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Strand-specific real-time RT-PCR for distinguishing influenza vRNA, cRNA, and mRNA

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Abstract

Real-time RT-PCR is used to quantify individual influenza viral RNAs. However, conventional real-time RT-PCR, using strand-specific primers, has been shown to produce not only the anticipated strand-specific products, but also substantial amounts of non-strand-specific products, indicating lack of specificity. Therefore, in this study, a novel strand-specific real-time RT-PCR method was established to quantify the three types of influenza viral RNA (vRNA, cRNA, and mRNA) separately. This method is based on reverse transcription using tagged primers to add a tag sequence at the 5' end and the hot-start method. Real-time PCR using the tag portion as the forward primer and a segment-specific reverse primer ensured the specificity for quantifying the three types of RNA. Using this method, specific target RNA was detected at 100 – 100,000 folds higher level than other types of RNA. This method was also used to evaluate the vRNA, cRNA, and mRNA levels of segments 5 and 6 in MDCK cells infected with influenza A virus at different time points post-infection. The cRNA level was 1/10 to 1/100 lower than that of the vRNA and mRNA. Moreover, different dynamics of vRNA, cRNA, and mRNA synthesis were observed; the copy number of the vRNA gradually increased throughout infection, the cRNA increased and then plateaued, while the mRNA increased and then decreased. This novel method thus provides data critical for understanding the influenza virus life cycle, including transcription, replication, and genome incorporation into virions.

Keywords

Influenza A virus; Quantitative real-time PCR; Strand-specific real-time RT-PCR; Detection of influenza vRNA; cRNA and mRNA

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1. INTRODUCTION

Influenza A virus is an enveloped negative-strand RNA virus, whose genome comprises eight single-stranded RNA segments. Each RNA segment forms a ribonucleoprotein (RNP) complex with the viral polymerase subunits (PB1, PB2 and PA) and the nucleoprotein (NP) (Palese and Shaw, 2007). The life cycle of influenza A virus begins with attachment to cell surface receptors, followed by internalization of virions into cells (Palese and Shaw, 2007). After uncoating, viral RNPs (vRNPs) are transported into the nucleus, where genome replication and transcription take place (Bouvier and Palese, 2008). The viral RNA (vRNA) is then transcribed into mRNA, which is used to produce viral proteins, and replicated via complementary RNA (cRNA). The viral polymerases and NP catalyze both genome replication and transcription (Neumann et al., 2004). The intracellular kinetics of these three types of RNA (i.e., vRNA, cRNA, and mRNA) differs. In the early phase of infection, viral mRNA is predominantly synthesized to produce viral proteins, whereas in the late phase of infection, mRNA synthesis stops and the replication reaction dominates (Shapiro et al., 1987). To explain this difference in the kinetics of mRNA, vRNA, and cRNA production, a switching mechanism from transcription to replication was proposed in which newly synthesized NP makes the transition from replication to transcription (Portela and Digard, 2002), replicative intermediate cRNA, stabilized by the newly synthesized NP and viral polymerase, regulates replication (Vreed et al., 2004), and viral matrix M1 protein and NEP/NS2 protein, which is responsible for vRNP nuclear export, inhibit viral transcription at the late phase of infection (Robb et al., 2009; Shapiro et al., 1987). However this mechanism has yet to be validated.

To understand the mechanisms of transcription and replication of the viral genome in more detail, it is important to quantify the three types of RNA (i.e., vRNA, cRNA, and mRNA) accurately. Traditionally, these RNAs have been quantified by using radioisotopes in northern blotting, RNase protection, and primer extension assays (Hatada et al., 1989; Lee and Seong, 1998; Shapiro et al., 1987; Vreede et al., 2004). Recently, real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) has been used not only to detect viral genes (Spackman et al., 2002) but also to quantify independent types of RNA (Ge et al., 2003; Karlas et al., 2010; Mackay, Arden, and Nitsche, 2002). Although real-time RT-PCR is a powerful method to detect viral RNA in terms of its high sensitivity, reproducibility, and throughput, its weakness is strand-specificity. This limitation was reported by Lanford et al (1994) for hepatitis C virus (HCV). They found that the specificity of reverse transcription was low because of cDNA synthesis even in the absence of primers and because of false annealing of primers (Lanford et al., 1994). In HCV research, some improvement in strand-specificity has been obtained by using tagged primers, a high temperature for cDNA synthesis (Lanford et al., 1994), and exonuclease I to remove non-incorporated RT primer (Craggs et al., 2001). In influenza research, however, this problem has not been addressed. Currently, to distinguish the three types of influenza RNA using conventional RT-PCR, a primer complementary to the 3' portion of vRNA is used to initiate cDNA synthesis from the vRNA. The primer complementary to the 3' portion of the cRNA is used for cRNA and Oligo dT (Ge et al., 2003) or the same primer as for the cRNA with 5' T additions is used for mRNA (Vester et al., 2010). However, with these primers, the three types of RNA may be indistinguishable. In fact, the level of cRNA in infected cells was estimated to be higher than that of vRNA in a quantification using conventional real-time RT-PCR (Vester et al., 2010). This result is incompatible with an earlier study that showed cRNA accumulation in infected cells to be less than that of vRNA and mRNA, based on an RNA-RNA hybridization technique (Hatada et al., 1989).

In this study, the lack of specificity of the conventional reverse transcription approach was revealed by using synthetic viral RNA as a standard. To discriminate between the three

types of influenza viral RNA accurately, a hot-start strand-specific reverse transcription method with tagged primers and trehalose was developed. This method allowed the successful determination of the copy numbers of viral RNAs in infected cells.

2. MATERIALS AND METHODS

2.1. Cell culture and virus infection

Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM), containing 5% newborn calf serum and antibiotics. A/WSN/33 virus (WSN; H1N1) was generated by reverse genetics, as described previously (Neumann et al., 1999) and propagated in MDCK cells. Viruses were titrated by plaque assay in MDCK cells. For virus infection, MDCK cells were washed with MEM containing 0.3% bovine serum albumin and antibiotics (MEM/BSA) and incubated with WSN at a multiplicity of infection (MOI) of 10 at 4°C for 1 h. Cells for further analysis were harvested at the indicated time points.

2.2. Extraction of viral RNA from purified virions and virus-infected cells

WSN virus was purified by differential sedimentation through 25%–50% sucrose gradients in a Beckman SW32 rotor at 28,000 rpm and 4°C for 4 h. RNA was extracted from purified virions by use of the RNeasy Mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Virus-infected cells were washed three times with ice-cold phosphate-buffered saline and lysed by direct addition of RLT buffer from the RNeasy kit (QIAGEN), followed by RNA extraction. The concentration of purified RNA was determined by use of spectrophotometry.

2.3. In vitro synthesis of RNA standard

The primers used to create templates containing a T7 phage promoter sequence (TAATACGACTCACTATAGGG) are summarized in Sup. Table 1. Viral gene sequences in pPolI plasmids (Neumann et al., 1999) were amplified by PCR using corresponding primer pairs and were purified by using the QIAquick PCR purification kit (QIAGEN). Purified PCR products were in vitro transcribed by using the RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI) at 37°C for 4 h followed by RQ1 DNase I (Promega) digestions of the DNA template at 37°C for 15 min. The transcript was purified with the RNeasy Mini kit (QIAGEN). Concentrations of purified RNA transcripts were determined by using spectrophotometry. The molecular copies of synthetic RNA were calculated from the total molecular weight of the segment. 5×10^9 copies of synthetic RNA (2.85 – 7.5 ng by weight, respectively) were separated by electrophoresis on a 4% polyacrylamide gel containing 7 M urea with 0.5 × TBE buffer at room temperature at 120 V for 5 h and visualized by silver staining using the CLEAR STAIN Ag kit (Nippon Gene, Tokyo, Japan). Single bands corresponding to the expected viral RNA segment size were observed when 50 ng of viral RNA from purified WSN was electrophoresed simultaneously as a molecular marker (Sup. Fig. 1).

2.4. Conventional real-time RT-PCR

Conventional real-time RT-PCR was performed as described (Vester et al., 2010). In brief, 4 µl of the RNA standard was mixed with 1.5 µl of 10 µM primer specific for the segment 5 vRNA (WSNseg5_1F: AGCAAAAGCAGGGTAGATAATCACTC), cRNA (WSNseg5_1565R: AGTAGAAACAAGGGTATTTTCTTT) or mRNA (WSNseg5_dTR: TTTTTTTTTTTTTTCTTTAATTGTC) and made up to 13 µl with RNase free water. The mixture was then incubated at 65°C for 5 min and was cooled to 4°C. The reaction mixture [5 µl of First Strand buffer (5 ×, Invitrogen, Carlsbad, CA), 4 µl of 25 mM MgCl₂, 2 µl of 0.1 M dithiothreitol and 1 µl of Superscript II RT (50 U/µl, Invitrogen)] was then

added. The RT reaction was carried out at 42°C for 60 min and was terminated by heating at 70°C for 5 min. After the RT reaction, 1 µl of cDNA was added to the qPCR reaction mixture [10 µl SYBR GreenER qPCR SuperMix for ABI PRISM (2 ×), 1.5 µl of forward primer (10 µM; WSNseg5_432F: AACGGCTGGTCTGACTCACATGAT), 1.5 µl of reverse primer (10 µM; WSNseg5_542R: AGTGAGCACATCCTGGGATCCATT), 3 µl of double-distilled water]. The cycle conditions of qPCR were 95°C 10 min, followed by 40 cycles of 95°C 15 sec and 60°C for 1 min.

2.5. Hot-start reverse transcription with a tagged primer

cDNAs complementary to the three types of influenza viral RNA were synthesized with tagged primers to add an 18–20 nucleotide tag that was unrelated to the influenza virus (Table 1: vRNA_{tag}; GGCCGTCATGGTGGCGAAT, cRNA_{tag}; GCTAGCTTCAGCTAGGCATC, and mRNA_{tag}; CCAGATCGTTCGAGTCGT), at the 5' end. Reverse transcription with the tagged primer was performed as described elsewhere (Lanford et al., 1994) with the hot-start modification of using saturated trehalose (Mizuno et al., 1999). A 5.5 µl mixture containing the approximately 200 ng of total RNA sample and 10 pmol of tagged primer were heated for 10 min at 65°C, chilled immediately on ice for 5 min, and then heated again at 60°C. After 5 min, 14.5 µl of preheated reaction mixture [4 µl First Strand buffer (5 ×, Invitrogen), 1 µl 0.1 M dithiothreitol, 1 µl dNTP mix (10mM each), 1 µl Superscript III reverse transcriptase (200 U/µl, Invitrogen), 1 µl RNasin Plus RNase inhibitor (40U/µl, Promega) and 6.5 µl saturated trehalose] was added and incubated for 1 h.

Real-time PCR (qPCR) was performed with SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) on an ABI PRISM 7900HT. Four microliters of a ten-fold dilution of the cDNA was added to the qPCR reaction mixture [10 µl SYBR GreenER qPCR SuperMix for ABI PRISM (2 ×), 1.5 µl forward primer (10 µM), 1.5 µl reverse primer (10 µM), 3 µl double-distilled water]. The cycle conditions of qPCR were 95°C 10 min, followed by 40 cycles of 95°C 15 sec and 60°C for 1 min. The primers used are listed in Table 1.

Ten-fold serial dilutions (10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 copies/µl) of synthetic viral RNA standards were used to generate a standard curve.

3. RESULTS

3.1. Low specificity of conventional real-time RT-PCR

First, the specificity of conventional real-time RT-PCR to distinguish between the three types of influenza viral RNA (i.e., vRNA, cRNA and mRNA) was evaluated. Synthetic vRNA, cRNA and mRNA were used as templates to amplify reverse transcripts, which were detected with real-time PCR by using the segment-specific primer set described in Fig. 1A and the Materials and Methods. When 1 ng each of synthetic vRNA, cRNA and mRNA was used as a template for reverse transcription with a primer for vRNA, substantial background amplification of cRNA (3% of vRNA) and mRNA (5% of vRNA) was observed. With primers for cRNA, more vRNA than cRNA and substantial amounts of mRNA (25% of cRNA) were detected. For the mRNA primers, no difference in the amounts of the three types of RNA was seen (Fig. 2). The amplification of vRNA was also observed even when no primer was added to the reverse transcription reaction (Fig. 2). Primer-independent vRNA amplification was reported in an HCV study by Lanford et al. (1994), who suggested that this event may be caused by priming by reverse transcriptase by direct interaction with the vRNA template and/or with degraded oligonucleotides serving as a primer. These data suggest low specificity of conventional real-time RT-PCR due to primer-independent cDNA synthesis and nonspecific annealing of primers.

3.2. Strand-specific real-time RT-PCR using a tagged primer and the hot-start method

To establish a method that can distinguish between vRNA, cRNA, and mRNA, two major modifications were made to the conventional real-time RT-PCR assay. First, a tagged primer for cDNA synthesis was used, as described elsewhere (Lanford et al., 1994). As shown in Fig 1B, real-time PCR was performed by using a forward primer identical to the tag portion in the RT primer and a primer complementary to the influenza viral RNA as a reverse primer. Thus, even if primer-independent cDNA synthesis occurred, only the cDNA generated with the tagged primer would be detected in the real-time PCR. The use of this tagged primer for cDNA synthesis improved the specificity of vRNA detection (Sup. Fig. 2). However, while vRNA detection was specific, the same approach did not amplify specifically cRNA and mRNA (Sup. Fig. 2). The cRNA and mRNA sequences are almost identical except for the cap structure of the 5' end and the poly A tail of the 3' end. Therefore, some overlap is inevitable between primers used to synthesize cDNAs for cRNA and mRNA. Accordingly, another modification was needed in order to eliminate nonspecific annealing of the primers. Therefore, the hot-start method with trehalose was applied, as described by Mizuno et al. (Mizuno et al., 1999). Trehalose heat-stabilizes many enzymes including Superscript III, allowing it to work optimally at 60°C, and reducing nonspecific primer annealing.

These two modifications allow the distinction between the three types of segment 5 RNA with high specificity (Fig. 3A). Using a tagged primer for vRNA (vRNA_{tag}_WSNseg5_740F), this RNA was detected at a 100,000-fold higher level relative to cRNA and mRNA. cRNA was detected at a 10,000-fold higher level than vRNA and mRNA with the tagged primer for cRNA (cRNA_{tag}_WSNseg5_1565R). The specificity using the tagged primer for mRNA (mRNA_{tag}_WSNseg5_dTR) was relatively low and cRNA was detected at 1/100 the level of mRNA. However, cRNA accumulation in infected cells is low compared with that of mRNA (Hatada et al., 1989), suggesting that this may have little effect on practical quantification. Significant specificity was also obtained with tagged primers for segment 6 (Fig. 3B). Standard curves had strong linear correlations (>0.99) and amplification efficiency was between 94% and 112% (Fig. 4, Table 2). The lowest concentration at which linearity was retained in the standard curve was $10^3 - 10^4$ copies/μl (Table 2). Assuming that RNA derived from approximately $10^4 - 10^5$ cells would be contained in 1 μl of sample used for reverse transcription, the minimum detectable quantity in this method is estimated to be 0.01 – 1 copies/cell.

3.3. Absolute quantitation of viral RNAs in infected cells

Next, the utility of this novel strand-specific real-time RT-PCR method was validated by measuring the intracellular dynamics of the three types of influenza viral RNA in virus-infected cells. MDCK cells were infected with influenza virus A/WSN/33 at an MOI of 10. The cells were harvested at 0, 1, 2, 4, 6, 8, 10, and 12 h post-infection, and total cellular RNA was extracted. Molecular numbers of vRNA, cRNA, and mRNA copies of viral segments in the total cellular RNA were quantified by use of this strand-specific real-time RT-PCR method. In this study, segments 5 and 6 were selected as representative segments. The values were expressed as numbers of RNA copies in an infected cell, assuming that a cell contains 10 pg of RNA (Hatada et al., 1989). The data suggest that the intracellular dynamics of the vRNA, cRNA and mRNA differ (Fig 5). The copy number of vRNA increased gradually (Fig. 5A), whereas that of cRNA increased until 4 h post-infection, at which point production slowed down for segment 5 and plateaued for segment 6 (Fig. 5B). mRNA increased exponentially until 4 h post-infection and then decreased from 6 h post-infection (Fig. 5C).

The amount of cRNA was relatively small compared to that of vRNA and mRNA throughout the infection (Fig. 5 and Sup. Fig. 3A). These ratios of vRNA, cRNA, and mRNA and the temporal changes in the amount of RNA in infected cells are essentially consistent with those of earlier studies (Hatada et al., 1989; Shapiro et al., 1987). In contrast, the levels of the three RNAs determined by the conventional real-time RT-PCR method (Sup. Fig. 3B) were different from those determined in previous studies (Hatada et al., 1989; Shapiro et al., 1987), indicating the low specificity of this method, as shown in Fig. 2. These results demonstrate that a strand-specific real-time RT-PCR method that distinguishes and absolutely quantifies the three types of influenza viral RNA was established successfully in this study.

The temporal changes among the three types of RNA were similar for segments 5 and 6. Although the molecular copy numbers of vRNA and cRNA of segment 5 and segment 6 were the same, there was about a 10-fold difference between the mRNA copies of the two segments (Fig. 5C). This result likely reflects a difference in the vRNA promoter. In the WSN/33 virus, segment 5 has a “U” at position 4 of the 3' vRNA end but segment 6 has a “C”. The C4-containing promoter has relatively weak transcription activity compared with that of the U4-containing promoter, which correlates with the lower mRNA and protein expression levels in infected cells (Lee and Seong, 1998). This finding indicates that this strand-specific real-time RT-PCR method is also applicable to comparisons of RNA segments.

4. Discussion

Currently, two major models have been proposed for the regulation of transcription (i.e., viral mRNA synthesis) and replication (i.e., vRNA synthesis) of the influenza viral genome. One of these models suggests that the RNA polymerase is switched from a transcriptase to a replicase, which is used for vRNA synthesis, and the switching event is triggered by the newly synthesized NP protein (reviewed in Portela and Digard, 2002). The other model suggests that replication of influenza virus is regulated by cRNA, which is stabilized by newly synthesized NP and polymerases (Vreede et al., 2004). Both models are based on the hypothesis that in the influenza virus life cycle, transcription occurs first, followed by viral genome replication. This hypothesis is supported by earlier findings that cRNA, unlike mRNA, is not detected in the early phase of infection (Shapiro et al., 1987; Vreede et al., 2004). However, it is possible that the methods used in these earlier studies (i.e., primer extension and RNase protection assays) were not sufficiently sensitive to detect small amounts of cRNA. In fact, the strand-specific real-time RT-PCR method (Fig 5) detected cRNA synthesis 2 hours post-infection, which is when the mRNA appeared. Thus, contrary to the previous two models, the data in this study suggest that both replication and transcription occur simultaneously in the early phase of infection. In the late phase of infection, the mRNA level decreased while vRNA increased throughout the infection (Fig 5), suggesting that viral replication, but not transcription, continues even in the late phase of infection. These results are consistent with data from the recent study suggesting a role for the NS2/NEP protein in the regulation of transcription and replication (Robb et al., 2009).

In summary, the novel strand-specific real-time RT-PCR method established in this study allows the quantification of specific types of viral RNA with accuracy and specificity. With this method, the RNA dynamics in virus-infected cells, including replication and transcription, transport and incorporation into virions, as well as the role of host factors in the influenza viral life cycle can be investigated closely.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1A

Figure 1B

Fig. 1.

Schematic diagram of conventional real-time RT-PCR and real-time RT-PCR with tagged primers. (A) Conventional real-time RT-PCR. Viral RNAs are reverse transcribed with specific primers. In real-time PCR, the cDNAs are then amplified with primer sets common to the three types of RNA. (B) Real-time RT-PCR with tagged primers. cDNA synthesis is performed using "tagged" primers complementary to each type of RNA. A tag of 18–20 nucleotides unrelated to the influenza virus, is shown as red, yellow, and green bars for the vRNA_{tag}, cRNA_{tag}, and mRNA_{tag}, respectively. The tagged cDNA is amplified by PCR using the tag portion of the cDNA primer as the forward primer and a segment-specific oligonucleotide as the reverse primer.

Fig. 2.

Low specificity of conventional real-time RT-PCR. 10^9 copies of synthetic viral vRNA, cRNA, and mRNA of segment 5 are used as standards in conventional real-time RT-PCR. Reverse transcription was performed with primers specific for vRNA, cRNA, and mRNA respectively or in the absence of primers [primer (-)], or the absence of reverse transcriptase [RTase (-)]. The average molecular number and standard deviation of triplicate experiments are presented as a percentage of the average value of the target type of RNA. Error bars represent the standard deviation of triplicate experiments.

Figure 3A

Figure 3B

Fig. 3.

Analysis of synthetic viral RNAs for segment 5(A) and segment 6 (B) by using strand-specific real-time RT-PCR with tagged primers and the hot-start method. cDNAs were synthesized with tagged primers to add an 18–20 nucleotide tag that was unrelated to the influenza virus (Table 1: vRNA tag; GGCCGTCATGGTGGCGAAT, cRNA tag; GCTAGCTTCAGCTAGGCATC, and mRNA tag; CCAGATCGTTCGAGTCGT), at the 5' end. The tagged cDNA was amplified by real-time PCR by using the tag portion of the cDNA primer as the forward primer and a segment-specific oligonucleotide as the reverse primer. The average molecular number and standard deviation of triplicate experiments are presented as a percentage of the average value of the target type of RNA. Error bars represent the standard deviation of triplicate experiments.

Figure 4A

Segment 5

Figure 4B

Segment 6

Fig. 4.

Standard curve for segment 5 (A) and segment 6 (B), generated by plotting the C_q values against the input synthetic RNA molecular numbers. Ten-fold serial dilutions ($10^3 - 10^9$ copies/ μ l for segment 5, $10^4 - 10^9$ copies/ μ l for segment 6) of synthetic viral RNA standard were used to generate a standard curve.

Figure 5A

Figure 5B

Figure 5C

mRNA

Fig. 5.

Kinetics of synthesis of (A) vRNA, (B) cRNA and (C) mRNA of segments 5 and 6 in MDCK cells infected with influenza virus (A/WSN/33 (H1N1) strain, MOI=10). The average molecules per cell were determined by strand-specific real-time RT-PCR using a tagged primer and the hot-start method with synthetic viral RNA as a reference standard. The values are expressed as numbers of RNA copies in an infected cell, assuming that a cell contains 10 pg of RNA. Error bars represent the standard deviation of triplicate experiments.

Characteristics of the primer sets for strand-specific real-time RT-PCR using tagged primers for quantification of the vRNA, cRNA, and mRNA of segments 5 and 6.

Table 1

Target	Purpose	Primer name	Sequence (5' - 3')	Position (nt)
Segment 5	vRNA	Reverse transcription	vRNAtag_WSNseg5_740F	740-763
		Real-time PCR	vRNAtag	
			WSNseg5_845R	845-822
	cRNA	Reverse transcription	cRNAtag_WSNseg5_1565R	1565-1541
		Real-time PCR	cRNAtag	
			WSNseg5_1466F	1466-1483
Segment 6	mRNA	Reverse transcription	mRNAtag_WSNseg5_dTR	1549-1534
		Real-time PCR	mRNAtag	
			WSNseg5_1466F	1466-1483
	vRNA	Reverse transcription	vRNAtag_WSNseg6_689F	689-712
		Real-time PCR	vRNAtag	
			WSNseg6_839R	839-816
Segment 6	cRNA	Reverse transcription	cRNAtag_WSNseg6_1413R	1413-1389
		Real-time PCR	cRNAtag	
			WSNseg6_1314F	1314-1339
	mRNA	Reverse transcription	mRNAtag_WSNseg6_dTR	1398-1382
		Real-time PCR	mRNAtag	
			WSNseg6_1314F	1314-1339

Table 2

Validation parameters of strand-specific real-time RT-PCR using tagged primers and the hot-start method for quantification of the vRNA, cRNA, and mRNA of segments 5 and 6.

Target	Sensitivity ^a (copies)	Linear regression ^b			R ²
		Slope	Intercept	Amplification efficiency (E%)	
Segment 5	vRNA	-3.3266	43.605	100	0.9977
	cRNA	-3.3844	44.188	97	0.9996
	mRNA	-3.4826	46.512	94	0.9988
Segment 6	vRNA	-3.3533	47.355	99	0.9997
	cRNA	-3.4675	47.587	94	0.9988
	mRNA	-3.0732	46.744	112	0.9949

^aSensitivity is shown as the minimum copy number of RNA molecules that was on the standard curve.

^bStandard curves were generated by the least-square method. Amplification efficiency was calculated with the function $E = (10^{-(1/\text{Slope})} - 1) * 100$