A One-enzyme RT-qPCR Assay for SARS-CoV-2, and Procedures for Reagent Production

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[Abstract] Given the scale of the ongoing COVID-19 pandemic, the need for reliable, scalable testing, and the likelihood of reagent shortages, especially in resource-poor settings, we have developed an RT-qPCR assay that relies on an alternative to conventional viral reverse transcriptases, a thermostable reverse transcriptase/DNA polymerase (RTX) (Ellefson *et al.*, 2016). Here we show that RTX performs comparably to the other assays sanctioned by the CDC and validated in kit format. We demonstrate two modes of RTX use – (i) dye-based RT-qPCR assays that require only RTX polymerase, and (ii) TaqMan RT-qPCR assays that use a combination of RTX and Taq DNA polymerases (as the RTX exonuclease does not degrade a TaqMan probe). We also provide straightforward recipes for the purification of this alternative reagent RTX. We anticipate that in low resource or point-of-need settings researchers could obtain the available constructs and begin to develop their own assays, within whatever regulatory framework exists for them.

Keywords: Coronavirus, SARS-CoV-2, Quantitative RT-PCR, Reverse transcriptase, TaqMan RT-qPCR, Nucleic acid diagnostics

[Background] While various virus detection methods have been implemented for the detection of SARS-CoV-2 infection, including a variety of molecular diagnostics and immunodiagnostic tests, the Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) remains the primary and most sensitive test for SARS-CoV-2 detection (D'Cruz *et al.*, 2020; Tang *et al.*, 2020). The primacy of RT-qPCR is in large measure because antibody-based tests as well as rapid nucleic acid diagnostic platforms, such as Abbott IDNow, often suffer from poor sensitivity, especially during early infection when viral loads are generally low in patients (Basu *et al.*, 2020; D'Cruz *et al.*, 2020). Given the importance of the early diagnosis in containing COVID-19 outbreak (Peck, 2020), the need for a rapid and scalable RT-qPCR setup is imminent. Unfortunately, there are increasing shortages of a wide variety of reagents necessary to scale RT-qPCR-based tests (Pettit *et al.*, 2020). The main manufacturers of PCR platforms cannot scale-up the production of tests in sufficient quantities to supply resource-poor settings. Even were resource-poor settings to attempt to develop their own solutions, researchers, clinicians, and public health officials often lack the necessary reagents, including enzymes, to develop testing programs (Kavanagh *et al.*, 2020).

Herein, we layout protocols for dye-based and TaqMan probe-based RT-qPCR assays for CDC-designed SARS-CoV-2 N gene tests (https://www.fda.gov/media/134922/download). We detail a one-enzyme RTX-based RT-qPCR protocol, as well as a two-enzyme RTX/Taq DNA polymerase-based RT-qPCR protocol. While the dye-based one enzyme and the TaqMan probe-based two enzyme RT-qPCR

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both exhibit comparable performances in the detection of viral RNA, the TaqMan probe-based assay exhibited lower background. In these, we compare our RTX enzyme preparations, for which we provide a detailed purification protocol, with commercially available reagents.

Our RTX-mediated protocols are as sensitive as those that rely on enzyme combinations or commercial enzymes, and can expedite SARS-CoV-2 tests by reducing the number of kits or key enzymes that must be purchased. These protocols can also form the basis for further assay simplification and democratization via the use of so-called 'cellular reagents': polymerase-overexpressing cells that can be directly added to molecular diagnostics assays without loss of sensitivity or specificity (Bhadra *et al.*, 2018).

Materials and Reagents

A. For RTX polymerase-based RT-qPCR analysis
1. Barrier tips for pipets
2. Tris-HCl pH7.5 and 8.0 (Sigma-Aldrich, catalog number: T1503)
3. $(NH_4)_2SO_4$ (Sigma-Aldrich, catalog number: A4413)
4. KCl (Sigma-Aldrich, catalog number: P3911)
5. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)
6. D-glucose (Sigma-Aldrich, catalog number: 47829)
7. 1% Triton® X-100 (New England Biolabs, B9004S), store at -20 °C
8. Forward PCR primers (Integrated DNA Technologies, **Table 1**) (Note a), store at -20 °C, 100 µM Stock
9. Reverse PCR primers (Integrated DNA Technologies, **Table 1**) (Note a), store at -20 °C, 100 µM Stock
10. TaqMan probes (Integrated DNA Technologies, **Table 1**) (Note a), store at -20 °C
11. 20x EvaGreen solution (Biotium, #31000), store at 4 °C
12. 4 mM deoxyribonucleotides (dNTP) (New England Biolabs, N0446S), store at -20 °C
13. 5 M Betaine (Sigma-Aldrich, B0300), store at 4 °C
14. 20 U/µl SUPERase·In RNase Inhibitor (Thermo Fisher Scientific, AM2694), store at -20 °C
15. 5 U/µl Taq DNA polymerase (New England Biolabs, M0267L), store at -20 °C
16. 0.2 mg/ml RTX polymerase (Note b) (purified by Ellington lab), store at -20 °C
17. Nuclease-free water (store at room temperature)
18. qPCR tubes or plates with optical covers (Note c) (Roche, 04729692001), store at room temperature
19. Cold-block or ice
20. 10x RTX buffer (see Recipes)
21. 10x ThermoPol buffer (see Recipes)
Notes:

a. *Individual primer and probe stocks or pre-made primer-probe mixes for the CDC N1, N2, and N3 assays (**Table 1**) available from Integrated DNA Technologies may be used.*

b. *RTX polymerase with proofreading capability (RTX), and RTX polymerase without proofreading capability (RTX Exo-) have been compared.*

c. *LightCycler 96 real-time PCR machine and 96-well plates with optical plastic film cover designed for use with LightCycler platform were used for these experiments.*

Table 1. CDC TaqMan RT-qPCR primers and probes for SARS-CoV-2[a]

Name	Description	Sequence[b]
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
2019-nCoV_N1-P	2019-nCoV_N1 Probe	5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
2019-nCoV_N2-F	2019-nCoV_N2 Forward Primer	5'-TTA CAA ACA TTG GCC GCA AA-3'
2019-nCoV_N2-R	2019-nCoV_N2 Reverse Primer	5'-GCG CGA CAT TCC GAA GAA-3'
2019-nCoV_N2-P	2019-nCoV_N2 Probe	5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'
2019-nCoV_N3-F	2019-nCoV_N3 Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'
2019-nCoV_N3-R	2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA GCA TTG-3'
2019-nCoV_N3-P	2019-nCoV_N3 Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'

[a]Primer and probe sequences adapted from https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html; last accessed on May 6th, 2020. According to the CDC, oligonucleotide sequences are subject to future changes as the 2019-Novel Coronavirus evolves. Refer to the CDC website for the latest updates.

[b]FAM: 6-carboxyfluorescein; BHQ-1: Black Hole Quencher 1

B. For purification of RTX DNA polymerase

1. Sterile culture tubes and flasks

2. Centrifugation tubes (Nalgene, catalog number: 3115-0050)

3. Sterile syringe filter 0.2 μm (PES, VWR, catalog number: 28145-501)

4. RTX or RTX Exo- polymerase expression plasmid (Addgene, Plasmid #102787 and #102786)

5. Competent *E. coli* T7 RNA polymerase-based protein expression strain, such as BL21 (DE3) (New England Biolabs, C2527H), store at -80 °C

6. Antibiotics such as ampicillin or carbenicillin (Goldbio, catalog number: 4800-94-6)

7. Superior Broth™ (Athena Enzyme Systems™ , catalog number: 0105), store at room temperature

8. 1 M Isopropyl-β-D-thiogalactoside (IPTG) (Sigma Aldrich), store at -20 °C

9. Ni-NTA agarose (Thermo Fisher Scientific, catalog number: 88223), store at 4 °C

10. Disposable protein purification columns (Thermo Fisher Scientific, catalog number: 29924)

11. 20,000 MWCO Dialysis cassette (Thermo Fisher Scientific, catalog number: 66012)

12. HiTrap™ Heparin HP column (GE Healthcare, catalog number: 17-0406-01)

13. Bis-Tris mini protein gel (Thermo Fisher, catalog number: NP0321BOX)

14. EDTA-free protease inhibitor tablet (Thermo Scientific, catalog number: A32965)

Note: All chemicals were molecular biology or analytical grade and purchased from Sigma-Aldrich (see below for catalog number) unless otherwise indicated.

15. NaCl (Sigma-Aldrich, catalog number: S7653)

16. Imidazole (Sigma-Aldrich, catalog number: I5513)

17. Igepal CO-630 (Sigma-Aldrich, catalog number: 542334)

18. $MgSO_4$ (Sigma-Aldrich, catalog number: M7506)

19. HEW Lysozyme (Sigma-Aldrich, catalog number: L6876)

20. Tris (Sigma-Aldrich, catalog number: T1503)

21. DTT (Sigma-Aldrich, catalog number: D9779)

22. KCl (Sigma-Aldrich, catalog number: P3911)

23. Tween-20 (Sigma-Aldrich, catalog number: P9416)

24. Purification buffers for RTX DNA polymerase purification, store at 4 °C (see Recipes)

    Resuspension Buffer

    Equilibration Buffer

    Lysis Buffer

    Wash Buffer

    Elution Buffer

    Heparin Buffer A

    Heparin Buffer B

25. Dialysis buffers for dialysis of RTX DNA polymerase, store at 4 °C (see Recipes)

    Ni-NTA Dialysis Buffer

    Heparin Dialysis Buffer

    Final Dialysis Buffer

Equipment

1. Magnetic stir bar

2. Magnetic stirrer

3. Real-time PCR machine

    Note: LightCycler96 real-time PCR machine from Roche was used for these experiments.

4. Refrigerated shaker incubator (New Brunswick Scientific, model: Innova44)

5. Sonicator (Fisher Scientific, Sonic Dismembrator Model 500)

6. Centrifuge (Beckman Coulter, model: Avanti JXN-26, Fixed angle)

7. Thermomixer (Eppendorf, model: Thermomixer C)

8. Fast protein liquid chromatography (FPLC) machine (GE Healthcare, model: AKTA pure)

9. Fraction collector (GE Healthcare, model: Fraction Collector F9-R)

Software

1. LightCycler96 software (Roche; available for download from https://lifescience.roche.com/en_us/brands/realtime-pcr-overview.html#software)

Procedure

A. Dye-based RT-qPCR reaction set up using only RTX DNA polymerase

1. Set up EvaGreen dye-containing RT-qPCR reactions for CDC SARS-CoV-2 N1, N2, and N3 assays as shown in **Table 2**. The three assays target three different regions within the nucleocapsid phosphoprotein (N) gene near the 3'-end of the ~30 kb viral genome. In particular, N1 primers (**Table 1**) amplify a 72 nt long region from position 28287 to 28358 of the genome (GenBank Accession No. MN985325.1). The N2 primers produce a 67 bp amplicon from the genomic position 29164 to 29230. Meanwhile, the N3 primers amplify a 72 nt long region from genomic position 28681 to 28752. RNA isolation should be performed before RT-qPCR.

Table 2. EvaGreen RT-qPCR reaction mix using only RTX DNA polymerase

	Volume (µl)	Final concentration
RNA sample[a]	5	
5 µM Forward PCR primer	2.5	500 nM
5 µM Reverse PCR primer	2.5	500 nM
20x EvaGreen	1.25	1x
10x RTX buffer	2.5	1x
4 mM dNTP	2.5	400 µM
20 U/µl SUPERase·In RNase Inhibitor	1	0.8 U/µl
RTX or RTX Exo- 0.2 mg/ml	0.5	4 ng/µl
5M Betaine	2.5	0.5 M
Water	4.75	
Total reaction volume	25	

[a]Full length SARS-CoV-2 or MERS-CoV N synthetic RNAs were used in these studies. The RNA copies per reaction (100 to 100,000 copies) are indicated in the relevant figures. Patient saliva is reported to have median SARS-CoV-2 viral loads of 3.3×10^6 copies/ml (To *et al.*, 2020).

2. Assemble all reaction mixes on a cold-block and directly transfer to a real-time PCR machine programmed to cycle through the steps depicted in **Table 3**. Include Melt curve analysis steps after qPCR amplification in order to distinguish target amplicons from spurious products.

Table 3. RTX polymerase-based EvaGreen RT-qPCR cycling conditions

Step	Temperature	Duration	Signal acquisition
1[a]	60 °C	30 min	None
2[a]	95 °C	10 min	None
55 cycles of Steps 3 and 4			
3	95 °C	15 s	None
4[b]	55 °C	30 s	Single
Melt curve analysis in Steps 5, 6, and 7			
5	95 °C	10 s	None
6	65 °C	60 s	None
7	97 °C	1 s	Continuous[c]

[a]Assay time may be reduced by shortening the reverse transcription step (step 1) to 15 min and the following denaturation step (step 2) to 2 min.

[b]Signal acquisition occurs in the FAM (fluorescein) channel during steps 4 and 7.

[c]20 measurements/°C temperature change

B. RTX polymerase-based TaqMan RT-qPCR reaction set up

1. In order to perform TaqMan RT-qPCR we developed a one-pot two enzyme system comprising RTX and Taq DNA polymerases. Set up RTX-based TaqMan RT-qPCR reactions for CDC SARS-CoV-2 N1, N2, and N3 assays as shown in **Table 4**.

2. Assemble all reaction mixes on a cold-block and directly transfer to a real-time PCR machine programmed to cycle through the steps depicted in **Table 5**.

Table 4. RTX polymerase-based TaqMan RT-qPCR reaction mix

	Volume (µl)	Final concentration
RNA sample	5	
6.7 µM Forward PCR primer	1.5	402 nM
6.7 µM Reverse PCR primer	1.5	402 nM
1.7 µM TaqMan probe	1.5	102 nM
10x Assay buffer[a]	2.5	1x
4 mM dNTP	2.5	400 µM
20 U/µl SUPERase·In RNase Inhibitor	1	0.8 U/µl
5 U/µl Taq DNA polymerase	0.5	0.1 U/µl
RTX or RTX Exo- 0.2 mg/ml or water[b]	0.5	4 ng/µl
5M Betaine	2.5	0.5 M
Water	6	
Total reaction volume	25	

[a]Assay buffer: Duplicate TaqMan RT-qPCR reactions were performed either in 1x ThermoPol buffer or in 1x RTX buffer as indicated to determine optimum buffer conditions.

[b]Control reactions lacking RTX polymerase were set up using the same recipe except RTX was replaced with water.

Table 5. RTX polymerase-based TaqMan RT-qPCR cycling conditions

Step 1[a]	60 °C	30 min
Step 2[a]	95 °C	10 min
55 cycles of Steps 3 and 4		
Step 3	95 °C	15 sec
Step 4[b]	55 °C	30 sec

[a]Assay time may be reduced by shortening the reverse transcription step (step 1) to 15 min and the following denaturation step (step 2) to 2 min without compromising results.

[b]Signal acquisition occurs in the FAM (fluorescein) channel during step 4.

C. SARS-CoV-2 RT-qPCR using commercial TaqPath[TM] 1-Step RT-qPCR Master Mix, CG

1. To perform CDC N1, N2, and N3 SARS-CoV-2 TaqMan RT-qPCR assays using commercial assay master mix, set up reactions using TaqPath[TM] 1-Step RT-qPCR Master Mix, CG (Thermo Fisher Scientific, Waltham, MA, USA) as shown in **Table 6**.

2. Assemble all reactions on a cold-block and then incubate at room temperature (25 °C) for 2 min prior to cycling on a real-time PCR machine using the steps indicated in **Table 7**.

Table 6. CDC N1, N2, N3 TaqMan RT-qPCR set up using commercial RT-qPCR master mix

	Volume (µl)
RNA sample	5
6.7 µM Forward PCR primer	1.5
6.7 µM Reverse PCR primer	1.5
1.7 µM TaqMan probe	1.5
4x TaqPath[TM] master mix	5
Water	5.5
Total reaction volume	20

Table 7. TaqPath[TM] TaqMan RT-qPCR cycling conditions

Step 1	50 °C	15 min
Step 2	95 °C	2 min
45 cycles of Steps 3 and 4		
Step 3	95 °C	3 s
Step 4	55 °C	30 s[a]

[a]Signal acquisition occurs in the FAM (fluorescein) channel during step 4.

D. Purification of RTX

1. Transform a T7-based *E. coli* expression strain, such as BL21(DE3), with a RTX expression plasmid that contains a carboxyl terminal six histidine tag for purification.

2. On the next day, pick an individual transformant colony and inoculate it into desired sterile media (10.Superior Broth™) with appropriate antibiotics (*e.g.*, Carbenicillin or Ampicillin 100 µg/ml) and 1-2% glucose Grow this starter culture overnight in shaking incubator at 37 °C.

3. On day three, inoculate 1 L sterile Superior Broth™ (Athena Enzyme Systems™) or other rich media with appropriate antibiotics (*e.g.*, Carbenicillin or Ampicillin 100 µg/ml) in a 4 L Erlenmeyer flask with 250-350 µl of the overnight starter culture. Incubate at 37 °C, 220-250 rpm until the culture reaches OD_{600} of 0.4-0.7 (0.5-0.6 is ideal).

4. When the desired OD_{600} is reached, place flask(s) in 4 °C cold room for 30-45 min and set shaking incubator to 18 °C. Following cold incubation, induce expression of RTX by adding 1 ml of 1 M IPTG to each 1 L of culture. Place flask(s) in the 18 °C shaking incubator for 16-18 h.

5. Following overnight expression, harvest cells by centrifuging cultures at 4 °C, 5,000 x g for 20 min.

6. On the ice, resuspend cell pellet in 30 ml cold **Lysis Buffer**. Transfer the resuspended cell pellet to a small 50 ml beaker with a clean stir bar and place securely in an ice bath. With moderate stirring, sonicate the sample using 40% amplitude and 1 s ON/4 s OFF for 4 min total sonication time.

7. Centrifuge the resulting lysate at 4 °C, 35,000 x g for 30 min. Carefully transfer the supernatant to a clean ultracentrifugation tube, shake in thermomixer at 400 rpm, 85 °C for 10 min, and then place on ice for 10 min.

8. Centrifuge the heat-treated lysate at 4 °C, 35,000 x g for 30 min. Carefully transfer the supernatant to a clean tube and filter the clarified lysate using a 0.2 µm filter.

9. Prepare Ni-NTA agarose columns with a final column volume (CV) of 1 ml using 10 ml Pierce™ disposable columns. Equilibrate column with 20 CV **Equilibration Buffer**.

10. Apply the clarified lysate from 1 L of expressed culture to a previously equilibrated column and collect the flow-through.

11. Wash column with 20 CV **Equilibration Buffer** and collect the flow-through. Wash column with 5 CV **Wash Buffer** and collect flow-through.

12. Elute RTX from a column with 5 ml **Elution Buffer** and transfer to a dialysis cassette with appropriate molecular weight cut-off. Dialyze eluate into 2 L of **Ni-NTA Dialysis Buffer** for 3-4 hours at 4 °C. Then dialyze eluate into a second 2 L of **Ni-NTA Dialysis Buffer** overnight at 4 °C.

13. Pass the dialyzed eluate over an equilibrated 5 ml heparin column (HiTrap™ Heparin HP) and elute along a sodium chloride linear gradient (100 mM to 2 M NaCl) generated from **Heparin buffer A** and **Heparin buffer B** mixed by an FPLC machine. The peak corresponding to RTX or its variants could be expected between 40-60% Buffer B.

14. Collect fraction tubes containing RTX peaks from the fraction collector. Pool the fractions and

dialyze into 2 L **Heparin Dialysis Buffer** for 3-4 h. Then transfer dialysis cassettes into 2 L **Final Dialysis Buffer** overnight where it is expected that the protein sample volume will decrease significantly. Following the final dialysis, recover the protein sample and determine protein concentration before storing at -20 °C. The RTX stock is typically kept concentrated (5-10 mg/ml) in the **Final Dialysis Buffer**. If a lower concentration is required (*e.g.*, 0.2 mg/ml working solution), **Final Dialysis Buffer** was used to dilute the RTX stock to the desired final concentration. An example of RTX purification is shown in **Figure 7**.

15. A similar protocol should work for Taq and other thermostable DNA polymerases, but may require different dialysis and storage buffers.

Data analysis

Analyze RT-qPCR data using LightCycler96 software.

Example data for dye-based RTX-only RT-qPCR

Representative results of SARS-CoV-2 RT-qPCR tests performed using synthetic RNA templates and only RTX or RTX Exo- DNA polymerases are depicted in **Figures 1 and 2**. These results demonstrate that RTX DNA polymerase alone, with or without proofreading capability, can support dye-based RT-qPCR analyses. In our hands, the full-length RTX DNA polymerase was slightly better than the Exo- version, especially in the N3 assay.
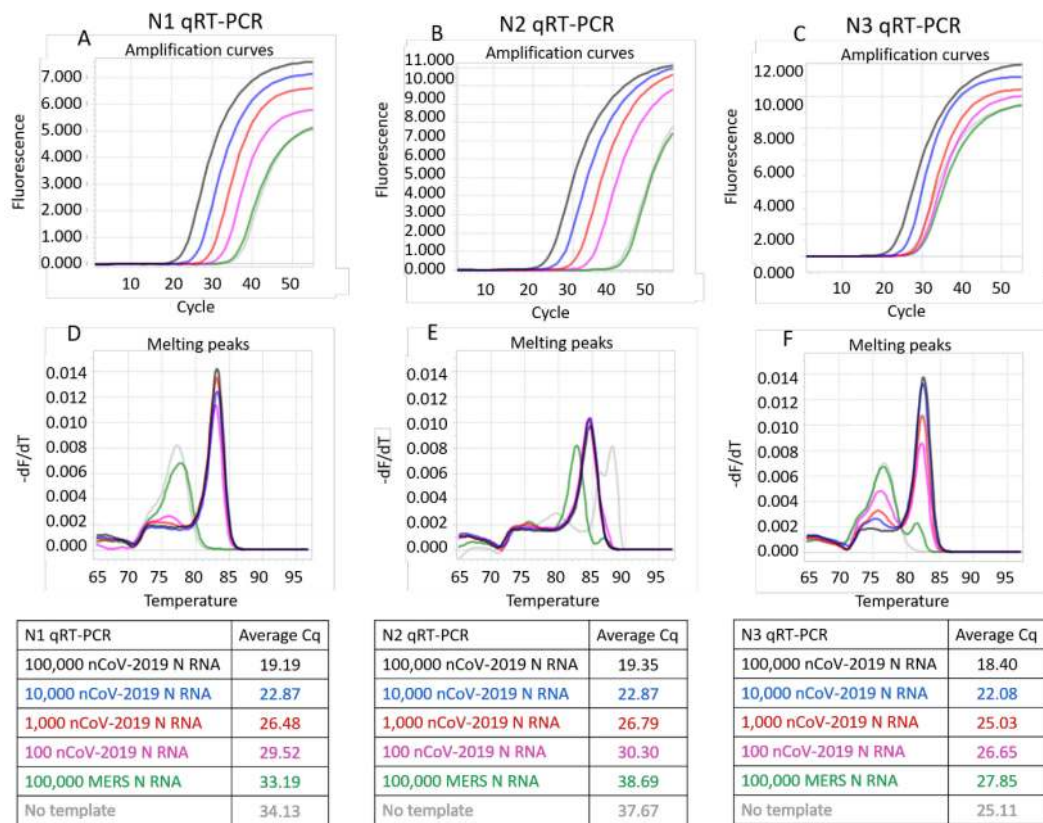
Figure 1. CDC SARS-CoV-2 N1, N2, and N3 RT-qPCR assays performed using indicated copies of synthetic RNA and RTX DNA polymerase. Panels A, B, and C depict N1, N2, and N3 assays measured in real-time using EvaGreen dye. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Panels D, E, and F depict melting peaks of amplicons determined using the 'Tm calling' analysis in the LightCycler96 software. Note that the amplicons observed in negative controls were spurious, as indicated by their distinct melting temperatures compared to target-derived amplicons. Average Cq values of all assays are tabulated.
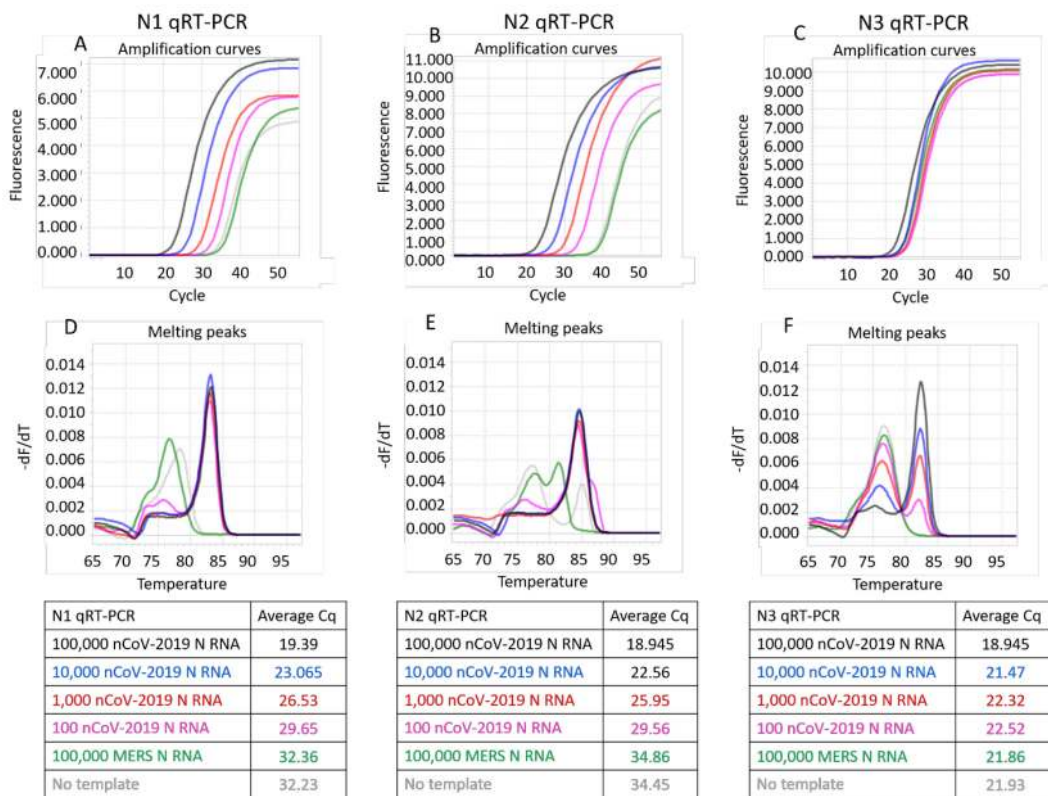
Figure 2. CDC SARS-CoV-2 N1, N2, and N3 RT-qPCR assays performed using indicated copies of synthetic RNA and RTX Exo- DNA polymerase. Panels A, B, and C depict N1, N2, and N3 assays measured in real-time using EvaGreen dye. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Panels D, E, and F depict melting peaks of amplicons determined using the 'Tm calling' analysis in the LightCycler96 software. Average Cq values of all assays are tabulated.

Example data for RTX polymerase-based TaqMan RT-qPCR

Representative results of RTX polymerase-based SARS-CoV-2 TaqMan RT-qPCR tests performed using synthetic RNA templates are depicted in **Figures 3 and 4**. Both RTX and RTX Exo- when combined with Taq DNA polymerase are able to support one-pot TaqMan RT-qPCR assays for all three CDC SARS-CoV-2 assays. Under both buffer conditions tested, 1x ThermoPol versus 1x RTX buffer, RTX polymerase yielded more consistent amplification curves for all three CDC assays compared to RTX Exo- polymerase.

N1 qRT-PCR in ThermoPol	RTX	Taq	RTX Exo-
100,000 nCoV-2019 N RNA	26.19	39.48	26.15
10,000 nCoV-2019 N RNA	29.71	42.74	29.71
1,000 nCoV-2019 N RNA	33.21	--	33.33
100 nCoV-2019 N RNA	36.29	--	36.99
100,000 MERS N RNA	--	--	--
No template	--	--	--

N2 qRT-PCR in ThermoPol	RTX	Taq	RTX Exo-
100,000 nCoV-2019 N RNA	28.81	45.56	--
10,000 nCoV-2019 N RNA	32.47	--	36.63
1,000 nCoV-2019 N RNA	35.78	--	40.48
100 nCoV-2019 N RNA	39.53	--	--
100,000 MERS N RNA	--	--	--
No template	--	--	--

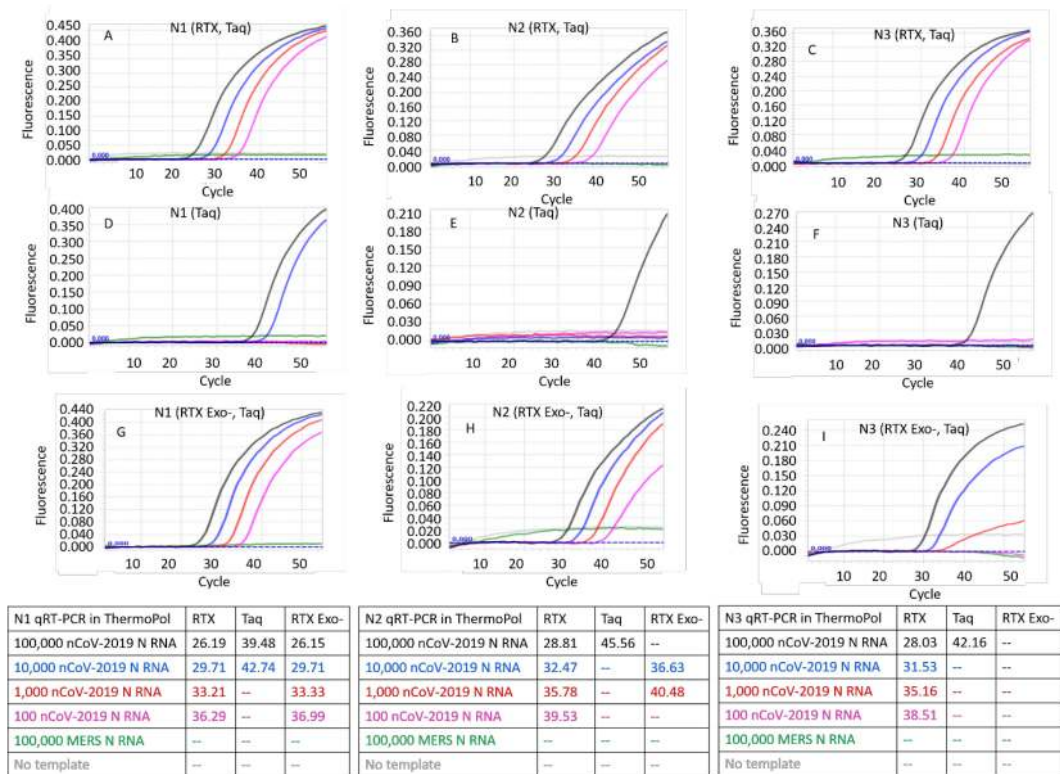| N3 qRT-PCR in ThermoPol	RTX	Taq	RTX Exo-
100,000 nCoV-2019 N RNA	28.03	42.16	--
10,000 nCoV-2019 N RNA	31.53	--	--
1,000 nCoV-2019 N RNA	35.16	--	--
100 nCoV-2019 N RNA	38.51	--	--
100,000 MERS N RNA	--	--	--
No template	--	--	--

Figure 3. CDC SARS-CoV-2 N1, N2, and N3 TaqMan RT-qPCR assays performed in 1X ThermoPol buffer using indicated copies of synthetic RNA and RTX or RTX Exo- and Taq DNA polymerases. Panels A, B, and C depict TaqMan assays containing both RTX and Taq DNA polymerases. Panels D, E, and F depict TaqMan assays containing only Taq DNA polymerase. Panels G, H, and I depict TaqMan assays containing RTX Exo- and Taq DNA polymerases. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Cq values of all assays are tabulated.

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N1 qRT-PCR in RTX buffer	RTX	RTX Exo-
100,000 nCoV-2019 N RNA	22.18	22.18
10,000 nCoV-2019 N RNA	25.54	25.64
1,000 nCoV-2019 N RNA	28.88	28.92
100 nCoV-2019 N RNA	32.06	32.14
100,000 MERS N RNA	--	--
No template	--	--

N2 qRT-PCR in RTX buffer	RTX	RTX Exo-
100,000 nCoV-2019 N RNA	23.77	23.27
10,000 nCoV-2019 N RNA	27.46	26.81
1,000 nCoV-2019 N RNA	31.09	30.43
100 nCoV-2019 N RNA	34.97	34.36
100,000 MERS N RNA	--	--
No template	--	--

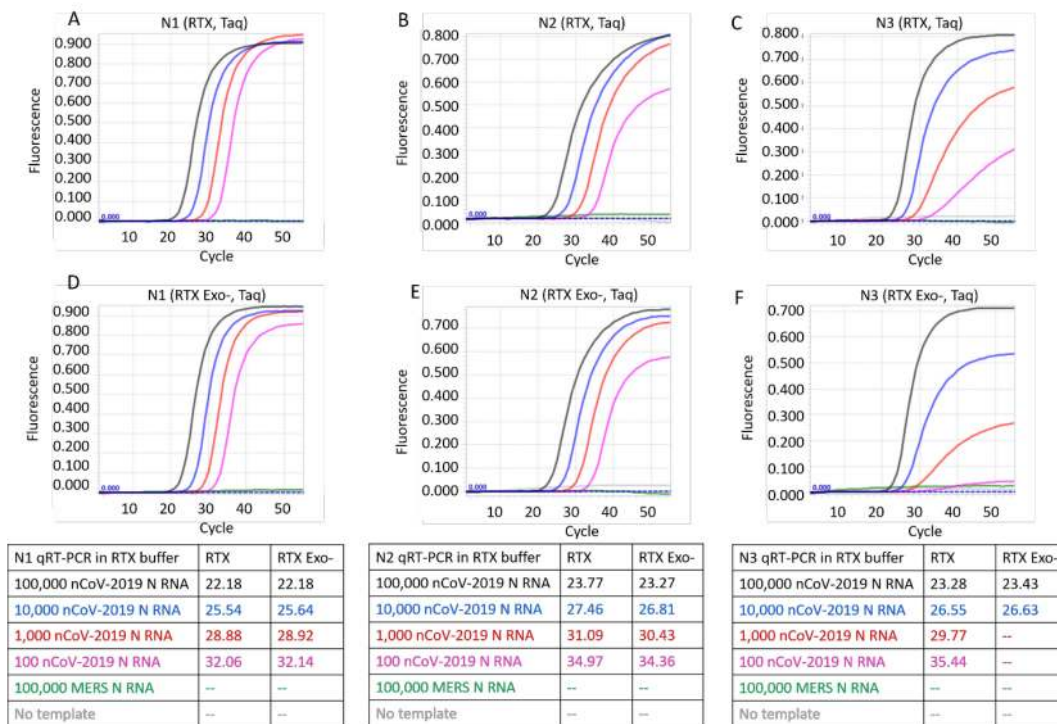| N3 qRT-PCR in RTX buffer	RTX	RTX Exo-
100,000 nCoV-2019 N RNA	23.28	23.43
10,000 nCoV-2019 N RNA	26.55	26.63
1,000 nCoV-2019 N RNA	29.77	--
100 nCoV-2019 N RNA	35.44	--
100,000 MERS N RNA	--	--
No template	--	--

Figure 4. CDC SARS-CoV-2 N1, N2, and N3 TaqMan RT-qPCR assays performed in 1X RTX buffer using indicated copies of synthetic RNA and RTX or RTX Exo- and Taq DNA polymerases. Panels A, B, and C depict TaqMan assays containing RTX and Taq DNA polymerases. Panels D, E, and F depict TaqMan assays containing RTX Exo- and Taq DNA polymerases. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Cq values of all assays are tabulated.

Example data for TaqPath^TM TaqMan RT-qPCR

Figure 5 depicts typical results from CDC N1, N2, and N3 TaqMan qRT-PCR reactions performed using synthetic RNA and TaqPath^TM commercial RT-qPCR mastermix.
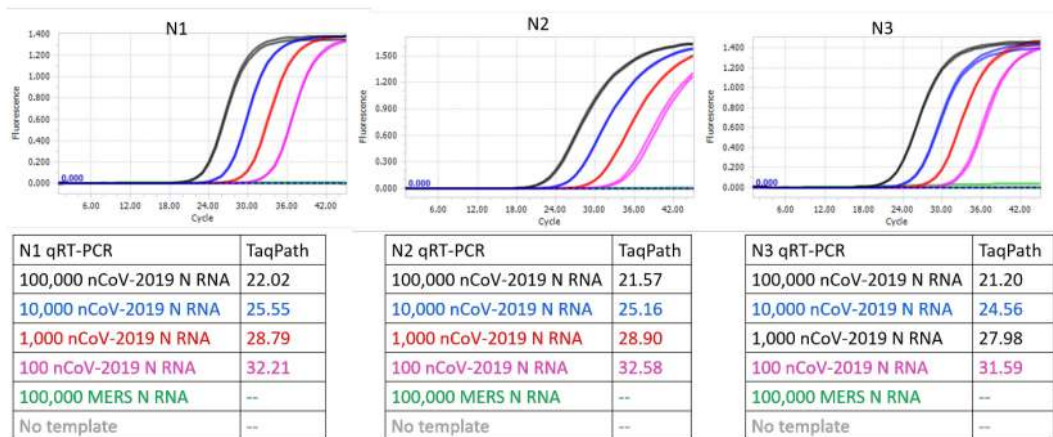
N1 qRT-PCR	TaqPath
100,000 nCoV-2019 N RNA	22.02
10,000 nCoV-2019 N RNA	25.55
1,000 nCoV-2019 N RNA	28.79
100 nCoV-2019 N RNA	32.21
100,000 MERS N RNA	--
No template	--

N2 qRT-PCR	TaqPath
100,000 nCoV-2019 N RNA	21.57
10,000 nCoV-2019 N RNA	25.16
1,000 nCoV-2019 N RNA	28.90
100 nCoV-2019 N RNA	32.58
100,000 MERS N RNA	--
No template	--

N3 qRT-PCR	TaqPath
100,000 nCoV-2019 N RNA	21.20
10,000 nCoV-2019 N RNA	24.56
1,000 nCoV-2019 N RNA	27.98
100 nCoV-2019 N RNA	31.59
100,000 MERS N RNA	--
No template	--

Figure 5. CDC SARS-CoV-2 N1, N2, and N3 TaqMan RT-qPCR assays performed using indicated copies of synthetic RNA and TaqPath^{TM} commercial RT-qPCR mastermix. Amplification curves from reactions containing 100,000 (black traces), 10,000 (blue traces), 1,000 (red traces), and 100 (pink traces) copies of SARS-CoV-2 synthetic N RNA are depicted. Negative control reactions either contained no templates (gray traces) or contained 100,000 copies of synthetic N RNA from MERS-CoV (green traces). Cq values of all assays are tabulated.

The results with either a viral RT (in TaqPath^{TM} commercial mastermix) or RTX are summarized in **Figure 6**. As can be seen, RTX-based TaqMan assays are of comparable sensitivity and specificity to the gold standard TaqPath assay. While all three RTX-based TaqMan assays performed in ThermoPol buffer consistently yielded Cq values, these were somewhat delayed compared to Cq values obtained with the commercial TaqPath^{TM} Master Mix. In contrast, RTX-based TaqMan assays when executed in RTX buffer yielded Cq values that were closer to Cq values obtained with the commercial mastermix.

N1 qRT-PCR	RTX+Taq in ThermoPol	RTX+Taq in RTX buffer	TaqPath™
100,000 nCoV-2019 N RNA	26.19	22.18	22.02
10,000 nCoV-2019 N RNA	29.71	25.54	25.55
1,000 nCoV-2019 N RNA	33.21	28.88	28.79
100 nCoV-2019 N RNA	36.29	32.06	32.21
100,000 MERS N RNA	--		--
No template	--		--

N2 qRT-PCR	RTX+Taq in ThermoPol	RTX+Taq in RTX buffer	TaqPath™
100,000 nCoV-2019 N RNA	28.81	23.77	21.57
10,000 nCoV-2019 N RNA	32.47	27.46	25.16
1,000 nCoV-2019 N RNA	35.78	31.09	28.90
100 nCoV-2019 N RNA	39.53	34.97	32.58
100,000 MERS N RNA	--		--
No template	--		--

N3 qRT-PCR	RTX+Taq in ThermoPol	RTX+Taq in RTX buffer	TaqPath™
100,000 nCoV-2019 N RNA	28.03	23.28	21.20
10,000 nCoV-2019 N RNA	31.53	26.55	24.56
1,000 nCoV-2019 N RNA	35.16	29.77	27.98
100 nCoV-2019 N RNA	38.51	35.44	31.59
100,000 MERS N RNA	--		--
No template	--		--

Figure 6. Comparison of Cq values of RTX-based and TaqPath-based SARS-CoV-2 TaqMan RT-qPCR assays
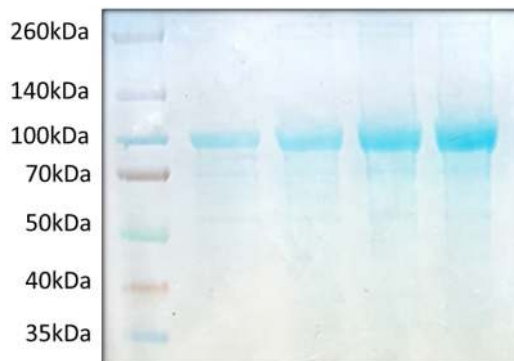

Figure 7. An example of RTX purification analyzed by SDS-PAGE. A total of ten to forty micrograms of purified RTX was developed on a NuPAGE™ 4 to 12%, Bis-Tris mini protein gel. RTX bands appear near the 100kDa size marker. 1st lane: 10 μg, 2nd lane: 20 μg, 3rd lane: 30 μg, 4th lane: 40 μg.

Notes

Overall, RTX performs well either as a single enzyme for RT-qPCR with intercalating dyes, such as EvaGreen, or as the RT component of a TaqMan based assay. At the lowest RNA concentrations examined (100 copies), the gold standard TaqPath assay generally gave a signal at a Cq value of ca. 32. The more robust full-length RTX on its own actually performed slightly better, with Cq values typically from 29-30 (although the N3 primer set gives a higher signal and higher background). When

used as a substitute for a viral RT, RTX is slightly less sensitive overall, with Cq values typically from 32-35.

By obtaining a RTX expression vector and purifying the thermostable reverse transcriptase it should prove possible to carry out RT-qPCR reactions with a sensitivity similar to that already observed for approved kits, with few or no false positive results. Plasmids and sequence information for 6xHis tagged   RTX and RTX Exo- protein expression are available from Addgene (pET_RTX: https://www.addgene.org/102787/ and pET_RTX_(exo-): https://www.addgene.org/102786/), or can be obtained via https://reclone.org/.

Recipes

1. 10x RTX buffer
   600 mM Tris-HCl
   250 mM $(NH_4)_2SO_4$
   100 mM KCl
   20 mM $MgSO_4$
   pH 8.4, 25 °C
2. 10x ThermoPol buffer
   200 mM Tris-HCl
   100 mM $(NH_4)_2SO_4$
   100 mM KCl
   20 mM $MgSO_4$
   1% Triton® X-100
   pH 8.8, 25 °C

Buffers for RTX DNA polymerase purification
3. Resuspension Buffer
   50 mM Phosphate Buffer, pH 7.5
   300 mM NaCl
   20 mM imidazole
   0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)
   5 mM $MgSO_4$
4. Equilibration Buffer
   50 mM Phosphate Buffer, pH 7.5
   300 mM NaCl 20 mM imidazole
5. Lysis Buffer
   30 ml Resuspension Buffer
   1x EDTA-free protease inhibitor tablet
   30-60 mg HEW Lysozyme

6. Wash Buffer

   50 mM Phosphate Buffer, pH 7.5

   300 mM NaCl

   50 mM imidazole

7. Elution Buffer

   50 mM Phosphate Buffer, pH 7.5

   300 mM NaCl

   250 mM imidazole

8. Heparin Buffer A

   40 mM Tris-HCl, pH 7.5

   100 mM NaCl

   0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

9. Heparin Buffer B

   40 mM Tris-HCl, pH 7.5

   2 M NaCl

   0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

Buffers for dialysis of RTX DNA polymerase

10. Ni-NTA Dialysis Buffer (2 L x2)

    40 mM Tris-HCl, pH 7.5

    100 mM NaCl

    10 mM beta-mercapto ethanol (BME) or 1 mM 1,4-Dithiothreitol (DTT)

    0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

11. Heparin Dialysis Buffer (2 L)

    50 mM Tris-HCl, pH 8.0

    50 mM KCl

    0.1% Tween-20

    0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

12. Final Dialysis Buffer (1 L)

    50% Glycerol

    50 mM Tris-HCl, pH 8.0

    50 mM KCl

    0.1% Tween-20

    0.1% Igepal CO-630 (non-toxic Non-idet P40 equivalent)

    1 mM DTT

Acknowledgments

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Competing interests

The Board of Regents of The University of Texas has licensed IP covering RTX to Promega Corporation.

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