Qiagen RGQ | SP/NP/LP/N | 100 |
| 25 | Un | Un | A | ABI stepone plus | SP/NP/N/N | 100 |
| 26 | Un | Un | A | ABI viia7 | SP/NP/LP/N | 100 |
| 27 | QIAamp | Spin column | A | ABI 7500 | SP/NP/LP/N | 100 |
| 28 | QIAamp | Spin column | B | ABI 7500 | SP/NP/N/N | 100 |
| 29 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |

**Table IV.** Continued.

| No. | NA extraction kit[a] | NA extraction method | NA detection method[b] | qPCR platform | Sample panel[c] | Agreement with the expected results (%) |
|---|---|---|---|---|---|---|
| 30 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 31 | High viral RNA | Magnetic bead | A | Roche LC480 | SP/NP/N/N | 100 |
| 32 | QIAamp | Spin column | B | ABI 7500 | SP/NP/N/N | 100 |
| 33 | ZJ bio-tech | Magnetic bead | A | ABI 7500 fast | SP/NP/LP/N | 100 |
| 34 | Un | Un | A | ABI 7500 | SP/NP/N/N | 100 |
| 35 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 36 | Un | Un | A | Biorad CFX96 touch | SP/NP/N/N | 100 |
| 37 | Un | Un | A | Roche LC480 | SP/NP/N/N | 100 |
| 38 | QIA EZ1 | Magnetic bead | E | ABI 7300 | SP/NP/LP/N | 100 |
| 39 | Un | Un | A | Roche LC 96 | SP/NP/LP/N | 100 |
| 40 | QIAamp | Colo | A | ABI 7500 | SP/NP/N/N | 100 |
| 41 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 42 | Tianlong | Magnetic beads | G | ABI 7500 | SP/NP/LP/N | 100 |
| 43 | QIAamp | Spin column | H | ABI 7500 | SP/NP/N/N | 100 |
| 44 | Un | Un | B | ABI 7500 | SP/NP/LP/N | 100 |
| 45 | Un | Un | A | ABI viia7 | SP/NP/LP/N | 100 |
| 46 | QIA EZ1 | Magnetic bead | A | ABI 7500 | SP/NP/LP/N | 100 |
| 47 | Un | Un | A | ABI 7500 | SP/NP/LP/N | 100 |
| 48 | MagMax | Magnetic bead | A | Roche LC480 | SP/NP/N/N | 100 |
| 49 | Un | Un | A | RGQ | SP/NP/LP/N | 100 |

*Notes*: [a]QIAamp, QIAamp viral RNA minikit (Qiagen); KINGFISHER, KingFisher pure RNA tissue kit (Thermo Scientific); PROMEGA, SV total RNA isolation system (Promega); Daan gene, Daan gene viral RNA kit; RNeasy, RNeasy minikit (Qiagen); ZJ Bio-Tech, ZJ Bio-Tech viral RNA kit; QIA EZ1, Qiagen EZ1 virus mini kit (Qiagen); High viral RNA, High pure viral RNA kit (Roche); Tianlong, Tianlong viral RNA kit; MagMax, ABI Life Technologies AM 1836 MagMAX-96 ViralRNA isolation kit. [b]A, ZJ Bio-Tech Co., Ltd., Shanghai, China; B, BioPerfectus Technologies, Jiangsu, China; C, Daan Gene Co., Ltd., Guangzhou, China; D, Primers and probes recommended by the US CDC; E, Primers and probes recommended by the China CDC; F: Mokobio Biotechnology Co., Ltd. Nanjing, China; G, TianLong Bio-Tech Co., Ltd., Suzhou, China; H, Shanghai Huirui Biotechnology Co., Ltd., Shanghai, China. [c]SP, SP sample; NP, NP sample; LP, LP sample; N, Negative sample; Un, Unknown.

### 3.3. Evaluation of MERS-CoV VLPs as the Reference Material in EQA

The EQA required a sample panel with different concentrations. Therefore, the homogeneity of the MERS-CoV VLP solutions reconstituted with the virus preservation solution was tested. As shown in Figure 3, repeated assays of the solutions showed very low variations, with CV less than 0.6%. The analysis of the stability of the sample panel under different storage temperatures documented that the MERS-CoV VLP solutions were stable after storing at −70 °C, −20 °C, and 4 °C for at least one week (Fig. 4). However, the Ct values of the MERS-CoV VLPs solutions stored at 37 °C increased gradually over time, indicating that the MERS-CoV VLPs were degraded slowly at 37 °C. These results showed that the sample panel solutions prepared from the lyophilized MERS-CoV VLP powders should be stored under cool conditions.

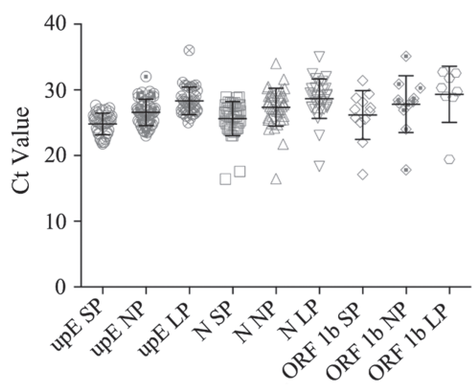


**Figure 5.** Scatter diagram showing the Ct values (*y*-axis) for different samples (*x*-axis) obtained by all the laboratories participating in the EQA.

### 3.4. EQA Results of the 49 Laboratories

As summarized in Table IV, after testing the sample panel consisting of four different concentrations of the MERS-CoV VLPs (negative control, LP, NP, and SP), all the 49 laboratories reported negative and positive results 100% that were correct. This high accuracy was achieved despite different extraction methods and detection kits being used. However, the Ct values between different labs varied significantly (Fig. 5) when testing the VLP solutions at the same concentration level.

## 4. CONCLUSION

Because RT-PCR detection of MERS-CoV involves many steps, from RNA extraction, to reverse transcription, and to PCR, any of the steps can cause the detection failure. Therefore, it is important to employ a positive control material, which can be used to monitor the entire process of the RT-PCR detection.

In this study, we have used the lentivirus system to pack extraneous MERS-CoV RNA fragments inside virus-like particles.[15,16] The first advantage of this approach is that the final VLPs are safe for *in vitro* use because they

lack the self-reproduction ability. Secondly, the lyophilized powders of the MERS-CoV VLPs are stable during storage at 4 °C, and even at 37 °C, for over a week. These results imply that the lyophilized powders are suitable for use as the positive control material in PCR detection kits. It should be noted that although the lentivirus system has a larger packing capacity (4 k maximum) than MS2 bacteriophages, and could be exploited to load more MERS-CoV genes, the whole MERS-CoV genome is still too large to be encapsulated into one HIV-1 VLP. However, since commercial diagnosis kits commonly target only 1 or 2 conserved gene fragments of MERS-CoV, the load capacity of the lentivirus packing system should not be a limiting factor in constructing positive controls in PCR kits.

Besides being used as the positive control material in PCR detection kits, the constructed MERS-CoV VLPs were also employed to prepare a sample panel for EQA. The VLP solutions obtained by reconstitution with the virus preservation solution were found to be homogenous and stable at 4 °C for over a week. The initial EQA results from 49 laboratories demonstrated that the constructed VLPs are well suited as the reference material for EQA. At the same time, the different Ct values reported from different sites for the same sample suggest the need to standardize the RNA extraction method and/or the PCR detection conditions among the laboratories. Nanotechnology has drawn a great attention of scientists, particularly in the biomedical field. There is a need to integrate nanotechnology with the findings of this study in future.

In conclusion, we have successfully constructed two MERS-CoV VLPs and demonstrated that they are stable and safe to be used as the positive control in PCR detection kits, and can serve as the reference material in EQA.

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