Sensitive Detection and Quantification of SARS-CoV-2 by Multiplex Droplet Digital RT-PCR

Remco de Kock (✉ remco.d.kock@catharinaziekenhuis.nl)

Catharina Hospital Eindhoven <https://orcid.org/0000-0003-3167-8706>

Mieke Baselmans

Catharina Hospital Eindhoven

Volkher Schamhorst

Catharina Hospital Eindhoven

Birgit Deiman

Catharina Hospital Eindhoven

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Abstract

Purpose. We aimed to develop a one-step droplet digital RT-PCR (RT-ddPCR) multiplex assay that allows for sensitive detection of SARS-CoV-2 RNA with respect to human derived RNA and could be used for triage and monitoring of Covid-19 patients.

Methods. A one step RT-ddPCR multiplex assay was developed for simultaneous detection of SARS-CoV-2 *E*, *RdRp* and *N* viral RNA, and human *Rpp30* DNA and *GUSB* mRNA, for internal nucleic acid (NA) extraction and RT-PCR control. Dilution series of viral RNA transcripts were prepared in water and total NA extract of Covid-19 negative patients. As reference assay, an *E-GUSB* duplex RT-PCR was used.

Results. Assay sensitivity of the RT-PCR assay drastically decreased when SARS-CoV-2 copies were detected in a background of total NA extract compared to water, while the sensitivity of the RT-ddPCR was not affected by the total NA background. *GUSB* mRNA detection was used to set validity criteria to assure viral RNA and RT-PCR assay quality. In a background of at least 100 *GUSB* mRNA copies, 5 copies of viral RNA are reliably detectable and 10 copies viral RNA copies are reliably quantifiable.

Conclusion. The present study describes a robust and sensitive one-step RT-ddPCR multiplex assay for reliable detection and quantification of SARS-CoV-2 RNA. By determining the fractional abundance of viral RNA with respect to a human housekeeping gene, viral loads from different samples can be compared, what could be used to investigate the infectiveness and to monitor Covid-19 patients.

Introduction

The outbreak of lung disease Covid-19 caused by SARS-CoV-2 has spread worldwide. Up to now, over 25 million confirmed cases have been reported. The US, Brazil and India are the most affected countries with the highest mortality rates due to Covid-19 [1].

The gold standard for the detection of SARS-CoV-2 is based on real-time reverse-transcriptase PCR (RT-PCR). In the Netherlands, a RT-PCR based assay for the detection of the envelope (*E*) gene, followed by confirmatory testing with a RNA-dependent RNA polymerase (*RdRp*) gene assay, is recommended [2]. Another approach is to detect the nucleocapsid (*N*) gene, and to use an open reading frame 1a/b (*ORF1b*) gene or *E* gene assay as a confirmatory test [3]. In addition, to improve assay sensitivity, other studies have been focusing on the detection of *N*, *E* or *ORF1b* using droplet digital polymerase chain reaction (ddPCR) [4–7].

Most assays are targeted against one single gene. As the RNA genome of SARS-CoV-2 mutates during virus replication false negative results could be obtained due to the loss of primer or probe binding [8, 9]. By using multiplex assays, targeting various SARS-CoV-2 genes, the chance of missing a positive sample in this way is reduced.

The amount and quality of viral RNA in the patient sample is an important factor for reliable virus detection. Also, most assays do not include a patient derived internal RNA control for the reverse-transcriptase step which is a critical step in viral RNA detection. The inclusion of a patient derived internal RNA control would enable for quality control of the (viral) RNA in the patient sample and the RT-PCR.

Finally, quantification of the SARS-CoV-2 viral load becomes more and more important to distinguish positive patients who are infective, from positive patients with only residual viral RNA who are probably not infective anymore (viral shedding) [10], and for monitoring Covid-19 patients during treatment.

The goal of this study is to develop a sensitive one-step droplet digital RT-PCR (RT-ddPCR) multiplex assay for simultaneous detection of multiple SARS-CoV-2 genes *N* (*N1+N2*), *E* and *RdRp*, including the detection of patient derived mRNA of a housekeeping gene to assure sample and assay quality and to enable quantification of viral RNA.

Methods

Samples

As reference standards, the Wuhan Coronavirus 2019 *E* gene control (EVAg, European Virus Archive Global, France), an in vitro transcript (100.000 copies/mL), was used directly for amplification, or transcript RNA of the *E*, *N*, *ORF1ab*, *RdRp* and *S* gene (all 200.000 copies/mL), extracted from the Exact Diagnostics SARS-CoV-2 Standard (EDx, Exact Diagnostics, Texas, USA) using the MP24 Total NA isolation Kit, on the MagNA Pure 24 system (Roche Diagnostics, Rotkreuz, Switzerland), was used for amplification.

Dilution series of the reference standards were prepared in nuclease-free water and in remnant total nucleic acid (NA) MagNaPure 24 extract from nasopharyngeal swabs of Covid-19 negative patients, to obtain an input of 5-500 copies per reaction for the EVAg control and 2,5-1000 copies per reaction for the EDx control.

One-step Reverse Transcriptase Real-Time PCR

The one-step reverse transcriptase reaction for the detection of *E* was performed in a 25 µL reaction volume as described previously [2]. The assay was also performed in a 10 µL reaction volume, consisting of 5 µL 2 x reaction buffer, 0.16 µL of a 50 mM magnesium sulphate solution and 0.40 µL of SuperScript™ III RT/Platinum™ Taq Mix (all provided by the SuperScript™ III One-Step RT-PCR with Platinum™ Taq Polymerase, Invitrogen, USA), 400 nM forward primer, 400 nM reverse primer, 200 nM probe (primers and probe provided by TIB MolBiol, Berlin, Germany), 0.4 µg of nonacetylated bovine serum albumin (Ultrapure™ BSA, Invitrogen, USA) and 2 µL of RNA.

The *E-GUSB* duplex RT-PCR was performed in a 25 µL and 10 µL reaction volume as described above, with the addition of 1.25 µL and 0.5 µL *GUSB* assay (Bio-Rad Laboratories, Hercules, CA), respectively.

All assays were performed on the LightCycler 480 II system (Roche Diagnostics) using the following cycling conditions: 10 min at 55 °C for reverse transcription, followed by 3 minutes at 95 °C, continuing with 15 seconds at 95 °C, and 30 seconds at 58 °C.

One-step Reverse Transcriptase Droplet Digital PCR Multiplex

The ddPCR multiplex assay allows for the simultaneous detection of *E*, *RdRp*, *N* (*N1+N2*), *Rpp30* and *GUSB*. For each assay, a reaction mixture of 22 µL was prepared with 17 µL amplification mix and 5 µL RNA extract. The amplification mix, based on the One-Step RT-ddPCR advanced Kit for probes (Bio-Rad Laboratories), consisted of 5.5 µL Supermix, 2.2 µL Reverse Transcriptase, 1.1 µL 300 mM DTT (Bio-Rad Laboratories). The primers and probes for the detection of *E* and SARS-CoV-2 specific *RdRp* were described previously [2] except for the *E* reverse primer, as using this primer false positive reactions were obtained. For *E*, 450 nmol forward primer [2], 450 nmol in-house reverse primer (5'-GGTTTACAAGACTCACGTTAACA-3') (TIB MolBiol), 250 nmol HEX-labeled and 250 nmol FAM-labeled probe [2] (Integrated DNA technologies, Coralville, USA) was added, for *RdRp*, 900 nmol forward and reverse primers and 250 nmol probe [2] was added, for *N*, 1.0 µL 2019-nCov CDC ddPCR Triplex Probe assay (Bio-Rad Laboratories) was added and 1.0 µL of *GUSB* assay (Bio-Rad Laboratories) was added.

The QX200 Droplet Digital PCR System (Bio-Rad Laboratories) was used for the quantification of SARS-CoV-2 RNA. For droplet generation 20 µL reaction mix was used and the droplets were transferred to a 96-well plate. Samples were amplified in the C1000 Touch Thermal Cycler (Bio-Rad Laboratories) according to following protocol: 50°C for 60 min (reverse transcription), 95°C for 10 min (enzyme activation), 40 cycles of 95°C for 30 sec (denaturation) and 55°C for 60 sec (annealing), and 98°C for 10 min (enzyme deactivation). Data were analyzed using QuantaSoft version 1.7.4.0917 (Bio-Rad Laboratories).

Results

Recently, a commercial RT-ddPCR assay, targeting two highly conserved regions of the SARS-CoV-2 *N* gene became available (2019-nCoV CDC ddPCR Triplex Probe assay, Bio-Rad Laboratories). This assay also includes an internal control for the detection of patient derived *Rpp30* as internal DNA control for the PCR step. Based on this assay a multiplex one-step RT-ddPCR test was developed for the detection of the *E*, *RdRp*, and *N* (*N1+N2*) gene. In addition, primers and probes for the detection of patient derived *GUSB* mRNA were added as reverse-transcriptase control. The multiplex was tested on the EDx reference standard, including transcripts of *E*, *RdRp* and *N* and human genomic total nucleic acid (NA). For each SARS-CoV-2 target a distinct cluster was identified in the two-dimensional scatterplot (Figure 1A). For a reproducibility analysis, 5 replicates of the EDx standard were tested and quantified, showing that the

SARS-CoV-2 concentrations were all in the same order of magnitude and in agreement with the input used (Table 1).

Assay Sensitivity

To determine assay sensitivity of the SARS-CoV-2 RT-ddPCR multiplex assay, the previously described one-step reverse-transcriptase RT-PCR targeting the *E* gene [2] was set up and used as reference assay. To ensure the correct performance of this reference assay, a dilution series of the EVAg standard was tested, showing that the *E* target could be detected down to an input of 5 copies per reaction (Figure 2A), which is in agreement with the performance of this assay as described previously [2].

To reflect a more realistic clinical setting, a comparable dilution series of the EVAg standard was prepared in total NA extract obtained from nasopharyngeal swabs of Covid-19 negative patients. Strikingly, for the EVAg standard diluted in NA extract, 5 SARS-CoV-2 copies per reaction was no longer detectable, only 1 out of 4 reactions was tested positive, suggesting loss of sensitivity when compared to the EVAg standard diluted in water (Figure 2 A and B).

As this reference assay does not include the detection of a patient derived internal control, a duplex reaction was developed combining the *E* gene reference assay [2] with the *GUSB* assay (Bio-Rad Laboratories). The sensitivity of the *E-GUSB* duplex RT-PCR was tested, using the previously described dilution series of the EVAg standard in water (Figure 2A). Results show that using the *E-GUSB* duplex RT-PCR, an input of 5 copies per reaction is still detectable, suggesting no loss of assay sensitivity compared to the *E* gene simplex reference assay. The *E-GUSB* duplex RT-PCR was further used as reference assay to investigate the assay performance of the SARS-CoV-2 RT-ddPCR multiplex assay.

First, the *E-GUSB* duplex PCR was tested using a dilution series of the EDx standard in water, including *GUSB* mRNA. Using an input of 10 SARS-CoV-2 EDx copies per reaction, 100% was tested positive (n=2), while using an input of 5 SARS-CoV-2 EDx copies per reaction, 50% tested positive (n=4, Figure 3 A and C, Table 2). EDx copies are not identical to EVAg copies as the EVAg standard is used directly in amplification, while the EDx standard requires extraction, like patient samples, during which some RNA will be lost. Again, when using dilution series of the EDx standard prepared in total NA extract from Covid-19 negative patients, a serious loss of sensitivity was observed, as 5 and 10 SARS-CoV-2 EDx copies per reaction were no longer detectable (Figure 3 B and D, Table 2).

The same EDx dilution series, in water and in total NA extract of Covid-19 negative patients, were used to investigate the sensitivity of the SARS-CoV-2 RT-ddPCR multiplex assay. Using 5 SARS-CoV-2 copies per reaction of the EDx standard diluted in water, SARS-CoV-2 RNA could be detected (n=4, Figure 1C and Table 2), which is comparable to the assay sensitivity of the *E-GUSB* duplex RT-PCR. Fortunately, when using the EDx standard diluted in total NA extract from Covid-19 negative patients, the SARS-CoV-2 multiplex RT-ddPCR assay could again detect 5 SARS-CoV-2 copies per reaction (n=4, Figure 1D and Table 2). For each dilution, in water or total NA extract, the detected SARS-CoV-2 concentration was in the

same order of magnitude (Figure 4 and Table 2), indicating that the sensitivity of the RT-ddPCR multiplex assay is not affected by the total NA extract. In addition, in the presence of total NA extract, the SARS-CoV-2 multiplex RT-ddPCR assay is approximately 10-fold more sensitive than the *E-GUSB* duplex RT-PCR assay. The limit of detection (LoD) of the SARS-CoV-2 multiplex RT-ddPCR assay is estimated to be 5 copies per reaction.

Quantification of viral RNA

To correct for the differences in RNA yield and quality obtained during sample collection and differences in efficiency of the RT-ddPCR, quantification of the viral load was performed relative to the patient derived *GUSB* mRNA (fractional abundance, FA). For the undiluted EDx standard a reproducible FA of approximately 90% was obtained, showing the robustness of the FA determination (Table 1).

For the EDx standard dilution series in total NA extract, obtained from Covid-19 negative patients, a *GUSB* mRNA concentration of at least 100 copies per reaction was detected (Table 2). In this background of total NA, down to 5 SARS-CoV-2 copies per reaction can be detected, but the limit of quantification (LoQ) is estimated to be 10 copies per reaction, as the FA obtained with 5 or 10 SARS-CoV-2 copies per reaction overlap (Table 2).

For 5 Covid-19 negative patient samples, the *GUSB* mRNA concentration was determined individually (Figure 1B and Table 1). Remarkably, for one sample a *GUSB* mRNA concentration of just 14 copies per reaction, so far below 100 copies per reaction, was found, implying poor sample collection or poor reverse-transcription that could have resulted in a false negative result.

Discussion

A novel RT-ddPCR multiplex assay was developed targeting three different genes of the SARS-CoV-2 virus. As the SARS-CoV-2 genome evolves rapidly [8, 9], it is of interest to screen multiple targets simultaneously to avoid possible mismatches of primers and probes, which could lead to false negative results [11]. The SARS-CoV-2 RT-ddPCR multiplex assay also includes a patient derived NA extraction control, and a reverse-transcriptase control to ensure adequate sample and assay quality required for reliable virus detection.

While reverse-transcriptase RT-PCR is still the gold standard, the findings in the present study indicate that the assay sensitivity of the RT-PCR assay is reduced due to background NA from the patient sample. By contrast, the sensitivity of the RT-ddPCR multiplex assay was not affected by background NA, and is more sensitive than the gold standard reverse-transcriptase RT-PCR [4–7] in the clinical setting.

As ddPCR enables absolute quantification, not only the viral RNA can be quantified but also the *GUSB* mRNA, that can be used to set validity criteria and to ensure reliable analysis, as false negative results may occur due to poor sample quality as a result of inappropriate sample collection, handling or

transportation [12]. For the SARS-CoV-2 RT-ddPCR developed in this study, 5 copies of viral RNA are reliably detectable and 10 copies viral RNA copies are reliably quantifiable in a background of at least 100 *GUSB* mRNA copies.

By quantification of SARS-CoV-2 relative to patient derived *GUSB* mRNA, the fractional abundance of the viral loads of different samples can be compared. This could be used to gain insights in the relation between viral load and infectivity, which is at this moment unclear [13–16].

Together, this study presents a sensitive one-step RT-ddPCR multiplex assay that allows for reliable detection and quantification of SARS-CoV-2 viral RNA with respect to patient derived mRNA of a house-keeping gene, what could be used for triage and enables disease monitoring of Covid-19 patients.

Declarations

Funding: Not applicable

Conflicts of interest/Competing interest: Not applicable

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Consent to participate: Not applicable

Availability of data and material: Upon request

Code availability: Not applicable

Authors' contributions: RdK: Conducted experiments, conceptualised the laboratory work, wrote the manuscript. MB: Conducted experiments, contributed to manuscript. VS: Contributed to manuscript. BD: Planned experiments, conceptualised the laboratory work, conceptualised the overall study, wrote the manuscript.

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Tables

Table 1 Validation samples tested with the SARS-CoV-2 RT-ddPCR multiplex assay

Sample	SARS-CoV-2 (Copies/Reaction)	<i>Rpp30</i> (Copies/Reaction)	<i>GUSB</i> (Copies/Reaction)	FA (%)
Positive control 1	1018 (946-1090)	488 (438-536)	116 (92-140)	90
Positive control 2	806 (732-882)	308 (262-356)	62 (42-84)	93
Positive control 3	868 (796-942)	472 (420-526)	64 (46-86)	93
Positive control 4	848 (776-922)	430 (378-482)	74 (54-98)	92
Positive control 5	896 (816-978)	452 (396-510)	110 (84-140)	90
Negative control 1	ND	512 (450-574)	370 (316-422)	
Negative control 2	ND	1580 (1460-1700)	14 (6-30)	
Negative control 3	ND	1800 (1700-1920)	108 (84-134)	
Negative control 4	ND	11660 (11320-12000)	466 (406-526)	
Negative control 5	ND	5000 (4820-5200)	128 (100-158)	

ND, not detected. FA, fractional abundance of viral RNA calculated with respect to the *GUSB* concentration. The undiluted EDx standard was used as positive control and total NA extract from remnant nasopharyngeal swabs from Covid-19 negative patients as negative control.

Table 2 EDx dilution series tested with the SARS-CoV-2 RT-PCR *E-GUSB* duplex and RT-ddPCR multiplex assay

EDx standard (Cp/reaction)	RT-PCR (Ct value)			RT-ddPCR (Copies/Reaction)				FA (%)
	SARS-CoV-2		<i>GUSB</i>	SARS-CoV-2		<i>Rpp30</i>	<i>GUSB</i>	
	Water	Total NA		Water	Total NA			
1000	31,3	NA	NA	1098 (1004-1192)	NA	NA	NA	NA
1000	31,6	NA	NA	1002 (916-1090)	NA	NA	NA	NA
100	34,4	33,1	32,2	108 (80-140)	94 (70-124)	4531 (4338-4726)	155 (123-192)	38
100	34,6	33,6	32,2	114 (86-150)	96 (74-124)	4343 (4164-4523)	115 (89-145)	45
10	36,3	ND	32,2	20 (10-34)	5.6 (1.4-14.8)	5028 (4830-5229)	160 (128-196)	3
10	36,8	ND	32,1	12 (4-24)	18 (8-32)	4990 (4788-5194)	152 (120-188)	10
5	ND	ND	31,9	9 (2.6-21)	9 (3.2-19.6)	5254 (5054-5457)	112 (86-142)	7
5	37,3	ND	32,0	2.4 (0.2-11.8)	3.2 (0.4-10)	4942 (4761-5125)	129 (103-159)	2
5	36,9	ND	32,0	7.6 (2.2-17.8)	11.6 (4.8-22.4)	4818 (4635-5002)	124 (98-154)	9
5	ND	ND	32,0	5.6 (1.4-14.8)	10.2 (3.6-22.4)	5256 (5041-5472)	148 (117-186)	6
2.5	ND	ND	32,1	ND	ND	4820 (4640-5020)	122 (96-154)	3
2.5	ND	ND	32,2	ND	ND	5160 (4960-5380)	106 (80-138)	
2.5	ND	ND	32,0	ND	3.4 (0.6-11)	4840 (4660-5040)	112 (86-142)	
2.5	ND	ND	32,3	ND	ND	4860 (4660-5060)	112 (86-144)	

NA, not applicable. ND, not detected. FA, fractional abundance of viral RNA calculated with respect to the *GUSB* concentration.

Figures

SARS-CoV-2 RT-ddPCR Multiplex Assay

Figure 1

Two-dimensional scatterplots of the SARS-CoV-2 RT-ddPCR multiplex assay targeting N, E and RdRp. Rpp30 is included as internal DNA control and GUSB as internal reverse-transcriptase control. a Undiluted EDx standard with 1000 SARS-CoV-2 copies per reaction (n=1). b Total NA extract from nasopharyngeal swabs from Covid-19 negative patients. c EDx standard, diluted in water, with 5 SARS-CoV-2 copies per reaction (n=4). d EDx standard, diluted in total NA extract from Covid-19 negative patients, with 5 SARS-CoV-2 copies per reaction (n=4)

Figure 2

Real-time amplification curves of the E gene RT-PCR reference assay tested on the EVAg standard. The reference assay was described previously [2]. a EVAg dilution series with 5-5000 SARS-CoV-2 copies/reaction diluted in water. The continuous lines represent the RT-PCR assay in 25 μ L reaction volume (n=1), the interrupted lines represent the RT-PCR assay in 10 μ L reaction volume (n=1) and the dotted lines represent the E-GUSB duplex RT-PCR assay (n=1). b EVAg dilution series in total NA extract from a Covid-19 negative patient tested with the RT-PCR assay. For 500-5000 copies/reaction n=1, for 50 copies/reaction n=2, for 5 copies/reaction n=4

Figure 3

Real-time amplification curves of the E-GUSB duplex RT-PCR reference assay tested on the EDx standard. A,C: EDx dilution series with 2.5-1000 SARS-CoV-2 copies/reaction diluted in water. B,D: EDx dilution series in total NA extract of a Covid-19 negative patient. *EDx dilution in water. For 10-100 copies/reaction (n=2), 2,5-5 copies/reaction (n=4)

Figure 4

Dilution series of the EDx standard diluted in water or total NA extract of a Covid-19 negative nasopharyngeal swab tested with the SARS-CoV-2 RT-ddPCR multiplex assay. For 10-100 copies/reaction (n=2), 2,5-5 copies/reaction (n=4)