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Identification and optimization of parameters for accurate quantification of RNA by RT–dPCR

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Abstract

Reverse transcription–digital PCR (RT–dPCR) is attracting attention as a method that enables SI-traceable RNA quantification without calibration, but its accuracy and bias have not been thoroughly studied. In this study, the accurate quantification of RNA by the RT–dPCR method was investigated using NMIJ CRM 6204-b, an RNA certified reference material whose certified value was assigned by orthogonal chemical measurement methods. Moreover, a two-step RT–dPCR method was adopted to examine in detail the conditions for the RT reaction process, which was expected to be the major uncertainty component in the RT–dPCR measurement. Optimization experiments revealed that the type of reverse transcriptase, the concentration of template RNA, and the type and concentration of primers in the RT reaction affected the value quantified by RT–dPCR. Under the optimal conditions, the value quantified by RT–dPCR, 76.4 ng/µL ± 6.7 ng/µL (the quantified value ± expanded uncertainty ($k = 2$)), was consistent with the certified value, 68.2 ng/µL ± 5.8 ng/µL, of NMIJ CRM 6204-b RNA 1000-A within the expanded uncertainty. From the results of the uncertainty evaluation, the relative combined uncertainty of the RT–dPCR method was 4.42%, and the major uncertainty components in the RT–dPCR method were the preparation of RT solution (3.68%), the inter-day difference (1.80%), and the RT reaction (1.30%). Together, the results suggested that the contribution of the RT reaction process to the total uncertainty was greater than that of the dPCR process.

Keywords RNA · Reverse transcription · Digital PCR · Absolute quantification · Uncertainty · CRM

Introduction

RNA is measured and quantified clinically to detect relevant pathogens such as influenza virus [1] and SARS-CoV-2 virus [2] and diagnose cancers on the basis of the abnormal expression of such blood cancer genes as BCR-ABL [3–5] and miRNA [6, 7].

The most frequently used method for these RNA measurements is the reverse transcription–quantitative polymerase chain reaction (RT–qPCR) [1, 2]. To determine the accurate concentration of target RNA molecules by RT–qPCR, a calibration sample with the same amplification efficiency as the target RNA is required. This is typically achieved by using an RNA reference material (RM) or a certified reference material (CRM) with the same sequence as the target RNA and an accurate concentration.

Some DNA or RNA RMs and CRMs have quantified values, such as mass concentration or copy number concentration [2, 8–10], and some value-assignment methods for DNA or RNA RM and CRM development are available. In one of those methods, the constituent nucleotide monomers derived from DNA and RNA are quantified by liquid chromatography–isotope dilution mass spectrometry (LC–IDMS) [11–15], or the constituent phosphorus derived from DNA or RNA is quantified by inductively coupled plasma–optical emission spectrometry (ICP–OES) [16–18] or ICP–mass spectrometry (ICP–MS) [14].

Because LC–IDMS and ICP–MS can accomplish accurate and SI-traceable DNA or RNA measurement without requiring RMs or CRMs to have the same sequence, we adopted them for the development of certified reference materials, NMIJ CRM 6204-b [19] and 6205-b [20]. These methods have drawbacks, namely, DNA or RNA sequence information is lost because it is decomposed into nucleotide monomers or phosphorus for quantification by these

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methods, and DNA or RNA cannot be quantified sequence-specifically. On the other hand, the quantification principles of LC–IDMS and ICP–MS differ significantly from PCR-based methods, and therefore, we thought that NMIJ CRM 6204-b and 6205-b can be employed to validate (RT–)qPCR and (RT–)dPCR.

RT–dPCR is also expected to be used for absolute quantification [21–26] and frequently used for the value assignment of RMs and CRMs of viral RNAs including SARS-CoV-2 RNA [2, 8–10]. RT reaction is an essential step for RT-dPCR, and maximizing the RT reaction efficiency is also expected to achieve the accurate quantification by RT-dPCR. However, in the previous report, the conditions such as primer type and their concentrations were optimized only for PCR process in RT-dPCR assay, but not for the RT reaction process. In addition, RT reaction efficiency has only been examined for differences in enzyme type [27, 28], and there was no discussion on how to make the RT reaction efficiency more efficient. Also, the accuracy of RT–dPCR is undergoing verification through various international comparisons between laboratories, and the parameters influencing the accuracy and bias of RT–dPCR have yet to be determined [29, 30] and there is still much to be confirmed in this regard [31].

RT–dPCR is classified by the reaction process into two methods called one-step RT–dPCR and two-step RT–dPCR. In the former, the reagents for the RT reaction and dPCR are prepared and partitioned at the same time, and then, the RT and PCR reactions are performed in the same partition. The risk of contamination is minimized because the reagents are prepared at once, and the positive partition signal detected in the final dPCR is considered to reflect the number of RNA molecules converted into cDNA [21]. In two-step RT-dPCR, the RT reaction is performed first, followed by the partitioning of cDNA and the PCR reaction. The reagents for the RT process and the dPCR process are prepared independently, and each reaction is carried out separately.

In both one-step and two-step RT–dPCR, a high RT reaction efficiency of approximately 100% is required for accurate quantification. However, RT reactions generally have low reaction efficiencies [21, 28, 32]. Therefore, we thought that the RT reaction conditions should be optimized to obtain a high RT reaction efficiency. On the other hand, commercially available one-step RT–dPCR reagents are difficult to change the parameters of the RT reaction, such as the type of enzyme and the reaction mixture composition. For this reason, we chose the two-step RT–dPCR because it is possible to use various reagents and optimize the parameters of the RT reaction.

In this study, we identified and optimized the parameters for accurate RNA quantification by the RT–dPCR method. We used NMIJ CRM 6204-b RNA1000-A and two-step RT–dPCR to examine our method. Using RNA1000-A, we evaluated the RT reaction by comparing the certified value with the quantified value under various conditions, such as the type of reverse transcriptase (RTase), the type and concentration of primers, and the concentration of template RNA. After the optimal conditions were determined, the accuracy of the method was evaluated.

Materials and methods

RNA CRM

As samples, 1033 mer RNA, RNA1000-A (68.2 ng/μL $\pm$ 5.2 ng/μL, certified value $\pm$ expanded uncertainty, $k = 2$), which is one of five RNA solutions in NMIJ CRM 6204-b [19], was used. NMIJ CRM 6204-b is composed of five RNA solutions having different sequences and two RNA lengths, 533-mer and 1033-mer containing the 33 mer of poly-A tail at the 3′ end. Each of five RNA solutions of NMIJ CRM 6204-b was synthesized by in vitro transcription and has artificial sequences with low homology to previously known sequences derived from the eukaryotic sequence, approximately 50% GC content, a low bias in nucleotide composition, and no more than four consecutive bases that are the same. The certified value of each RNA is determined as the mass concentration of total RNA quantified by two SI-traceable methods, LC–IDMS with enzymatic digestion and ICP–MS based on the amount of phosphorus. High-performance liquid chromatography-size exclusion columns (HPLC-SEC) were used to evaluate its purity. In all chromatograms analyzing RNA1000-A, only single peaks were observed. RNA 1000-A solution was gravimetrically diluted 10^6-fold with water for the optimization of RT–dPCR measurement conditions, or to 1500 copies/μL for the accuracy evaluation of the RT–dPCR method.

Reagents

For the RT process, three types of RTases were used: SuperScript™ IV Reverse Transcriptase (SSIV, Super-Script IV First-Strand Synthesis System, Thermo Fisher Scientific, Invitrogen, Tokyo, Japan), MultiScribe Reverse Transcriptase (MSRT, High-Capacity cDNA Reverse Transcription kit, Thermo Fisher Scientific, Applied Biosystems, Tokyo, Japan), and ReverTra Ace (RTA, ReverTra Ace -a-, TOYOBO, Osaka, Japan). Three types of primers used in the RT reaction were oligo d(T)$_{20}$ primer, random hexamer, and RNA1000-A specific primer (GATATTCATTAGAGGACAGTCCGCATA, Eurofins Genomics K. K., Tokyo, Japan). Oligo d(T)$_{20}$ primer and random hexamer included in each kit were used in the experiments.

The digital PCR master mix used in the dPCR process was QuantStudio 3D Digital PCR Master Mix v2 (Thermo

Fisher Scientific). The primers and the probe (454F-primer: AGTTCTAAGGTCGTCGGGTCTAT; 534R-primer: the same sequence as that of the gene-specific primer for the RT reaction; 505probe: FAM-CACACGGTCGTATTATT-MGB, the number indicates the starting position in the RNA1000-A sequence and the amplicon size is 81 mer) were also purchased from Thermo Fisher Scientific. RNA1000-A specific primer sequences were designed using the sequence design service of Thermo Fisher Scientific. Water used for dilution and negative control of the RT-dPCR experiment was distilled water (Nippon Gene, Tokyo, Japan).

Equipment

A Takara PCR Thermal Cycler (Takara Bio Inc., Shiga, Japan) was used in the RT reaction. A QuantStudio 3D Digital PCR System and a QuantStudio 3D Digital PCR Chip v2 (Thermo Fisher Scientific) were used in the dPCR reaction. Nucleic acid low-adsorption tubes and chips (Maxymum Recovery, Axygen, Corning, NY, USA) were used in all experiments.

Reverse transcription reaction

The three RTases described in the "Reagents" and "Reverse transcription reaction" sections were used in the RT reaction. In the experiments using each RTase, the amounts of template RNA and R-primer were changed to determine the optimal conditions.

The preparation of the RT reaction solution and the reaction conditions followed the instruction manual for each enzyme. The protocol for each enzyme is described briefly as follows. As regards the protocol using SSIV: 4 µL of 5 × SSIV buffer, 1 µL of 100 mM DTT, 1 µL of ribonuclease inhibitor, 1 µL each of 200 U/µL SSIV and primer (50 µM oligo d(T)$_{20}$ primer, 50 ng/µL random hexamer or gene-specific primer), the appropriate amount of template RNA, and water were added to make 20 µL of the RT reaction solution. The RT reaction was performed at 55 °C for 10 min, followed by enzyme inactivation at 80 °C for 10 min.

As regards the protocol using MSRT, 2 µL of 10 × RT buffer, 0.8 µL of 25 × dNMP mix (100 mM), 1 µL of RNase inhibitor, 1 µL each of MSRT and primer, the appropriate amount of template RNA, and water were added to make 20 µL of the RT reaction solution. The RT reaction was performed at 25 °C for 10 min, at 37 °C for 120 min, and then at 85 °C for 5 min to inactivate the enzyme.

As regards the protocol using RTA, 4 µL of 5 × buffer, 2 µL of 10 mM dNMP mix, 1 µL of RNase inhibitor, 1 µL each of RTA and primer, the appropriate amount of template RNA, and water were added to make 20 µL of the RT

reaction solution. The RT reaction was performed at 42 °C for 20 min, followed by enzyme inactivation at 99 °C for 5 min.

Digital PCR reaction

For dPCR, 7.5 µL of QuantStudio 3D Digital PCR Master Mix, 1.35 µL of each primer, 0.75 µL of probe premix, cDNA sample after RT reaction, and water were mixed to make 15 µL of the dPCR reaction mix. A large-scale reaction solution for four dPCR reactions was prepared at one time and divided into three chips and measured, respectively. PCR was performed at 96 °C for 10 min, followed by 40 cycles at 60 °C for 2 min and at 98 °C for 30 s. This was followed by 60 °C for 2 min and kept at 10 °C. All steps for sample dilution, the preparation of the RT reaction mix, and the preparation of the dPCR reaction mix were gravimetrically performed.

The analytical software was used, QuantStudio 3D AnalysisSuite Cloud Software, and the thresholds were determined manually.

The quantified value by RT–dPCR was calculated by using Eqs. (1) and (2).

$$\lambda = -\ln(1 - \frac{k}{n}) \tag{1}$$

$$C_{RNA} = \lambda \times \frac{1}{V} \times D \tag{2}$$

where n is the number of partitions, k is the number of positive partitions, C_{RNA} is the RNA1000-A concentration, v is the volume of partitions, and D is the dilution factor calculated from the gravimetrical dilution. The partition volume, 753 pL ± 5 pL (the value ± standard uncertainty), was evaluated by scanning electron microscopy (SEM). The partition number was 16,063 ± 1735 (average ± SD), and l was 0.188 ± 0.009 (average ± SD).

Calculation of reaction efficiency (recovery rate)

The RT reaction efficiency was evaluated on the basis of the recovery rate using Eq. (3).

$$R = \frac{C_{RNA}}{C_{CRM}} \times 100 \tag{3}$$

where R is the recovery rate and C_{CRM} is the certified value of RNA1000-A.

Uncertainty evaluation of RT–dPCR

All uncertainties were calculated following GUM (Guide to the Expression of Uncertainty in Measurement, ISO).

To evaluate the uncertainty of RT–dPCR measurement, four experiments were designed and performed. The number of replications for each process was changed, and the uncertainty of each process was evaluated by ANOVA. Through four different experiments, the uncertainty components beyond the measurement variability of dPCR were extracted in each experiment. Each uncertainty component was evaluated by more than two experiments, and the largest value was chosen as the uncertainty of the component.

Experiment 1 was designed to evaluate the uncertainties of RT reaction and dPCR measurement: one replication of RT reaction mix preparation, nine replications of the RT reaction, one replication of dPCR reaction mix preparation, and three replications of dPCR measurements.

Experiment 2 was designed to evaluate the uncertainties of RT reaction mixture, RT reaction, and dPCR measurement: three replications of RT reaction mix preparation, three replications of the RT reaction, one replication of dPCR reaction mix preparation, and three replications of dPCR measurements.

Experiment 3 was designed to evaluate the uncertainty of RT reaction mixture, dPCR reaction mix preparation, and dPCR measurement: three replications of RT reaction mix preparation, one replication of the RT reaction, three replications of dPCR reaction mix preparation, and four replications of dPCR measurements.

Experiment 4 was designed to evaluate the uncertainties of RT reaction, dPCR reaction mixture, and dPCR measurement: one replication of RT reaction mix preparation, three replications of the RT reaction, three replications of dPCR reaction mix preparation, and three replications of dPCR measurements.

All experiments were performed independently, and the inter-day difference was calculated from the standard deviation of four quantified values. The combined uncertainty ($u_{\text{RT–dPCR}}$) was calculated by using Eq. (4), combining the relative standard uncertainties associated with "the RT reaction solution preparation (u_{RTprep})," "the RT reaction (u_{RT})," "the dPCR reaction mix preparation (u_{dPCRprep})," "the dPCR measurement ($u_{\text{measurement}}$)," "the weighing of RT reaction mix and dPCR reaction mix (u_{RTmix} and u_{dPCRmix})," "the partition volume ($u_{\text{partition}}$)," and "the difference between days ($u_{\text{between\_days}}$)."

$$u_{\text{RT–dPCR}} = \sqrt{\left(\frac{u_{\text{RTprep}}}{C_{\text{1000-A}}}\right)^2 + \left(\frac{u_{\text{RT}}}{C_{\text{1000-A}}}\right)^2 + \left(\frac{u_{\text{dPCRprep}}}{C_{\text{1000-A}}}\right)^2 + \left(\frac{u_{\text{measurement}}}{C_{\text{1000-A}}}\right)^2 + \left(\frac{u_{\text{RTmix}}}{w_{\text{RTmix}}}\right)^2 + \left(\frac{u_{\text{dPCRmix}}}{w_{\text{dPCRmix}}}\right)^2 + \left(\frac{u_{\text{partition}}}{V_{\text{partition}}}\right)^2 + \left(\frac{u_{\text{between days}}}{C_{\text{1000-A}}}\right)^2} \tag{4}$$

Results and discussion

Optimization of reverse transcription reaction

Study design

The RT reaction is an essential step for PCR-based RNA analysis even for RT–dPCR. Therefore, we thought that maximizing the efficiency of the RNA reaction would be the key to achieving the accurate measurement of RNA. It has been pointed out that the accuracy and the reaction efficiency for the conversion of RNA into cDNA vary depending on the type of RTase [21, 28]. Various RTases have been explored, modified, and improved, and many RTases and kits are now available commercially. We performed RT–dPCR using RTase derived from Moloney murine leukemia virus (MMLV), the most common RTase available on the market. Three RTases were used: one was an unmodified MMLV-RTase (MSRT), and the others were modified MMLV-RTases (SSIV and RTA).

In the RT reaction, three types of primers are generally used: an oligo d(T)$_{20}$ primer, a random hexamer, and a gene-specific primer. In the one-step RT–dPCR, a gene-specific primer is generally used because the RT reaction and the PCR reaction are performed consecutively in the same vial. On the other hand, in the two-step RT–dPCR, one of three types of primers described above is selected and used depending on the purpose because the RT reaction and the PCR reaction are performed separately in different vials. Because RNA1000-A, one of five RNA solutions in NMIJ RM 6204-b, has the 33-mer of poly-A tail at the 3′ end, the three types of primers can be used in the RT reaction. We used RNA-1000-A to examine and optimize the type of RTase, the concentration of template RNA used, and the type and concentration of primers for the accurate quantification of RNA by RT–dPCR.

Concentration of template RNA

Firstly, we conducted experiments using two kinds of sample RNA diluted at different timing to determine the optimal timing for performing template RNA dilutions in two-step RT-dPCR. The manufacturer has indicated that the appropriate amount of template RNA used in the RT reaction is dependent on the type of RTase: < 500 ng of mRNA for SSIV, < 2 µg of total RNA for MSRT, and < 10–100 ng of mRNA for RTA. One milliliter

of RNA1000-A stock solution (68.2 ng, i.e., 1.05×10^{11} copies/reaction) or 1 µL of 10^6-fold diluted RNA1000-A solution (1.05×10^5 copies/reaction) was used as the template RNA in the RT reaction to achieve the optimal concentration of 2000 copies/µL for dPCR measurements. After the RT reaction, the RT reactant solution using the RNA1000-A stock solution was diluted 10^6-fold so that the amount of cDNA in the dPCR reaction mix would be the same as that using the 10^6-fold diluted RNA1000-A solution. The above experiments were performed with the systematic combination of three enzymes, three primers, and two concentrations of template RNA.

Figure 1 shows the recovery rates for each RT enzyme when three primers and two concentrations of template RNA were used in the RT reaction. The recovery rate was higher in the sample using 10^6-fold diluted RNA1000-A solution than in the sample using RNA1000-A stock solution, regardless of the type of RTase or the type of primer used in the RT reaction (Fig. 1). The sample using the 10^6-fold diluted RNA1000-A solution had a higher RT reaction efficiency than the sample using the RNA1000-A stock solution. These results indicated that the amount of template RNA contained in the RT reaction solution affected the efficiency of the RT reaction and the more cDNA could be obtained by dilution before RT reaction. When the concentration of template RNA in the RT reaction solution is high, the rate of association between primer and RNA template will be low, even if there is a sufficient amount of primer.

Primer type

We evaluated the influence of RTase and primer type on the RT reaction efficiency by calculating the recovery rate in the RT reaction. We used three RTases and three primers to make nine combinations for the recovery rate evaluation. The three primers used in this study are representative of those used in RT reaction, and the RT reaction temperature is also the optimal temperature for the enzyme, as specified by the manufacturers. Therefore, the primer melt temperature did not seem to influence on RT efficiency. Figure 1 shows that the recovery rate of the sample using oligo d(T)$_{20}$ primer was the lowest, ranging from 2.0 to 44.6%. Because the oligo d(T)$_{20}$ primer annealed to the poly-A tail at the 3′ end of the sample RNA, the RT reaction was initiated from the 3′ end. The low quantitative value of the sample using the oligo d(T)$_{20}$ primer indicated that a low yield of synthesized cDNA was detected by dPCR with the gene-specific primer and probe positioned at approximately 500-mer from the 3′ end of RNA1000-A. The manufacturer claims that each RTase used in this study can synthesize approximately 5000-mer of cDNA, and the low yield of approximately 500-mer cDNA was unexpected. As a result, it was suggested that the oligo d(T)$_{20}$ primer was not suitable for RNA quantification by RT–dPCR when the detection position by dPCR is
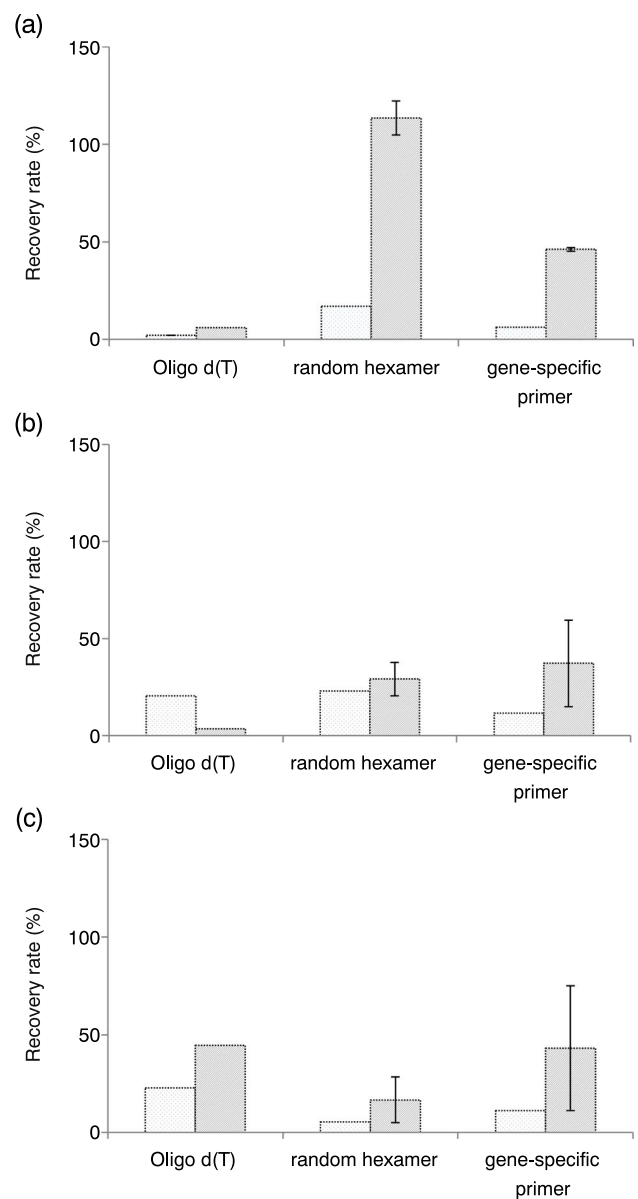

Fig. 1 Effects of template RNA concentration, type of primer, and type of RTase in the RT reaction on the recovery rate. Error bar shows SD calculated from triplicate RT–dPCR measurements. Each graph shows the results of three RTases: (a) SSIV, (b) MSRT, and (c) RTA. In each graph, the dotted bar shows the result obtained with 1.05×10^{11} copies of RNA 1000-A/reaction, and the striped bar shows that obtained with 1.05×10^6 copies of RNA 1000-A/reaction used as template RNA concentration

far from the 3′ end of poly-A tail, although it is frequently used for cDNA synthesis in the relative quantification of mRNA by RT–qPCR.

The random hexamer showed the highest recovery rate, exceeding 100% at times (Fig. 1). After experimenting multiple times, we found that the recovery rate of the random hexamer was always much higher than 100%, although its reproducibility was poor (120–180%). This exceedingly

high recovery rate of the random hexamer was pointed out in several studies on the quantification by RT–qPCR and RT–dPCR. According to Zhang and Byrne (1999) [33] and Sanders et al. (2013) [21], the random hexamer, which usually consists of six nucleotides, is annealed to multiple sites on the same template RNA, leading to a large increase in cDNA yield and causing an exceeded recovery of the RNA copy number at times. The results of our experiment were consistent with those studies. As more cDNAs were converted than the substantial copy number of RNA1000-A added to the RT reaction solution, the quantified value of dPCR was thought to be higher than the certified value when using the random hexamer. Therefore, the random hexamer also was not suitable for the RNA quantification by RT–dPCR.

From these results, we concluded that the gene-specific primer that specifically annealed to the measurement target was most suitable for the accurate RNA quantification by RT–dPCR.

Reverse transcriptase (RTase)

We also investigated the RT reaction efficiency and the reproducibility of the three types of RTases (SSIV, MSRT, and RTA). RT reactions were performed in triplicate and evaluated using the 10^6-fold diluted RNA1000-A solution as the template RNA, the gene-specific primer, and the three

RTases. The amount of gene-specific primer used in the RT reaction was 10 pmol/reaction. The results are shown in Table 1. All the recovery rates were around 40%, but the degree of reproducibility of the RT reactions varied markedly among the RTases used. SSIV showed the highest RT reaction efficiency and reproducibility, where the recovery rate was $46.2\% \pm 0.9\%$ (recovery rate $\pm$ SD). The recovery rates of MSRT and RTA were $37.2\% \pm 22.2\%$ and $43.3\% \pm 32.0\%$, respectively. The recovery rates of those two enzymes were not significantly different from that of SSIV, but the reproducibility of the RT reaction was poor. Because the reproducibility of the RT reaction was critical for accurate quantification, we decided to use SSIV in later experiments to determine other conditions that would enhance the recovery rates.

Optimization of gene-specific primer concentration in SSIV

The RT reactions using gene-specific primers showed low recovery rates of around 40%, suggesting that the amount of synthesized cDNA was low. Although it was not known why the RT reaction lacked efficiency, the reproducibility of the experiment using SSIV was good, and the amount of synthesized cDNA seemed to remain low. From these findings, we hypothesized that the shortage of gene-specific primers would result in the termination of the RT reaction, the stoppage of cDNA production, and the low amount of cDNA. Therefore, we evaluated the effect of the amount of the gene-specific primer on the recovery rate of the RT reaction. Then, we changed the amount of the gene-specific primer from 10 pmol/reaction to 200 pmol/reaction by 10 pmol/reaction steps. As shown in Fig. 2, the recovery rate exhibited a concentration-dependent increase between the primer concentration of 10 and 100 pmol/reaction and reached a plateau between 100 and 200 pmol/reaction. From these results, a higher than 100 pmol/reaction primer concentration in the

Table 1 Recovery rates of three RTases with gene-specific primer. The concentration of the gene-specific primer was 10 pmol/reaction. SD was calculated by performing the RT reaction in triplicate
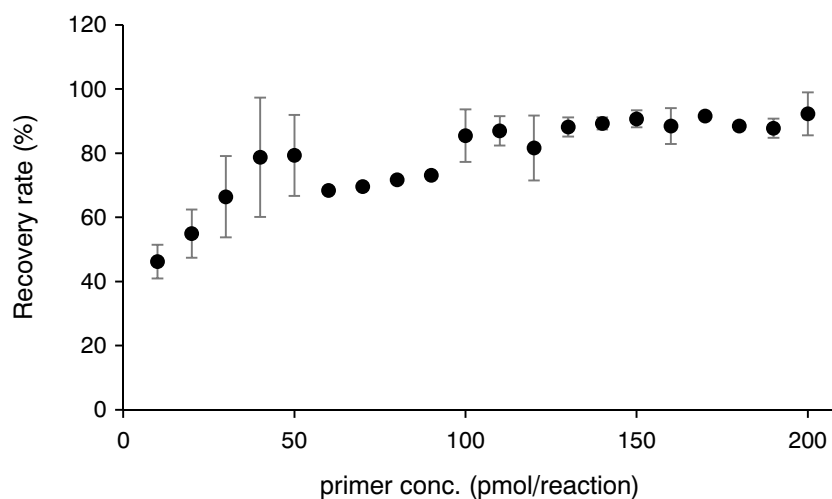
	SSIV	MSRT	RTA
Recovery rate	46.2	37.2	43.3
SD	0.9	22.2	32.0

Fig. 2 Effects of the amount of gene-specific primer on the recovery rate of the RT reaction. The error bar shows SD calculated from triplicate RT–dPCR measurements

RT reaction was sufficient, and we set 200 pmol/reaction as the optimal primer concentration. It was clarified that the amount of primer used in the RT reaction critically affected the quantified value. Therefore, the optimization of the amount of primer is essential, and the amount that optimizes the RT reaction should be selected as the optimal one.

Accuracy of the method

On the basis of the above optimization experiments, the optimal RT reaction conditions for the quantification of RNA1000-A by two-step RT–dPCR are as follows: approximately 1500 copies/µL of RNA1000-A as template RNA, SSIV as RTase, and 200 pmol/reaction of gene-specific primer. Under the optimal conditions, replicate experiments were performed as described in the "Uncertainty evaluation of RT–dPCR" section, and the following uncertainty components were evaluated by ANOVA: preparation of RT reaction solution, RT reaction, preparation of dPCR reaction mix, between chip, and between days. The concentration of RNA1000-A in the sample solution was calculated from the quantified value by RT–dPCR and each dilution factor in the gravimetric sample preparation. The quantified value by RT–dPCR was 76.4 ng/µL ± 6.7 ng/µL (value ± expanded uncertainty ($k=2$)). To confirm whether there is a significant difference between the certified value of CRM and the measurement value, we calculated their equivalence using Eq. (5) [34].

$$|C_{m} - C_{CRM}| \leq 2\sqrt{u_{m}^{2} + u_{CRM}^{2}} \qquad (5)$$

where C_m is the quantified value by RT–dPCR, C_{CRM} is the certified value of CRM, u_m is the standard uncertainty of RT–dPCR measurement, and u_{CRM} is the standard uncertainty of CRM. It was confirmed that the quantified value of the sample solution was consistent with the certified value within the expanded uncertainty. Without the optimization, the recovery rates using gene-specific primers tended to be much lower than 100%, but the RT reaction under the optimal conditions achieved a recovery rate of nearly 100%.

The uncertainty budget of the quantified value of the sample solution is shown in Table 2. Because Niu et al. [10] and Van Heuverswyn et al. [22] did not evaluate the uncertainty components for RNA quantification by RT-dPCR, we were not able to compare the uncertainty components obtained in this study with those of the two studies. However, in each previous study, the expanded measurement uncertainty for synthetic RNA quantification was approximately 5 to 10%, and the present method was able to quantify RNA with the same extent of uncertainty as the previous method without bias. The uncertainty budget showed that the major uncertainty components in RNA1000-A quantification by two-step RT–dPCR were,

Table 2 Quantified value and uncertainty budget for the quantification of RNA1000-A by two-step RT–dPCR

	Quantified value by RT–dPCR	Certified value of RNA1000-A
Mean value (ng/µL)	76.4	68.2
Expanded uncertainty (ng/µL, $k=2$)	6.7	5.8
Uncertainty component	Relative uncertainty (%)	
Between day	1.80	
Preparation of RT reaction mix	3.68	
RT reaction	1.30	
Preparation of dPCR reaction mix	0.29	
Between chip	0.71	
Weighting (RT reaction mix)	0.01	
Weighting (dPCR reaction mix)	0.01	
Chip volume	0.64	
Combined standard uncertainty	4.42	
Expanded uncertainty ($k=2$)	8.83	

in order of magnitude, the preparation of the RT reaction solution (3.68%), the inter-day difference (1.80%), and the RT reaction (1.30%). The largest uncertainty component in the two-step RT–dPCR is the one related to the RT reaction. Increasing the number of replications for the RT reaction is expected to accomplish more accurate RNA quantification by two-step RT–dPCR.

Quantification in the low concentration range

For practical use, we examined the quantitative performance of two-step RT–dPCR using a low-concentration RNA sample. The RT reaction was performed with 100, 400, 700, and 1000 copies of RNA 1000-A solution/reaction, and
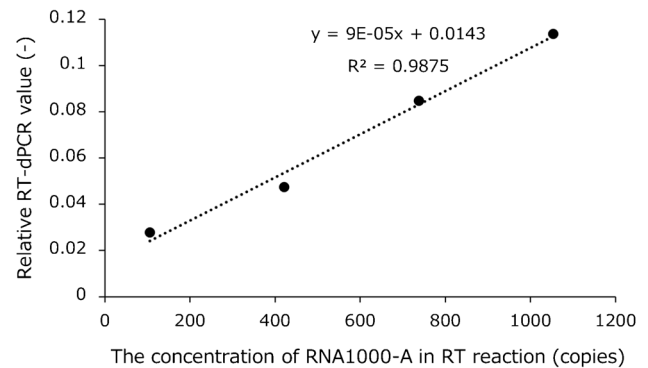

Fig. 3 Correlation between the amount of template RNA in the RT reaction and the quantified value by RT–dPCR. One measurement was performed at each concentration

RT–dPCR was performed with a total of 15 µL of the dPCR reaction mix using 4.125 µL of the RT reactant solution. As a result, a linear correlation between the quantitative value and the concentration of RNA 1000-A in the RT reaction solution was observed in the range of 100 copies to 1000 copies (Fig. 3). In other words, RT reaction efficiency remained constant between 100 and 1000 copies of RNA 1000-A in the RT reaction solution. This result verified that quantification by two-step RT–dPCR is possible if there are at least 100 copies of template RNA in the RT reaction solution.

Conclusion

We conclude that accurate RNA quantification by RT–dPCR requires optimization of the RT reaction conditions and that the RT reaction step significantly impacts the measurement uncertainty of RT–dPCR.

The parameters to be optimized in the RT reaction are as follows:

- The type of RTase
- The concentration of template RNA
- The type and concentration of R-primer used in the RT reaction

Without the optimization of these parameters, bias in the quantified values may occur, which can be either exceeded recovery (above 180% in this study) or failed recovery (below 10% in this study). By using the optimal condition, the quantified value of NMIJ CRM 6204-b RNA1000-A was 76.4 ng/µL ± 6.7 ng/µL (quantified value ± expanded uncertainty, $k = 2$, relative expanded uncertainty was 8.8%), and in good agreement with the certified value within the expanded uncertainty. The major uncertainty components in RNA1000-A quantification by two-step RT–dPCR were the preparation of the RT reaction solution, the inter-day difference, and the RT reaction, all of which were related to the RT reaction. Among the parameters identified in this study, the primer concentration in the RT reaction can be optimized even in one-step RT–dPCR.

Declarations

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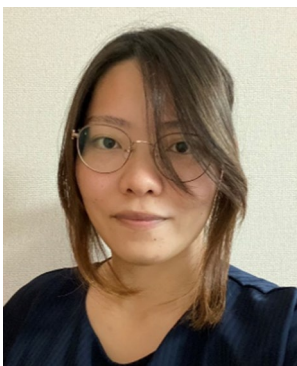
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