

Duplex RTqPCRs for detection and relative quantification of SARS-CoV-2 variants of concern (VOC)

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Method Article

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Abstract

During the evolution of the SARS-CoV-2 pandemic, new variants of the virus have emerged and spread worldwide. The increased transmissibility and proclivity of some of these variants to cause more serious disease threatens public health responses against the virus, and they are classified as variants of concern (Variants of Concern, VOCs). While Next-Generation-Sequencing (NGS) is the gold standard to identify VOC, it cannot always be rapidly implemented in some settings to provide information as an early warning tool. Duplex quantitative real time RTqPCR assays offer a sensitive and easy-to-use tool to detect, discriminate, and estimate relative proportions of SARS-CoV-2 variants containing VOC-specific signature mutations from variants lacking it, using allelic discrimination probes. We developed seven multiplexed RTqPCR assays that can detect Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Omicron (B.1.1.529), Omicron BA.1 (B.1.1.529.1) and Omicron BA.2 (B.1.1.529.2) VOCs by targeting 21765_21770DelTACATG, 22281_22289DelCTTTACTTG, 28262_28263InsAACA, 22029_22034DelAGTTCA, 28362_28370DelGAGAACGCA, 22121InsGAGCCAGAA, and 21633_21641DelTACCCCCTG deletions/insertions in their genomes, respectively. Alpha, Beta, Delta, Omicron BA.1 and Omicron BA.2 markers are mapped to the S gene (residues 69/70, 241/243, 157/158, 214, and 25/27 respectively), Gamma insertion is located between the end of ORF8 and the beginning of N gene, and Omicron (B.1.1.529) deletion is mapped to the N gene (residues 31/33). The duplex RTqPCR assay targeting 21765-21770DelTACATG mutation affecting residues HV69/70 has been previously used to estimate the relative proportion of Alpha VOC (Carcereny et al., 2021), and is also suitable to estimate the proportions of Omicron BA.4 and BA.5. All duplex RTqPCR assays targeting signature mutations of VOCs may be used as a complementary tool to NGS for rapid variant tracking and surveillance in wastewater-based epidemiology.

Introduction

Reagents

- One Step PrimeScript™ RT-PCR Kit (Perfect Real Time) (RR064A, Takara)
- Twist Synthetic SARS-CoV-2 RNA Control 2 (MN908947.3) (102024, Twist Bioscience)
- Twist Synthetic SARS-CoV-2 RNA Control 16 (EPI_ISL_678597) (104043, Twist Bioscience)
- Twist Synthetic SARS-CoV-2 RNA Control 17 (EPI_ISL_792683) (104044, Twist Bioscience)
- Twist Synthetic SARS-CoV-2 RNA Control 23 (EPI_ISL_1544014) (104533, Twist Bioscience)
- Twist Synthetic SARS-CoV-2 RNA Control 48 (EPI_ISL_6841980) (105204, Twist Bioscience)
- Twist Synthetic SARS-CoV-2 RNA Control 50 (EPI_ISL_7190366) (105345, Twist Bioscience)
- Nuclease-free water

- Primers/Probes (**Table 1**)

Equipment

- Bio-Rad CFX96 touch real-time PCR detection system

Procedure

- Composition of duplex RTqPCR mastermixes (final volume of RTqPCR reaction of 20 µl)

Alpha^a, Beta, Delta, Omicron (B.1.1.529), Omicron BA.1 and Omicron BA.2 RTqPCR

2x One Step: 10 µl

Forward primer 10µM: 0.8 µl (400 nm)

Reverse primer 10µM: 0.8 µl (400 nm)

Probe_Variant 10µM: 0.4 µl (200 nm)

Probe_NoVariant 10µM: 0.4 µl (200 nm)

Takara Ex Taq: 0.4 µl

Prime Script Enzyme: 0.4 µl

H₂O PCR: 1.8 µl

Extracted RNA: 5 µl

^a21765_21770DeITACATG (deletion 69/70) is also a marker for Omicron BA.4 and BA.5

Gamma RTqPCR

2x One Step: 10 µl

Forward primer 10µM: 0.8 µl (400 nm)

Reverse primer 10µM: 0.8 µl (400 nm)

Probe_Gamma 10µM: 0.4 µl (200 nm)

Probe_NoGamma1 10 μ M: 0.2 μ l (100 nm)

Probe_NoGamma2 10 μ M: 0.2 μ l (100 nm)

Takara Ex Taq: 0.4 μ l

Prime Script Enzyme: 0.4 μ l

H₂O PCR: 1.8 μ l

Extracted RNA: 5 μ l

· Controls to be included in each RTqPCR assay:

- 2 wells with 5 μ l of nuclease-free water (RTqPCR negative controls)

- 2 wells with 5 μ l of RNA extraction negative control (RNA extraction negative controls)

- 1 well with 5 μ l of each corresponding synthetic SARS-CoV-2 RNA controls at 1000 copies/ μ L (RTqPCR positive controls)

· For each sample, 2 wells of undiluted RNA and 2 wells of 1/10 dilution are analyzed.

· For quantification of each target, standard curves are constructed, using a minimum of 5 10-fold dilutions and 3 wells for each dilution, using 2 synthetic SARS-CoV-2 RNA controls as reference materials (one corresponding to the variant containing the specific mutation and another corresponding to a variant without the specific mutation).

· Thermocycler conditions (common for all duplex RTqPCR assays except for BA.2)

- 10 min at 50°C (x1)

- 3 min at 95°C (x1)

- 3 sec at 95°C and 30 sec at 60°C (x45)

- Thermocycler conditions for BA.2
- 10 min at 50°C (x1)
- 3 min at 95°C (x1)
- 3 sec at 95°C and 45 sec at 58°C (x45)

INTERPRETATION OF RESULTS

a) RTqPCR controls (see Tables 2 to 8)

b) Samples

- The calculation of each specific SARS-CoV-2 target concentration in genome copies per reaction (gc/rxn) in each well is performed using the standard curve.
- Occurrence of inhibition and calculation of mean viral titers are estimated by comparing concentrations obtained from duplicate wells tested for the two RNA dilutions (undiluted RNA and 1/10 dilution), as described in Carcereny et al., 2021 (1). Mean concentration of samples and standard error are calculated using as many data as possible, taking into consideration the following steps:
 - a. Calculate mean concentration as gc/rxn for each RNA dilution:
 - ai. Data from RNA dilutions containing "No Cq" or $Cq \geq 40$ in both wells are not be used for calculation.
 - aii. When in the analysis of the 2 wells of any RNA dilution, one of the wells has a Cq value < 40 and the other has "No Cq" or $Cq \geq 40$, this last well is assigned a concentration equal to the theoretical limit of detection (LoD) of 1 gc/rxn.
 - b. Calculate mean concentration as gc/rxn for each sample:
 - bi. When the difference between the concentration estimated from undiluted RNA and 1/10 dilution is $< 0.5 \log_{10}$, mean concentration of sample is calculated using data from the 4 wells.

bii. When the difference between the concentration estimated from undiluted RNA and 1/10 dilution is $\geq 0.5 \log_{10}$, inhibition is considered and mean concentration of sample is calculated using data from the 1/10 dilution.

· The proportion of SARS-CoV-2 genomes corresponding to variants containing the specific signature mutation is calculated using the formula (example):

Delta Duplex RTqPCR:

$$\% = \text{gc/rxn (Probe_Delta)} / [\text{gc/rxn (Probe_Delta)} + \text{cg/rxn (Probe_NoDelta)}] \times 100$$

· Cases in which any of the concentrations fall below the limit of quantification (LoQ), the percentage is calculated using the corresponding LoQ. When both concentrations are $< \text{LoQ}$, percentage cannot be estimated.

Troubleshooting

Time Taken

Anticipated Results

References

1. Carcereny A. et al. Monitoring Emergence of the SARS-CoV-2 B.1.1.7 Variant through the Spanish National SARS-CoV-2 Wastewater Surveillance System (VATar COVID-19). *Environ. Sci. & Technol.* 55(17):11756–11766 (2021). Available from: <https://doi.org/10.1021/acs.est.1c03589>

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables.pdf](#)