Simple, Inexpensive RNA Isolation and One-Step RT-qPCR Methods for SARS-CoV-2 Detection and General Use

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The most common method for RNA detection involves reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR) analysis. Commercial one-step master mixes—which include both a reverse transcriptase and a thermostable polymerase and thus allow performing both the RT and qPCR steps consecutively in a sealed well—are key reagents for SARS-CoV-2 diagnostic testing; yet, these are typically expensive and have been affected by supply shortages in periods of high demand. As an alternative, we describe here how to express and purify *Taq* polymerase and M-MLV reverse transcriptase and assemble a homemade one-step RT-qPCR master mix. This mix can be easily assembled from scratch in any laboratory equipped for protein purification. We also describe two simple alternative methods to prepare clinical swab samples for SARS-CoV-2 RNA detection by RT-qPCR: heat-inactivation for direct addition, and concentration of RNA by isopropanol precipitation. Finally, we describe how to perform RT-qPCR using the homemade master mix, how to prepare in vitro−transcribed RNA standards, and how to use a fluorescence imager for endpoint detection of RT-PCR amplification in the absence of a qPCR machine In addition to being useful for diagnostics, these versatile protocols may be adapted for nucleic acid quantification in basic research.

Keywords: direct RT-qPCR • M-MLV reverse transcriptase purification • one-step RT-qPCR master mix • SARS-CoV-2 testing • *Taq* polymerase purification

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INTRODUCTION

Detection of viral RNA in patient samples provides a sensitive means of diagnostic testing for SARS-CoV-2 infection. The most common method for RNA detection involves reverse transcription (RT) of sense-strand viral RNA followed by the quantitative polymerase chain reaction (RT-qPCR), also referred to as real-time RT-PCR (rRT-PCR) (Centers for Disease Control and Prevention, 2020). The essentials of RT-qPCR have been extensively reviewed elsewhere (see Current Protocols article: Fraga, Meulia, & Fenster, 2008). "One-step" RT-qPCR master mixes, which include both a reverse transcriptase and a thermostable polymerase, allow both the RT and PCR steps to occur in a single tube, reducing the required number of pipetting steps and consumables (Wong & Medrano, 2005). The composition of such qPCR master mixes and the parameters for thermal cycling must be carefully optimized so that both reverse transcription and DNA amplification occur efficiently. In the initial reverse-transcription step, incubation at ∼50°C partially denatures RNA secondary structure to facilitate the annealing of the reverse PCR primer and extension by a reverse transcriptase, such as Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Fig. 1A). Exponential PCR amplification then follows during subsequent cycling between a higher temperature (to denature nucleic acid duplexes) and a lower temperature (to permit primer annealing and extension) by a thermostable DNA polymerase such as *Thermus aquaticus* (*Taq*) DNA polymerase (Fig. 1A). Amplification may be detected by including in the reaction an intercalating dye such as SYBR Green, whose fluorescence increases upon binding to double-stranded products (Higuchi, Dollinger, Walsh, & Griffith, 1992; Higuchi, Fockler, Dollinger, & Watson, 1993; Morrison, Weis, & Wittwer, 1998; Wittwer et al., 1997; also see Fig. 1). Alternatively, amplification may be detected by including a third oligonucleotide, known as a hydrolysis probe (e.g., "TaqMan" probe), labeled with a fluorophore-quencher pair, which anneals inside the amplicon (Bustin et al., 2009; Heid, Stevens, Livak, & Williams, 1996; Wittwer et al., 1997; Wong & Medrano, 2005; Fig. 1). During amplification of the target, the TaqMan probe is degraded by the 5′-3′ exonuclease activity of the polymerase, de-quenching the fluorescence of the fluorophore. Repeated cycles of amplification lead to degradation of the probe, resulting in increased fluorescence. TaqMan detection is, therefore, more specific than dye-based detection, because an increase in fluorescence occurs only upon binding of the probe to a specific sequence and subsequent de-quenching, while no signal is produced by amplification of off-target sequences that do not bind the probe. Multiple targets may be quantified simultaneously by including multiple TaqMan probes, labeled with different fluorophores, in the same reaction (Grace et al., 2003). Multiplexing may also be achieved in dye-based reactions by using melting curve analysis to distinguish amplicons of different sizes (Beuret, 2004; Wan et al., 2016).

Quantitative PCR makes it possible to quantify the amount of a DNA (or cDNA) target in a sample based on the number of PCR cycles required to detect amplification (Higuchi et al., 1993). This may be determined based on the "threshold cycle" (Ct), that is, the cycle at which the fluorescence intensity crosses a user-specified threshold. More generally, a quantification cycle (Cq) may be defined using threshold-independent features of the amplification curve, such as the peak of the second derivative (Bustin et al., 2009; Rasmussen, 2001). Comparison of the Cq values of test samples with those of

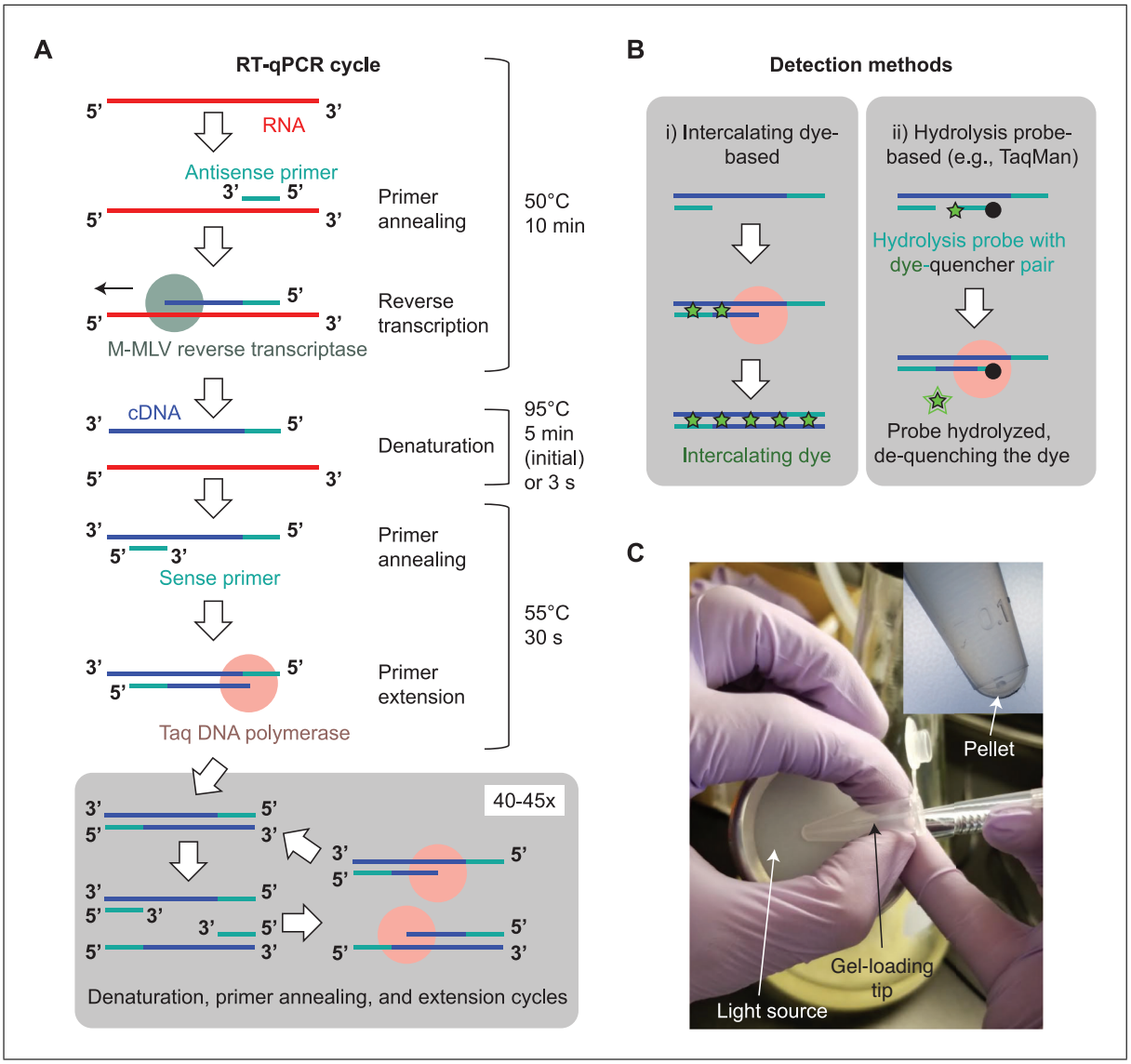

Figure 1 (**A**) Schematic of RT-PCR. An antisense primer anneals to the RNA, priming reverse transcription by M-MLV reverse transcriptase (green circle). The resulting complementary DNA (cDNA) is amplified in a polymerase chain reaction, which involves 40-45 rounds of denaturation, primer annealing, and extension by *Taq* DNA polymerase (pink circle). (**B**) Real-time fluorescence readouts for qPCR: (i) An intercalating dye becomes more fluorescent upon binding double-stranded DNA products of PCR. (ii) Cleavage of a hydrolysis probe (e.g., TaqMan) oligonucleotide by the 5′-3′ exonuclease activity of *Taq* DNA polymerase releases a fluorophore from a quencher, increasing its fluorescence. (**C**) Aspiration of residual 75% ethanol from an RNA pellet using a gel-loading tip. The tip is held close to the bottom of the tube without touching the pellet. Holding the tube against a light (turned off for clarity in this photograph) makes it easier to see the pellet. Inset: Image of an RNA and linear polyacrylamide pellet after the 75% ethanol wash step. Pellets from swab samples are sometimes larger than the pellet shown in this image, likely due to the abundance of human nucleic acids in the sample.

known-concentration standards (see Support Protocol) allows absolute quantification of the target. Because PCR amplifies DNA exponentially, Cq values are linearly proportional (within some finite range) to the logarithm of the starting nucleic acid concentration. Thus, qPCR may be used to quantify nucleic acid concentrations over several orders of magnitude.

The composition of commercial one-step RT-qPCR master mixes is proprietary, making labs and clinical testing centers dependent on black-box products that may cost hundreds of dollars per milliliter. Self-sufficient production of RT-qPCR reagents by academic laboratories could guard against supply shortages and provide options that are cheaper

Table 1 Options for SARS-CoV-2 RT-PCR Testing Workflow[a]

Step	Options
Sample collection	Collect swab sample in: 1) Proteinase K solution (Basic Protocol 2) 2) Commercial buffered saline-based solution (e.g., UTM or V-C-M) 3) Nucleic acid preservation solution with protein denaturant (e.g., DNA/RNA Shield (Zymo Research) or 4 M guanidinium)[a]
RNA extraction	1) Direct addition to RT-qPCR after proteinase K treatment and heat-inactivation (Basic Protocol 2) 2) Isopropanol precipitation (Alternate Protocol 1) 3) Commercial RNA purification kit
RNA amplification	1) RT-PCR with BEARmix (Basic Protocol 3) 2) RT-PCR with commercial master mix
Detection	1) Real-time fluorescence detection using a qPCR machine (Basic Protocol 3) 2) Endpoint fluorescence detection using a fluorescence imager (Alternate Protocol 2)

[a]Collection in a denaturant solution is incompatible with direct addition to RT-qPCR, and samples must be purified using either a commercial RNA purification kit or isopropanol precipitation (Alternate Protocol 1).

than those based on commercial reagents. In addition to being useful for diagnostics, protocols to produce RT-qPCR reagents could be adapted for general-purpose RNA and DNA quantification, providing substantial cost savings for basic research.

In Basic Protocol 1, we provide a detailed protocol for purifying *Taq* polymerase and M-MLV reverse transcriptase enzymes and assembling them into a homemade RT-qPCR master mix, the Basic Economical Amplification Reaction mix (BEARmix). Next, we describe simple methods for extraction of SARS-CoV-2 genomic RNA from patient swab samples by heat inactivation (Basic Protocol 2) and isopropanol precipitation (Alternate Protocol 1), which are compatible with downstream RT-qPCR detection and circumvent costly, time-consuming, and potentially scarce commercial RNA purification kits. Finally, we describe how to detect SARS-CoV-2 RNA by RT-qPCR with the homemade master mix, using in vitro−transcribed control RNAs as a concentration standard (Basic Protocol 3 and Support Protocol). We also provide a simple alternative protocol for endpoint detection of RT-PCR amplification in the absence of a qPCR machine (Alternate Protocol 2). These protocols can be used together or in combination with commercial options (Table 1).

STRATEGIC PLANNING

For SARS-CoV-2 testing, multiple options are available at the sample collection, RNA extraction, and RT-qPCR steps, and these may be mixed and matched depending on reagent availability, budget, and other constraints. The various alternatives at each step are presented in Table 1. Note that samples collected in DNA/RNA Shield (Zymo) or guanidinium-based denaturing buffers are not compatible with direct RT-qPCR, and RNA must, instead, be purified using either a commercial RNA purification kit or isopropanol precipitation (Alternate Protocol 1).

If a solution of proteinase K (PK) is used for sample collection (Basic Protocol 2), then it is a good idea to verify the stability of the enzyme under the storage conditions used by mixing samples of stored PK with bovine serum albumin and checking for efficient protein degradation by SDS-PAGE (Graham et al., 2021). It has been shown that PK diluted in water is stable for at least 3 weeks at room temperature (Graham et al., 2021).

The protocols for purification of *Taq* DNA polymerase and M-MLV reverse transcriptase enzymes involve the use of a fast performance liquid chromatography (FPLC) system, and assume that the user has familiarity with its operation.

PREPARATION OF A ONE-STEP RT-qPCR MASTER MIX USING HOMEMADE ENZYMES

BASIC PROTOCOL 1

This section details how to prepare a one-step RT-qPCR master mix using homemade enzymes. First, this protocol describes how to express His-tagged *Taq* DNA polymerase in *E. coli* and purify the enzyme using a combination of nickel-NTA and heparin chromatography. *Taq* DNA polymerase is well expressed in *E. coli* and easy to purify, with a yield of 6-8 mg/L of culture. Because the enzyme is thermostable, an initial step of high-temperature incubation of the bacterial lysate can be used to denature most *E. coli* proteins, leaving soluble *Taq* polymerase in the supernatant. The purified *Taq* polymerase may optionally be converted into a "hot-start" version using formaldehyde crosslinking (Buratowski, 2015). This inactivates the enzyme, which can be reactivated by incubating at 95°C to reverse the crosslinks. While homemade hot-start *Taq* polymerase permits reaction setup at room temperature, it has the drawback of lower amplification efficiency. This tradeoff should be considered when deciding whether to produce regular or hot-start enzyme. We describe both protocols below.

Second, we describe how to express and purify His-tagged M-MLV reverse transcriptase. Like *Taq* DNA polymerase, this enzyme is well expressed in *E. coli* and straightforward to purify with a yield of several milligrams per liter. The details of this protocol are very similar to those of the *Taq* DNA polymerase protocol. Finally, we describe how to prepare the two components of the Basic Economical Amplification Reaction mix (BEARmix; i.e., one-step RT-qPCR master mix): a 4× buffer and an enzyme mix containing the two enzymes purified, *Taq* polymerase and M-MLV reverse transcriptase.

It is crucial when preparing these components to avoid contamination with in vitro−transcribed RNA or amplified products of previous RT-qPCR reactions. This can be prevented by working in an area of the lab separate from where concentrated in vitro−transcribed RNAs or PCR products have been handled, preferably in a dedicated laminar flow hood such as a biosafety hood or enclosed PCR workstation, and by using a dedicated set of pipettes and filter tips, if possible.

Materials

Plasmid pET-28a_6H-TAQ_E602D (Addgene, #166944)
BL21 (DE3) or Rosetta (DE3) competent *E. coli*, or other T7-based protein expression strain
Luria-Bertani (LB) agar plates (see recipe)
Kanamycin, 50 mg/ml (1000×) stock solution
Chloramphenicol, 34 mg/ml (2000×) stock solution in ethanol [if using Rosetta (DE3) cells]
Luria-Bertani (LB) liquid medium (see recipe)
1 M isopropyl β-D-1-thiogalactopyranoside (IPTG; GoldBio, 12481C25) in water
1× phosphate buffered saline (PBS; see recipe)
Liquid nitrogen
Taq lysis buffer (see recipe)

20 mg/ml lysozyme (Sigma, L6876-10G) in 1× PBS (see recipe)
Glycerol
2 M imidazole (Sigma, 12399-500G), adjusted to pH 8 using hydrochloric acid
β-mercaptoethanol (Sigma, 444203-250ML)
Ni-NTA agarose (Qiagen, 30230)
Taq buffer W500 (see recipe)
Taq buffer W100 (see recipe)
Taq elution buffer (see recipe)
SDS-PAGE gels and running buffer (see Current Protocols article: Gallagher, 2012, for details)
4× SDS-PAGE sample buffer (see recipe)
Coomassie blue stain or rapid protein gel stain, such as InstantBlue (Expedeon, ISB1L)
Heparin dialysis buffer (see recipe)
Heparin buffer A (see recipe for heparin buffer A/B)
Heparin buffer B (see recipe for heparin buffer A/B)
Taq storage buffer (see recipe)
Taq crosslinking buffer (see recipe)
16% formaldehyde (Polysciences, Inc., 18814-10)
2 M Tris·HCl, pH 8 (see, e.g., Moore, 1996)
Taq post-crosslinking dialysis buffer (see recipe)
Plasmid pET-28a_6H-MMLV_RT_D524N-6H (Addgene, #166945)
M-MLV RT lysis buffer (see recipe)
5 M NaCl
M-MLV RT buffer W500 (see recipe)
M-MLV RT elution buffer (see recipe)
SP buffer A (see recipe)
SP buffer B (see recipe)
M-MLV RT storage buffer (see recipe)
1 M Tris·HCl, pH 8.4 (see, e.g., Moore, 1996)
2 M KCl
1 M $MgCl_2$
D-(+)-trehalose dihydrate (Sigma, T0167)
1 M dithiothreitol (DTT; GoldBio, DTT50)
0.5 M ethylenediaminetetraacetic acid (EDTA), pH 7.5 (see, e.g., Moore, 1996)
10 mM dNTP solution (New England Biolabs, N0447L)
DNase/RNase-free water

Optional: Laminar flow hood (e.g., biosafety cabinet or enclosed PCR workstation)
Shaker set to 37°C
5-ml plastic culture tubes with caps (Falcon, 352063)
Four 2.8-liter Fernbach flasks
1-liter centrifuge bottles
Spectrophotometer
15- and 50-ml conical tubes (e.g., Corning Falcon)
Refrigerated high-speed centrifuge with rotors for 1-liter bottles and 30-ml tubes
Rotator
Heat block set to 75°-80°C with wells large enough to accommodate 5-ml plastic culture tubes
30-ml screw-top centrifuge tubes
250-ml glass bottle
Empty glass or plastic column for gravity flow chromatography (e.g., Qiagen, 34964)
Support stand and clamp for holding column

Magnetic stir plate and stirrer
Vacuum line (optional)
Dialysis tubing, MWCO 12-14 kDa (Spectra/Por 2, 132678)
Dialysis clips (Spectrum, 142153)
Heparin affinity column compatible with the FPLC system, e.g., 1-ml HiTrap Heparin HP affinity column (Cytiva, # 17040701)
Fast performance liquid chromatography (FPLC) system, e.g., ÄKTA pure (Cytiva)
4°C microcentrifuge
1.7-ml microcentrifuge tubes
250-ml metal beaker
Tip sonicator (Branson Sonifier 450 or equivalent)
250-ml glass beaker
1-ml HiTrap SP HP column (Cytiva, 29-0513-24)

Additional reagents and equipment for introduction of DNA into cells (see Current Protocols article: Seidman, Struhl, Sheen, & Jessen, 2001), SDS-PAGE (see Current Protocols article: Gallagher, 2012), staining gels (see Current Protocols article: Sasse & Gallagher, 2009), and dialysis (see Current Protocols article: Zumstein, 2001)

Purification of Taq DNA polymerase and preparation of hot-start Taq polymerase

Protein expression

1. Transform expression plasmid pET-28a_6H-TAQ_E602D using your method of choice (see Current Protocols article: Seidman et al., 2001) into competent cells of an *E. coli* strain optimized for T7-based protein expression, such as BL21 (DE3) or Rosetta (DE3). Plate on LB agar supplemented with 50 µg/ml kanamycin [as well as 17 µg/ml chloramphenicol for Rosetta (DE3)], and allow to grow overnight at 37°C.

2. Inoculate four 5-ml starter cultures of LB liquid medium supplemented with 50 µg/ml kanamycin [as well as 17 µg/ml chloramphenicol for Rosetta (DE3)] each with a single colony from the plate. Grow overnight in a 37°C shaker.

3. Pool the four 5-ml starter cultures and use 4 ml to inoculate 1 liter of LB liquid medium + 50 µg/ml kanamycin [+ 17 µg/ml chloramphenicol for Rosetta (DE3)] in each of four 2.8-liter Fernbach flasks.

4. Grow in a 37°C shaker at 200 rpm until the optical density at 600 nm reaches 0.8-1.0.

   This typically takes 2-3 hr.

5. Add IPTG to a final concentration of 1 mM (1 ml of a 1 M stock per liter of culture), and grow for 2 hr at 37°C.

6. Pellet cells by centrifugation for 10 min at 4000 × *g*, room temperature. Resuspend the pellets in 20 ml of 1× PBS each, pool the four resuspended pellets for a total of 80 ml, and transfer 40 ml of the resuspended cells to each of two 50-ml conical tubes. Pellet again by centrifugation for 10 min at 4000 × *g*, room temperature, and flash-freeze pellets in liquid nitrogen in the 50-ml conical tubes. Store at −80°C until ready for the next step.

   The cell pellets are likely stable for months or years when stored at −80°C.

Protein purification by nickel affinity

Frozen pellets may be thawed at room temperature.

7. Resuspend two cell pellets (each coming from the equivalent of 2 L of culture) in 40 ml of *Taq* lysis buffer each.

8. Add 2 ml of a 20 mg/ml lysozyme solution to each tube (i.e., 1 ml per liter of culture), and rotate the 50-ml conical tubes at room temperature for 15 min.
9. Distribute the lysate among 5-ml plastic culture tubes, adding approximately 4 ml per tube.
10. Seal the plastic culture tubes and heat to between 75°C and 80°C in a heat block for 1 hr.
11. Pool the heated lysates into a 250-ml glass beaker and add 60 ml of *Taq* lysis buffer (i.e., 15 ml per equivalent liter of culture). Mix by stirring, either on a stir plate or with a pipette.
12. Distribute the solution between 30-ml screw-top centrifuge tubes, filling each tube at least 3/4 full. Balance the tubes, and centrifuge for 30 min at 38,000 × *g*, 4°C.
13. Transfer the supernatant to a 250-ml glass bottle. Add the following and mix well:

    a. 8 ml glycerol (∼10% final concentration)
    b. 0.4 ml of 2 M imidazole, pH 8 (∼10 mM final concentration)
    c. 30 µl of β-mercaptoethanol (∼5 mM final concentration)

14. Use a gravity flow column to equilibrate the Ni-NTA agarose slurry with *Taq* lysis buffer. To do this, thoroughly resuspend the Ni-NTA agarose slurry by repeatedly inverting the bottle. Pipette 8 ml of Ni-NTA agarose slurry (4 ml bed volume) into a gravity flow column in a clamp on a support stand. Uncap the bottom of the column, and wash with 40 ml of water, then 40 ml of *Taq* lysis buffer.

    This may be done by gravity flow, or alternatively, a vacuum may be applied to the bottom of the column to cause liquid to flow through more quickly.

15. Cap the bottom of the column. Using 10 ml of the lysate, resuspend the Ni-NTA agarose and transfer from the column to the glass bottle containing the rest of the lysate. Stir on a magnetic stir plate at 4°C for 1 hr to allow the protein to bind to the Ni-NTA.
16. Pour the mixture of lysate and Ni-NTA agarose into the column, and allow it to drain either by gravity flow or by applying a vacuum to the bottom of the column.
17. Wash with 80 ml of *Taq* buffer W500 and 80 ml of *Taq* buffer W100, and allow to drain either by gravity flow or by applying a vacuum to the bottom of the column.
18. Add 2 ml of *Taq* elution buffer, and collect the flowthrough (void fraction) in a 15-ml conical tube.
19. Add 4 ml of *Taq* elution buffer, and collect the flowthrough (elution fraction) in another 15-ml conical tube. Repeat this step four additional times, for a total of 5 elution fractions in different 15-ml conical tubes. Keep the elution fractions on ice.
20. Cap and invert each of the 15-ml conical tubes several times to mix the contents. Mix 15-µl samples of the void and each of the elution fractions with 5 µl of 4× SDS sample buffer in separate tubes, and heat to 95°C for 5 min. Separate 5 µl of each sample on an SDS-PAGE gel, and visualize by staining with a protein gel stain (see Fig. 2A).

    Essential protocols for SDS-PAGE can be found in Current Protocols article Gallagher, 2012)

    More details about analysis of proteins by SDS-PAGE can be found in other Current Protocols articles (Gallagher, 2012; Sasse & Gallagher, 2009).

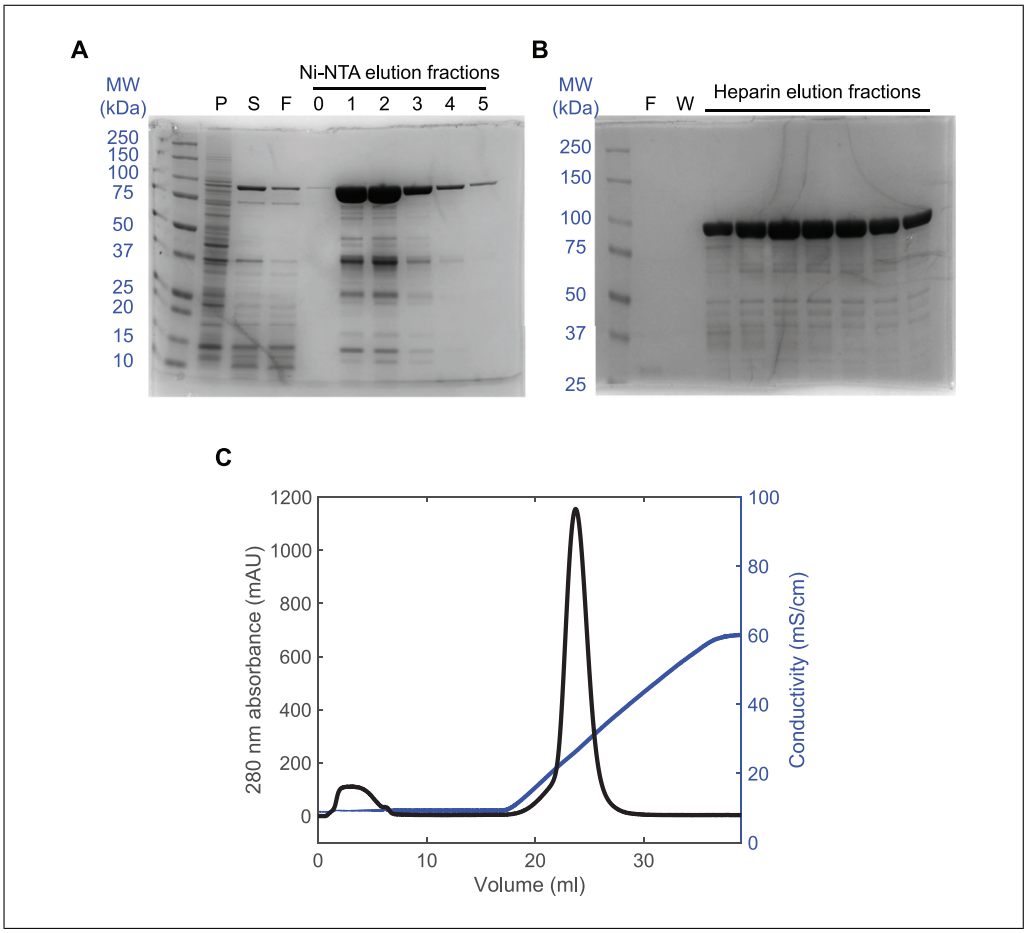

Figure 2 Purification of *Taq* polymerase. (**A**) Coomassie-stained 10% SDS-PAGE gel of initial Ni-NTA purification. P, insoluble pellet after heat denaturation. S, supernatant after heat denaturation. F, Ni-NTA column flowthrough. 0-5, Ni-NTA elution fractions. (**B**) Coomassie-stained 10% SDS-PAGE gel of flowthrough (F), wash (W), and elution peak fractions from heparin purification step. (**C**) Chromatogram of HiTrap heparin purification. Black curve, 280 nm absorbance. Blue curve, conductivity. In this example, the initial peak is the column flowthrough, and the peak of *Taq* elution occurred at 30% SP Buffer B.

21. Pool the fractions that contain protein. Wash dialysis tubing with water and heparin dialysis buffer. Use a dialysis clip to seal one end of the dialysis tubing, transfer the protein to the dialysis tubing, and seal the other end with a second clip. Transfer the sealed dialysis tubing containing the protein to a beaker with 2 L of heparin dialysis buffer, and incubate with gentle stirring on a magnetic stir plate overnight at 4°C.

    See Current Protocols article: Zumstein (2001) for additional detail on dialysis.

Protein purification on a heparin column

22. Connect a 1-ml HiTrap heparin column to an FPLC system, according to the manufacturers' instructions, and equilibrate with at least 10 column volumes of heparin buffer A.

    We use a 1-ml HiTrap heparin column, but a larger column may be required if scaling up the protocol.

23. Remove the protein solution from the dialysis tubing by detaching one of the two dialysis clips and pouring the solution from the open end of the tubing into a 50-ml conical tube. Distribute the solution among 1.7-ml microcentrifuge tubes, and microcentrifuge 5 min at 16,000 × *g*, 4°C. Transfer the supernatant to a new tube, being careful not to transfer any pelleted material.

24. Load the centrifuged protein onto the heparin column according to the manual for your FPLC system.

25. Elute with a 0%-100% gradient of heparin buffer B, according to the manual for your FPLC system (see Fig. 2C).

26. Based on the 280 nm absorbance readout from your FPLC system, determine which fractions have the highest total protein concentration. Analyze samples of these fractions by SDS-PAGE (see Fig. 2B).

27. Based on the SDS-PAGE gel, pool the fractions that contain the highest concentration of pure *Taq* DNA polymerase, i.e., those that exhibit a strong band at the correct molecular weight (~94 kDa) with minimal contaminant bands of other molecular weights. Proceed as described below, depending on the type of *Taq* you are preparing:

    a. If preparing non-hot-start *Taq*, dialyze overnight against *Taq* storage buffer (at least 100 sample volumes). The next day, transfer dialysis tubing to a second beaker of *Taq* storage buffer, and dialyze for an additional 2-3 hr. Store aliquots at −80°C for long-term storage or −20°C for short-term use. Measure the absorbance of the protein at 280 nm with a spectrophotometer, using the dialysis buffer in the beaker as a blank. Use the Beer-Lambert law, $C = A/(x\varepsilon)$, to determine the molar protein concentration, where C is the concentration, A is the absorbance, ε is the extinction coefficient (112760 $M^{-1}cm^{-1}$ for *Taq* DNA polymerase), and x is the pathlength of your spectrophotometer (in cm). Convert mol/L into g/L by multiplying by the molecular weight, 93910 g/mol.
    b. If preparing hot-start *Taq*, dialyze overnight against *Taq* crosslinking buffer (at least 100 sample volumes). The next day, transfer tubing to a second beaker of *Taq* crosslinking buffer, and dialyze for an additional 2-3 hr.

    It is critical in the crosslinking step to use a buffer that does not contain primary amines (i.e., HEPES, not Tris). Proceed to step 28.

Preparing hot-start Taq polymerase by formaldehyde crosslinking

Protocol adapted from Buratowski (2015).

28. Transfer the dialysate from step 27b to a 50-ml conical tube, add 16% formaldehyde to a final concentration of 0.45%, and mix well.

29. Incubate for 45 min at 37°C.

30. Add 75 µl of 2 M Tris·HCl, pH 8, per ml of dialysate, mix well, and incubate for 2 min at room temperature to quench the crosslinking reaction.

31. Dialyze three times (first dialysis for 30 min, then for 60 min, and finally for 90 min) against *Taq* post-crosslinking dialysis buffer (at least 100 sample volumes for each dialysis).

32. Dialyze overnight against *Taq* storage buffer (at least 100 sample volumes).

33. The next day, dialyze 2-3 hr against at least 100 sample volumes of *Taq* storage buffer. Determine the protein concentration as described in step 27a above. Store aliquots at −80°C for long-term storage or −20°C for short-term use.

Purification of M-MLV reverse transcriptase

Protein expression

34. Grow four 1-liter cultures of *E. coli* expressing M-MLV reverse transcriptase, following steps 1-6 of the *Taq* polymerase purification protocol (substitute plasmid

pET-28a_6H-MMLV_RT_D524N-6H for the *Taq* plasmid used in step 11). Induce M-MLV RT cultures with 1 mM IPTG for 3 hr, rather than 2 hr, at 37°C.

Protein purification using Ni-NTA

35. Resuspend pellets in 20 ml of M-MLV RT lysis buffer per liter of culture (80 ml total for 4 L of culture).

36. Add lysozyme to a final concentration of 1 mg/ml, and incubate on a rotator at 4°C for 30 min.

37. Transfer to a metal beaker on ice and lyse cells by sonication.

    We use a 1-cm-diameter tip on setting 7 of a Branson Sonifier 450, 5 cycles of 30 s on, 30 s off. Place the sonicator tip approximately 5 mm from the bottom of the beaker. Adjust the vertical position of the sonicator tip after 2-3 rounds of sonication to compensate for downward motion of the beaker due to melting of the ice.

38. Add 9.6 ml of 5 M NaCl and 40 ml of M-MLV lysis buffer.

39. Distribute between 30-ml screw-cap tubes, filling each at least 3/4 full, and centrifuge 30 min at 37,000 × *g*, 4°C.

40. Wash 4 ml bed volume of Ni-NTA agarose (8 ml slurry volume) with 40 ml each of water and M-MLV RT lysis buffer.

41. Mix supernatant with Ni-NTA agarose in a beaker, and stir on a stir plate for 1 hr at 4°C.

42. Pour slurry into a gravity column and allow the liquid to drain.

43. Wash with 40 ml of M-MLV RT buffer W500 followed by 40 ml of M-MLV RT lysis buffer.

44. Elute with M-MLV RT elution buffer. Collect one 2-ml void fraction followed by five 4-ml elution fractions in 15-ml conical tubes.

45. Mix 15-µl samples of the void and elution fractions with 5 µl of 4× SDS sample buffer in separate tubes, and heat to 95°C for 5 min. Separate 5 µl samples of each elution fraction mix on an SDS-PAGE gel (see Current Protocols article: Gallagher, 2012) and stain using a protein gel stain (see Current Protocols article: Sasse & Gallagher, 2009) (Fig. 3A).

46. Pool peak fractions, and dialyze them overnight against at least 100 volumes of SP buffer A.

Protein purification on a HiTrap SP column

47. Connect a HiTrap SP column to an FPLC system, according to the manufacturers' instructions, and equilibrate with 10 column volumes of SP buffer A.

48. Microcentrifuge the dialysate from step 46 for 5 min at 16,000 × *g*, 4°C, and transfer the supernatant to a fresh tube, avoiding any pelleted material.

    While we typically observe a pellet after centrifugation, the bulk of the protein remains in the supernatant.

49. Load the supernatant on the column, wash with 10 column volumes of SP Buffer A, and elute with a 0%-100% gradient of SP Buffer B (balance SP buffer A), according to the manual from the FPLC manufacturer.

50. Mix 15 µl samples of input, flowthrough, and peak fractions (determined by 280 nm absorbance) with 5 µl of 4× SDS-PAGE sample buffer, heat to 95°C for 5 min, and separate by SDS-PAGE (see Fig. 3B).

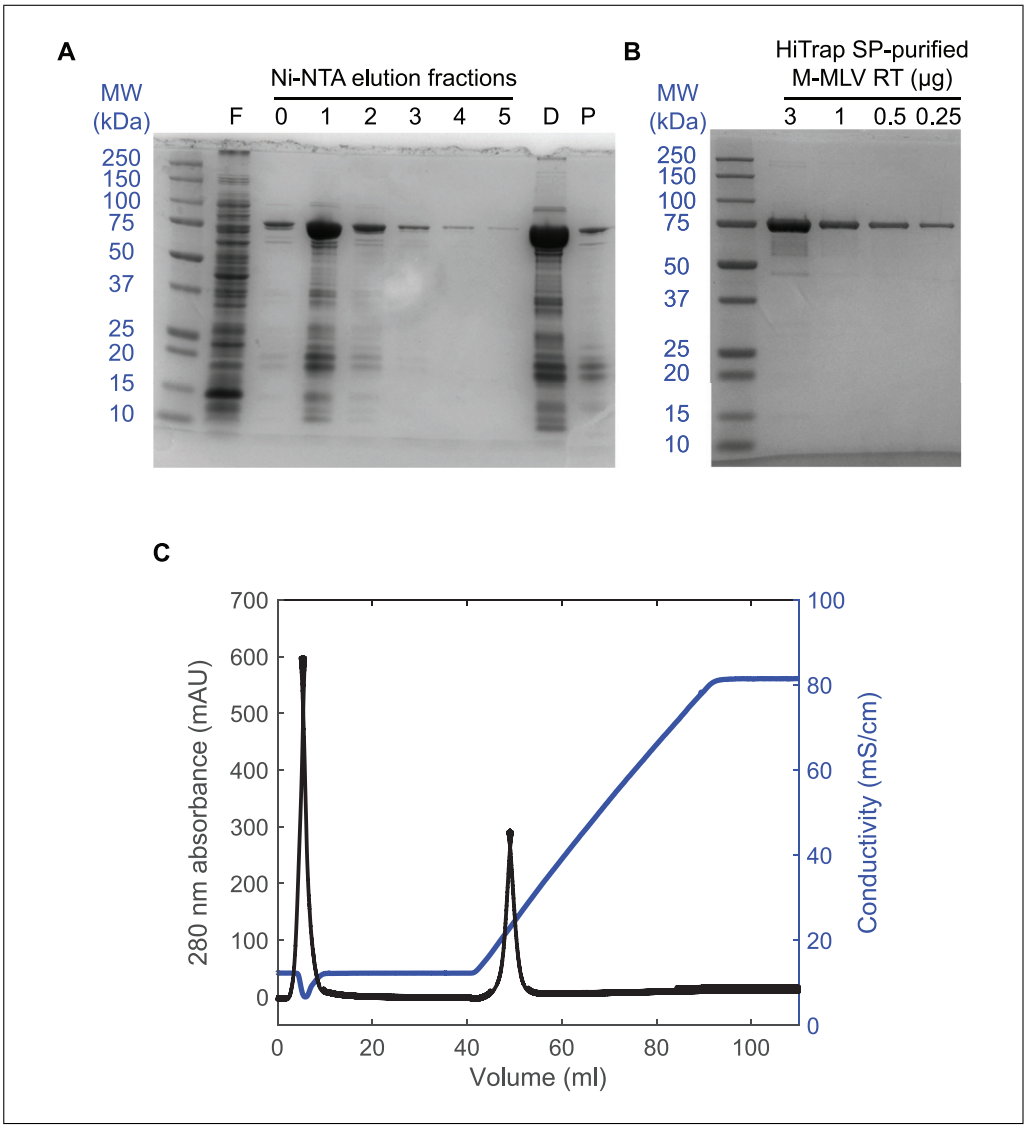

Figure 3 Purification of M-MLV reverse transcriptase. (**A**) Coomassie-stained 10% SDS-PAGE gel of Ni-NTA purification. F, Ni-NTA flowthrough. 0-5, Ni-NTA elution fractions. D, soluble dialysate after overnight dialysis. P, precipitate after overnight dialysis. (**B**) Coomassie-stained 10% SDS-PAGE gel of the indicated microgram quantities of purified M-MLV protein after HiTrap SP purification and dialysis against M-MLV RT storage buffer. (**C**) Chromatogram of purification on a 5-ml HiTrap SP column. Black curve, 280 nm absorbance. Blue curve, conductivity. In this example, the initial tall peak is the column flowthrough, and M-MLV reverse transcriptase elution occurred at 15% SP Buffer B.

See Current Protocols articles: Gallagher (2012) for SDS-PAGE and Sasse & Gallagher (2009) for staining gels.

51. Pool the peak fractions containing protein (typically ~5 ml), and dialyze overnight against at least 100 volumes of M-MLV RT storage buffer. The next day, transfer dialysis tubing to a second beaker of M-MLV RT storage buffer, and dialyze for an additional 2-3 hr. Store aliquots at −80°C for long-term storage or −20°C for short-term use.

See Current Protocols article: Zumstein (2001) for additional detail on dialysis.

52. Determine the concentration, as described in step 27a, of the *Taq* polymerase purification protocol, using an extinction coefficient at 280 nm of $\varepsilon = 79870\ M^{-1}cm^{-1}$ and a molecular weight of 63700 g/mol for M-MLV reverse transcriptase (Fig. 3C).

Preparation of the BEARmix one-step RT-qPCR master mix

53. Prepare 4× BEARbuffer by combining the ingredients below in a 15-ml conical tube. Add water to around the 11 ml mark on the tube and invert the tube repeatedly to dissolve all the trehalose. Then, add water to bring the total volume up to the 12.5 ml mark on the tube.

Composition	Per 12.5 ml
200 mM Tris·HCl, pH 8.4	2.5 ml of 1 M stock
300 mM KCl	1.875 ml of 2 M stock
12 mM $MgCl_2$	150 µl of 1 M stock
40% trehalose	5 g
40 mM DTT	500 µl of 1 M stock
0.4 mM EDTA	10 µl of 0.5 M stock
1.6 mM dNTPs	2 ml of 10 mM stock
DNase/RNase-free water	To 12.5 ml

54. Using the concentrations determined in the preceding steps (step 27a for non-hot-start *Taq* DNA polymerase, step 33 for hot-start *Taq* DNA polymerase, and step 52 for M-MLV reverse transcriptase), prepare a 100× BEAR enzyme solution by mixing the two enzymes with MMLV storage buffer to obtain final concentrations of 1.6 mg/ml for *Taq* DNA polymerase and 0.17 mg/ml for M-MLV reverse transcriptase.

55. Store 500 µl aliquots of 100× BEAR enzymes at −20°C.

The 100× BEAR enzyme mix will not freeze when stored at −20°C, due to the presence of 50% glycerol in M-MLV storage buffer.

PREPARATION OF SWAB SAMPLES FOR DIRECT RT-PCR

BASIC PROTOCOL 2

RNA purification is an expensive and time-consuming step in SARS-CoV-2 diagnostic testing. While direct addition of clinical samples to RT-qPCR reactions, also known as "direct RT-qPCR," provides a simple alternative (Bruce et al., 2020; Esbin et al., 2020; Graham et al., 2021; Hasan et al., 2020; Smyrlaki et al., 2020), the composition of standard commercial swab collection buffers such as UTM and V-C-M is not optimized for compatibility with direct RT-qPCR (Graham et al., 2021). Here, we describe a simple alternative collection solution for the subsequent detection of SARS-CoV-2 RNA from nasopharyngeal swab samples, which does not inhibit RT-qPCR when added at volumes of up to 6.65 µl per 10 µl reaction. Additionally, this method is compatible with heat-inactivation of SARS-CoV-2 in sealed tubes, to protect testing center personnel (Graham et al., 2021).

IMPORTANT NOTE: Samples should be collected by qualified medical personnel in compliance with all applicable biosafety and other regulations. In our previous work, we have tested direct RT-qPCR of patient swab samples in UTM and evaluated our proteinase K collection solution using "contrived" swab samples comprising cultured SARS-CoV-2 and human nasal mucus (Graham et al., 2021). However, collection of patient swab samples into PK solution has not yet been evaluated clinically.

Materials

200 µg/ml proteinase K (PK) (Millipore, 70663-5)

The solution can be stored at −20°C or 4°C, and is also stable at room temperature for at least 2 weeks (Graham et al., 2021). We have found that solutions of Millipore proteinase K are stable and active when the lyophilized powder is reconstituted in distilled water. Other manufacturers (e.g., Worthington Biochemical Corporation) recommend that lyophilized PK be reconstituted in other solutions such as 50 mM Tris·HCl, pH 8.0, with 1 mM $CaCl_2$.

Nasopharyngeal collection swabs (e.g., Typenex Medical, SW0102)
A method for 3D printing of swabs has also been described (Ford et al., 2020).
Swab collection tubes (e.g., Typenex Medical, TC0201)

Incubator set at 37°C
Incubator set at 75°C

1. Collect patient NP swab samples and deposit directly into 2 ml of 200 µg/ml PK in screw-cap swab-collection tubes.

   For safety, tubes may be sealed and the outside of each tube disinfected with 70% ethanol. While we have not assessed the long-term stability of viral RNA for real patient swab samples in PK solution, we have found that SARS-CoV-2 genomic RNA was stable for at least 3 days at room temperature in "contrived" swab samples consisting of a mixture of cultured virus and human nasal fluid diluted in PK solution (Graham et al., 2021).

2. Incubate samples at 37°C for 30 min.

3. Incubate samples at 75°C for 30 min to inactivate both virus and proteinase K. Samples can now be used in RT-qPCR.

   Incubation at 75°C for 30 min was found to inactivate cultured SARS-CoV-2 (Graham et al., 2021).

   Heat-inactivated samples may be added directly to an RT-qPCR mix (up to 6.75 µl per 10 µl reaction; see Basic Protocol 3). Alternatively, samples collected in commonly used commercial collection solutions, such as UTM or V-C-M, may be directly added to RT-qPCR reactions to a maximum of ∼5%-10% of the total reaction volume. However, we have found that addition of larger quantities of UTM or V-C-M inhibits RT-qPCR using either BEARmix or TaqPath One-Step Master Mix (Thermo Fisher).

ALTERNATE PROTOCOL 1

CONCENTRATION OF VIRAL RNA FROM SWAB SAMPLES BY ISOPROPANOL PRECIPITATION

If patient samples were not collected in a buffer compatible with direct RT-qPCR analysis (such as that described in Basic Protocol 2), Alternate Protocol 1 can be used instead to prepare RNA samples for subsequent analysis.

Denaturing swab collection solutions such as DNA/RNA Shield (Zymo Research) inactivate SARS-CoV-2 and preserve RNA integrity, but also strongly inhibit RT-qPCR. Non-denaturing swab collection solutions such as UTM and V-C-M may be added to RT-qPCRs in small quantities (5%-10% of the total reaction volume), but these solutions inhibit RT-qPCR when added in larger quantities. This creates a problem because achieving maximal detection sensitivity requires adding as much RNA as possible per reaction. The isopropanol precipitation procedure described below provides a simple and inexpensive means of isolating and concentrating RNA from swab samples, which can then be used for RT-qPCR. One pitfall of this method is that it concentrates the RNA into a pellet, which may be lost during the aspiration step. Thus, it is essential to include a recovery control (e.g., human RNase P RNA or exogenously added bacteriophage MS2 genomic RNA) to verify successful RNA recovery in each sample.

IMPORTANT NOTE: Samples should be collected by qualified medical personnel in compliance with all applicable biosafety and other regulations.

Materials

Proteinase K solution (see Basic Protocol 2 materials list)
2× DNA/RNA Shield (Zymo Research #R1200) or DNA/RNA Shield Collection Tube with Swab (Zymo Research #R1107-E)
3 M sodium acetate solution, pH 5.2 (see recipe)

Linear polyacrylamide (Invitrogen, AM9520)
Alternatively, linear polyacrylamide can be synthesized in the laboratory by polymerizing monomeric acrylamide (Gaillard & Strauss, 1990).
100% isopropanol (room temperature)
75% ethanol (room temperature)
DNase/RNase-free water

1.7-ml microcentrifuge tubes
Filter pipette tips
Gel loading tips (e.g., Flat Gel Loading Tip, Micro, 0.3 mm flat 83 × 0.33 mm, Denville #P3082)
Microcentrifuge set to 4°C
Thermomixer, incubator, heat block, or water bath set to 30°C

1. Collect swab samples in either of the following ways:
   a. Follow steps 1-3 of Basic Protocol 2 to collect a swab sample into 2 ml of proteinase K solution.
   b. Collect a swab sample in 2 ml of 1× DNA/RNA Shield (Zymo Research), which inactivates SARS-CoV-2 while preserving RNA.
2. Transfer 400 µl of inactivated swab sample liquid to a 1.7-ml microcentrifuge tube, and add 40 µl of 3 M sodium acetate (pH 5.2) and 1 µl of 5 mg/ml linear acrylamide carrier. Mix well by pipetting up and down five times.

   To concentrate the RNA further than described here, start with a larger initial volume of inactivated swab sample and rescale the quantities of isopropanol and sodium acetate accordingly.
3. Add 440 µl of isopropanol. Mix well by inverting the tubes five times.
4. Incubate at −20°C for 30 min.

   Incubation at −80°C for the same amount of time may further improve recovery.
5. Microcentrifuge 15 min at 16,000 × *g*, 4°C.
6. Carefully aspirate the supernatant, being careful not to disturb the pellet on the bottom of the tube (see Fig. 1C).

   The pellet may or may not be clearly visible at this stage. The use of fine gel-loading tips helps to avoid aspirating the pellet; it may also help to hold the tubes in front of a light (Fig. 1C). Hold the aspiration tip slightly above the bottom of the tube, on the side of the tube that was oriented toward the axis of the centrifuge rotor. Be sure to use a fresh tip for each sample to avoid cross-contamination.
7. Add 1 ml of 75% ethanol to each tube.
8. Microcentrifuge for 5 min at 16,000 × *g*, room temperature, and carefully aspirate the pellet again.

   It helps to aspirate all but ∼10-20 µl, briefly centrifuge the samples again, and then thoroughly aspirate the remaining supernatant.
9. Add 50 µl of RNase-free water directly to each pellet.

   To achieve a higher concentration of RNA, resuspend in a smaller volume of water.
10. Heat the sample at 30°C for 10 min to redissolve RNA.

    If using a thermomixer, shaking the samples may help to dissolve the RNA.

ONE-STEP RT-qPCR OF RNA SAMPLES USING A REAL-TIME THERMOCYCLER

Below we describe how to set up RT-qPCR reactions using the Basic Economical Amplification Reaction mix (BEARmix) described in Basic Protocol 1. RT-qPCR reactions may be set up in 96- or 384-well plates by adding either purified RNA or other RNA-containing samples (e.g., inactivated swab samples from Basic Protocol 2 or Alternate Protocol 1) to a mixture of buffer, dNTPs, enzymes, and primers. Real-time amplification is detected by including either an intercalating dye or hydrolysis probe in the reaction mixture. It is most efficient to array samples in multiwell plates and transfer them to a master mix plate using either a multichannel pipette or a pipetting robot. Reactions must be prepared on ice if using non-hot-start *Taq* polymerase, but may be prepared at room temperature if using hot-start *Taq* polymerase.

While we developed this master mix to quantify SARS-CoV-2 RNA, it can be used to quantify other RNAs as well, either for clinical diagnostics or basic research. The same mix without reverse transcriptase can be used as a qPCR master mix for DNA quantification. Whatever the application, it is important to validate the performance of your laboratory-made master mix by analyzing nucleic acid standards of known concentration (see Support Protocol).

Materials

Gene-specific PCR primers
For **hydrolysis probe-based reactions** only: TaqMan probe [here we use a primer and probe pre-mix in the 2019-nCoV EUA Kit from Integrated DNA Technologies (cat no. 10006770)]
We have tested BEARmix using the CDC-recommended SARS-CoV-2 N1 and N2 primer/probe sets and the human RNase P control primer/probe set, which can be obtained pre-mixed in the 2019-nCoV EUA Kit from Integrated DNA Technologies (cat no. 10006770).
For **intercalating dye-based reactions** only: SYTOX Orange (Thermo Fisher, cat. no. S11368), SYBR Green (Thermo Fisher, cat. no. S7563), or comparable dsDNA-binding fluorescent dye, 100 μM solution in DMSO
Nuclease-free water
4× BEARbuffer (Basic Protocol 1)
100× BEAR enzymes (Basic Protocol 1)

In vitro-transcribed RNA (see Support Protocol)
96-well or 384-well qPCR plates (Thermo Scientific, AB-0800/W and AB-3384)
Ice bucket
96-well or 384-well metal block (Universal Medical, cat no. 81001)
Multichannel (8-channel or 12-channel) 10-μl or 20-μl pipette
Real-time thermocycler
We have tested this protocol on BioRad CFX 96 and CFX 384 instruments.

1. Prepare solutions of primers and fluorogenic reagent:

   a. For **hydrolysis probe-based reactions**, prepare or obtain a primer/probe mixture containing forward and reverse gene-specific PCR primers at 6.7 μM each and TaqMan probe at 1.7 μM in nuclease-free water.
   b. For **intercalating dye-based reactions**, dilute SYTOX Orange to a working stock of 100 μM in dimethylsulfoxide (DMSO). In addition, prepare a mixture of forward and reverse PCR primers at a concentration of 6.7 μM each in nuclease-free water.

2. Prepare master mix:

a. For **hydrolysis probe-based reactions**, mix the following on ice in the order listed, rescaling the volumes as needed for the desired total number of reactions. "X" represents the sample volume, which may be up to 6.65 µl. We typically use 6.65 µl of sample volume.

	Per reaction	110-reaction master mix (for a full 96-well plate)
Water	(6.65 − *X*) µl	110 * (6.65 − *X*) µl
4× BEARbuffer	2.5 µl	275 µl
100× BEAR enzymes	0.1 µl	11 µl
Primer/probe mixture	0.75 µl	82.5 µl

b. For **intercalating dye-based reactions**, mix the following on ice in the order listed, rescaling the volumes as needed for the desired total number of reactions. Mix thoroughly by pipetting or vortexing after addition of SYTOX Orange, to avoid exposing the enzymes to a high concentration of DMSO at the bottom of the tube. "X" represents the sample volume, which may be up to 6.55 µl.

	Per reaction	110-reaction master mix (for a full 96-well plate)
Water	(6.55 − *X*) µl	110 * (6.55 − *X*) µl
4× BEARbuffer	2.5 µl	275 µl
100 µM SYTOX Orange	0.1 µl	11 µl
100× BEAR enzymes	0.1 µl	11 µl
Primer mixture	0.75 µl	82.5 µl

IMPORTANT NOTE: *Prepare the master mix immediately before use, and keep it on ice. Do not subject the master mix to freeze-thaw cycles after adding BEAR enzymes.*

3. Aliquot master mix for the desired number of reactions (samples + controls) into multiwell qPCR plates placed in a metal cooling block on ice.

4. Distribute samples and controls into a new multiwell plate, and then transfer them to the reaction plate using a multichannel pipette. For each RT-qPCR plate, include a positive control reaction with in vitro−transcribed RNA (see Support Protocol) and a negative control reaction with water only.

   It is also a good idea to include positive and negative RNA extraction controls in which no-RNA and positive control RNA samples are processed in the extraction step.

5. *Optional:* To quantify the absolute number of RNA molecules per sample, include a 10-fold dilution series of known concentrations of an in vitro−transcribed standard (see Support Protocol).

   For accurate quantification, the Cq values of this dilution series should span a range that encompasses the Cq values of the unknown samples. While Cq values will likely vary between different preparations of master mix and different thermocyclers, representative results are shown in Figure 4, where a range of template concentrations between 10^3 and 10^6 molecules gave Cq values between 35 and 23 cycles.

6. Run the following cycling program in a qPCR instrument:
   i. 50°C for 10 min.
   ii. 95°C for 5 min.

      Extend this incubation to 20 min when using hot-start Taq polymerase.

   iii. 95°C for 3 s.
   iv. 55°C for 30 s; read plate fluorescence in appropriate fluorescence channel(s).
   v. Repeat steps (iii) and (iv) 44 additional times.

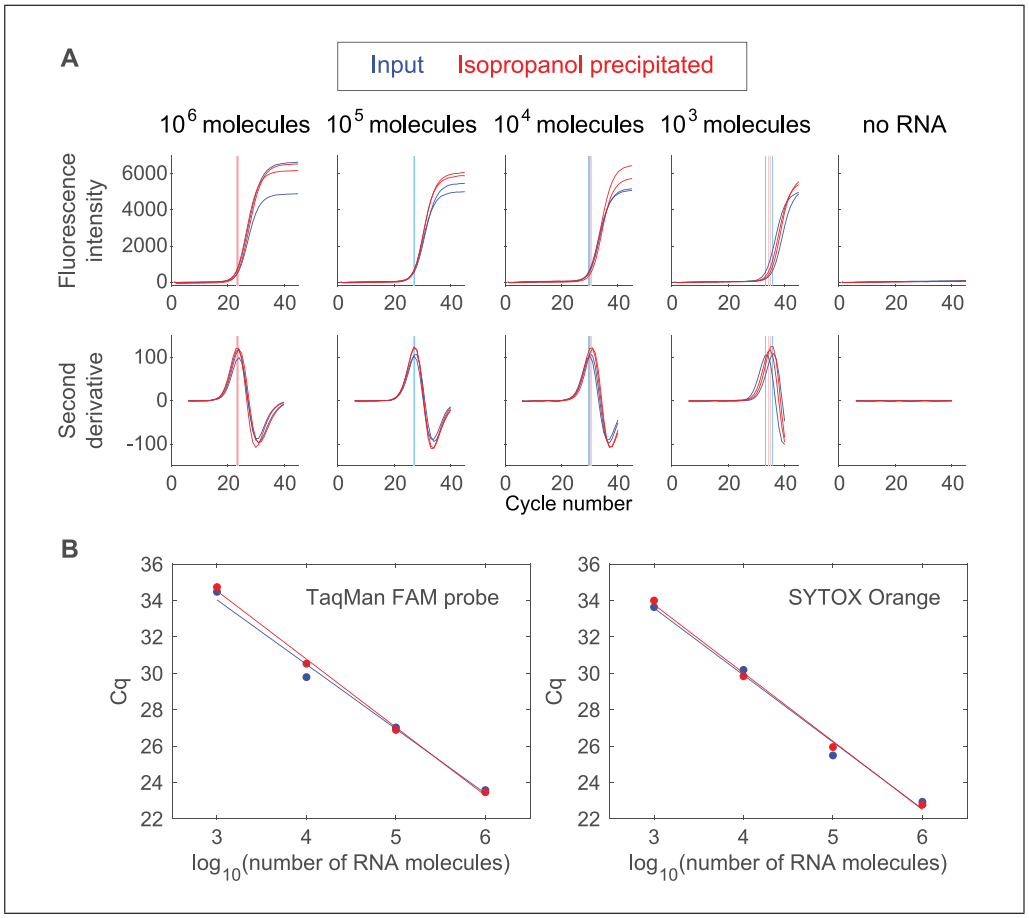

Figure 4 Sample BEARmix RT-qPCR reactions of in vitro–transcribed SARS-CoV-2 N gene RNA. A series of dilutions was prepared and subjected to isopropanol precipitation following Alternate Protocol 1. Isopropanol-precipitated samples (red) and non-precipitated standards (blue) were analyzed by RT-qPCR. (**A**) Top panel: Fluorescence traces from individual TaqMan RT-qPCR reactions using the CDC SARS-CoV-2 N2 primer/probe set. Results were consistent between technical duplicates, and precipitated samples gave comparable traces to non-precipitated samples, indicating essentially complete RNA recovery. Bottom panel: Second derivative of the curves in the top panel. Cq values (indicated by vertical lines) were determined by fitting the peak of the second derivative to a parabola. The PCR cycle number is shown on the *x*-axis. (**B**) Plots of Cq value (number of cycles, *y*-axis) vs. base-10 logarithm of RNA molecule number (*x*-axis) with best-fit lines by linear regression in MATLAB. Left panel: TaqMan fluorescence readout (same reactions as in (**A**). Right panel: SYTOX Orange dsDNA dye-based readout using the N2 primer set. Each data point is the mean of two technical duplicates. Control experiments (not shown) confirmed that there is negligible fluorescence bleed-through from TaqMan FAM signal into the HEX channel used to detect SYTOX Orange.

When using non-hot-start Taq polymerase, it is best to transfer the reactions directly from ice to a PCR block pre-heated to 50°C, without allowing them to sit at room temperature. On BioRad CFX96 and CFX384 qPCR instruments, close the lid and start the PCR cycle without the reaction plate. When the block temperature reaches 50°C, open the lid (which will automatically pause the program), insert the plate, and close the lid to resume the program.

The appropriate channels to use for measuring fluorescence will depend on the instrument and intercalating dye or hydrolysis probe(s) used. Fluorescence from SYTOX Orange can be measured in the HEX (hexachlorofluorescein) channel on BioRad instruments.

7. Determine Cq values using one of two methods:

   a. *Threshold-based (Ct):* Using the software for your qPCR thermocycler, determine at which cycle the fluorescence intensity crosses a user-specified threshold.

Using positive and negative controls as benchmarks, choose a threshold that clearly distinguishes amplification from non-amplification.

b. *Second derivative-based:* Determine the cycle at which the second derivative of fluorescence with respect to cycle number is maximal (Rasmussen, 2001). Local fitting to a parabola may be used to define the peak position with sub-cycle precision. Some qPCR thermocycler software packages include a built-in second-derivative Cq option, or the data may be exported (without baseline subtraction) and analyzed using custom code. Sample code for second-derivative analysis can be found at *https://gitlab.com/tjian-darzacq-lab/second-derivative-cq-analysis*.

8. For clinical diagnosis, first analyze numerous known-positive and known-negative clinical specimens by RT-qPCR, and choose a Cq value that reliably separates positive from negative samples (IGI Testing Consortium, 2020). Score unknown samples as positive if their Cq value falls below this threshold and negative if their Cq value falls above this threshold.

9. *Optional:* To quantify the absolute number of RNA molecules per sample, plot the Cq values of the standard curve (see step 5) against the logarithm of the number of input RNA molecules (see Fig. 4B), and use a best-fit line to determine the number of RNA molecules in unknown samples.

PREPARATION OF RNA CONCENTRATION STANDARDS BY in vitro TRANSCRIPTION

SUPPORT PROTOCOL

This protocol describes how to prepare a stock of in vitro−transcribed (IVT) RNA of a known concentration to serve as a standard for determining the absolute number of RNA molecules in a sample by RT-qPCR. IVT RNA is also invaluable for troubleshooting primer design and master mix performance. Due to the high concentrations of RNA produced by in vitro transcription, it is critical to avoid contaminating RT-qPCR reagents with IVT RNA, for instance, through dispersal by aerosols. Aerosol-free filter tips should be used when pipetting all reagents. Ideally, preparation of IVT RNA should occur in a separate area of the laboratory from where other RT-qPCR reagents are prepared, and a different set of pipettes should be used. One solution is to set up RT-qPCR reactions and aliquot RT-qPCR reagents in a dedicated qPCR setup hood or tissue culture hood.

Materials

Sample to use as a template for PCR (e.g., 2019-nCoV_N_Positive Control, Integrated DNA Technologies, 10006625)
PCR primers flanking the amplicon(s) of interest (see step 1)
PCR master mix [e.g., BEARmix (see Basic Protocol 1) or NEB 2× Q5 Master Mix]
Agarose gel with ethidium bromide or other fluorescent DNA dye (see Current Protocols article: Voytas, 2000)
Gel extraction kit (e.g., Zymoclean Gel DNA Recovery Kit; Zymo Research, D4007)
HiScribe T7 Quick Kit (NEB)
T7 RNA polymerase from other suppliers may be used, following the manufacturer's instructions, or T7 RNA polymerase may be purified according to published protocols (e.g., He et al., 1997). Also see http://archive.today/QOVhS.
RNA purification kit, such as Qiagen RNeasy Mini kit (cat. no. 74104) or TRIzol RNA purification kit (Invitrogen, 12183555)
Nuclease-free water

Razor blades

UV-vis spectrophotometer (e.g., Nanodrop)
UV transilluminator or other gel illuminator

Additional reagents and equipment for BEARmix single-step reaction (Basic Protocol 1), agarose gel electrophoresis (see Current Protocols article: Voytas, 2000), and RNA purification (see Current Protocols article: Dowhan, 2012)

1. Design forward and reverse primers flanking the amplicon(s) of interest. At the 5′ end of the forward primer, include the T7 promoter sequence 5′-TAATACGACTCACTATAGGG-3′. For instance, for the SARS-CoV-2 N gene, we amplified the entire 1260-bp open reading frame using the following primers:

a.	SARS-CoV-2_N_forward	5′ TAATACGACTCACTATAGGGttaggcctg agttgagtcagc 3′
b.	SARS-CoV-2_N_reverse	5′ ttaggcctgagttgagtcagc 3′

2. Amplify the target from RNA or cDNA/DNA in a 50-µl PCR using either a BEARmix single-step reaction (Basic Protocol 1) or a commercial PCR master mix, respectively.

   If using BEARmix, include a 68°C extension step of 1 min per kb of the desired product. M-MLV RT may be omitted from the BEARmix reaction if cDNA or a cDNA clone is used as a template.

3. Separate the PCR product on an agarose gel (see Current Protocols article: Voytas, 2000), and excise the full-length product band using a razor blade.

4. Extract DNA from the product band using a gel extraction kit.

5. Determine the concentration of the PCR product by measuring absorbance at 260 nm using a spectrophotometer.

   Typical concentrations are on the order of 10 ng/µl.

6. Set up a 20-µl in vitro transcription reaction using the HiScribe T7 Quick Kit (NEB) by mixing:

   a. 10 µl HiScribe T7 NTP buffer mix.
   b. 8 µl template DNA (typically at ∼10 ng/µl).
   c. 2 µl HiScribe T7 RNA polymerase mix.

   The manufacturer recommends a maximum of 1 µg of DNA per 20 µl reaction, but purified PCR products are seldom this concentrated, in our experience.

7. Incubate the reaction overnight at 37°C.

8. Purify RNA using the RNA purification kit of your choice.

   We typically use the TRIzol RNA purification kit, but a spin column kit, or phenol-chloroform precipitation and isopropanol precipitation, could also be used (see Current Protocols article: Dowhan, 2012).

9. Determine the concentration of purified RNA on a spectrophotometer by measuring absorption at 260 nm and using the formula $C = A/(\varepsilon x)$, where A is the absorbance, $\varepsilon = 0.025$ $(ng/\mu l)^{-1}cm^{-1}$ is the extinction coefficient of ssRNA, and x is the pathlength of the spectrophotometer.

   Alternatively, use the "single-stranded RNA" setting within the Nanodrop spectrophotometer software to calculate the RNA concentration.

10. Convert the concentration in ng/µl to molecules/µl.

Multiply the number of nucleotides by 330 g/mol/nucleotide (3.3×10^{11} ng/mol/nucleotide) to get the molecular weight of the ssRNA product. Divide the concentration in ng/µl by the molecular weight, and multiply by Avogadro's number (6.022×10^{23} molecules/mol) to get the concentration in molecules/µl.

11. Dilute the RNA in nuclease-free water to the nearest whole-number power of 10, and then prepare a series of further 10-fold serial dilutions in water down to a working stock concentration of 10^6 molecules/µl. For instance, if the starting concentration is 1.4×10^{12} molecules/µl, prepare an initial dilution to 10^{12} molecules/µl, and then prepare further serial dilutions, of 10^{11} molecules/µl, 10^{10} molecules/µl, etc., down to 10^6 molecules/µl. Store the original RNA stock, intermediate dilutions, and working stock aliquots at −80°C.

ONE-STEP RT-PCR USING ENDPOINT FLUORESCENCE DETECTION

ALTERNATE PROTOCOL 2

The protocol below provides an alternative to Basic Protocol 3 for detection of RT-PCR amplification, and involves the use of a conventional thermocycler and a fluorescent gel imager in place of a real-time qPCR thermocycler. Real-time qPCR thermocyclers are expensive instruments that are not universally available. Conventional thermocyclers are cheaper and more ubiquitous and, importantly, they make it possible to distinguish the presence or absence of SARS-CoV-2 RNA by imaging PCR plates or tube strips at the end of the PCR run using a standard fluorescence gel imager. Such an endpoint assay makes it possible to score positive and negative samples without an expensive real-time PCR instrument.

The detection threshold may be adjusted in this protocol by varying the number of PCR cycles. While increasing the total cycle number may permit detection of smaller amounts of RNA, this can also increase the background fluorescence of negative samples. An optimal cycle number may be chosen by performing a series of reactions for a set of known-negative and known-positive samples for a different numbers of cycles, to determine at which cycle number the fluorescence of known-positives and known-negatives is most distinct. To test the reliability of scoring positive and negative samples by eye, the evaluator should be blinded to the identity of positive and negative samples.

Additional Materials *(also see Basic Protocol 3)*

PCR tube strips or 96- or 384-well plates.
PCR thermocycler
Fluorescence gel imager: a commercial gel imager (e.g., BioRad Chemidoc) or a home-built fluorescence imaging system (Nuñez et al., 2017).
Optional: ImageJ (*https://imagej.net/Fiji*) or similar software for quantifying intensity of regions of interest within images

1. Set up reactions according to Basic Protocol 3, steps 1 to 4, then proceed to Basic Protocol 3, step 6, and run the PCR reaction using a regular thermocycler instead of a real-time qPCR thermocycler.

2. Image the RT-PCR plates or tubes on an imager in the appropriate fluorescence channel(s).

Adjust the exposure settings so that the fluorescence intensity inside the positive control wells is not saturated and positive and negative controls are clearly distinguishable. The outlines of the wells may be saturated if the plastic is autofluorescent, but this is not a problem as long as the fluorescence inside the positive and negative control wells is clearly distinguishable. Some background fluorescence will likely be observed due to nonspecific TaqMan probe degradation by BEARmix, but this can be distinguished from true amplification based on comparison with negative and positive controls (Fig. 5).

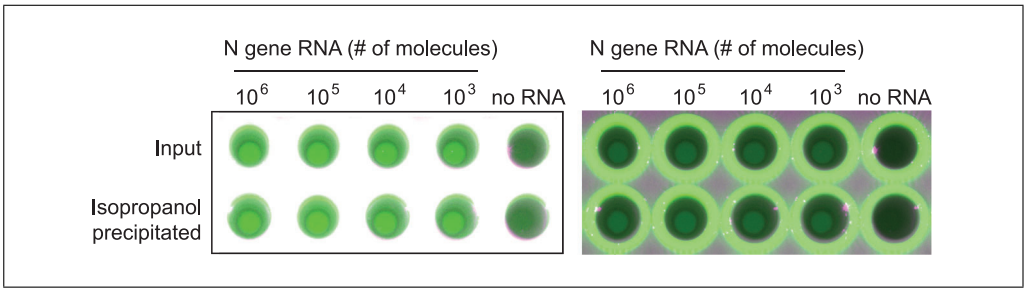

Figure 5 Quantification by endpoint detection of SARS-CoV-2 N gene RNA. The qPCR plate used for the TaqMan reactions in Figure 4 was imaged in the fluorescein channel on a BioRad Chemidoc imager with an exposure time of 75 ms (left panel) or 50 ms (right panel), and in the white light channel with an exposure time of 25 ms. Shown is an overlay of the fluorescein channel in green and the white light channel in magenta for one replicate set, such that white pixels indicate saturation of both channels. Contrast is enhanced in the left panel to display more clearly the fluorescence of the well contents, causing the outline of the plate to be saturated (white). A lower-contrast overlay is shown on the right panel. The outlines of the wells appear as green circles due to autofluorescence of the plastic. The number of RNA molecules per reaction is indicated above each column. Reactions containing RNA are clearly distinguishable from control reactions without RNA.

3. Score positive and negative samples by eye. Alternatively, to quantify the fluorescence intensity within each well, use the "ROI Manager" function in ImageJ to draw identically sized circular regions of interest and calculate their integrated intensity.

   While exact quantification of ROIs is more cumbersome, it may allow for less subjective scoring of positives and negatives.

REAGENTS AND SOLUTIONS

Buffers for Taq polymerase purification

Taq lysis buffer

Composition	Per 100 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	5 ml of 1 M
500 mM NaCl	10 ml of 5 M
0.1% NP-40	1 ml of 10% (v/v)
0.1% Triton X-100	1 ml of 10% (v/v)
Water	To 100 ml
Store up to 1 week or longer at 4°C	

Taq buffer W500

Composition	Per 100 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	5 ml of 1 M
500 mM NaCl	10 ml of 5 M
0.05% NP-40	0.5 ml of 10% (v/v)
5% glycerol	10 ml of 50% (v/v)
10 mM imidazole	0.5 ml of 2 M
5 mM β-mercaptoethanol (add immediately before use)	36 µl
1 mM benzamidine (add immediately before use)	100 µl of 1 M
Water	To 100 ml
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	
1 mM DTT may be substituted for 5 mM β-mercaptoethanol in *Taq* purification buffers.	

Taq buffer W100

Composition	Per 100 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	5 ml of 1 M
100 mM NaCl	2 ml of 5 M
0.05% NP-40	0.5 ml of 10% (v/v)
5% glycerol	10 ml of 50% (v/v)
10 mM imidazole	0.5 ml of 2 M
5 mM β-mercaptoethanol (add immediately before use)	36 µl
1 mM benzamidine (add immediately before use)	100 µl of 1 M
Water	To 100 ml
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

Taq elution buffer

Composition	Per 10 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	0.5 ml of 1 M
100 mM NaCl	0.2 ml of 5 M
0.05% NP-40	0.05 ml of 10% (v/v)
5% glycerol	1 ml of 50% (v/v)
300 mM imidazole	1.5 ml of 2 M
5 mM β-mercaptoethanol (add immediately before use)	3.6 µl
1 mM benzamidine (add immediately before use)	10 µl of 1 M
Water	6.7 ml
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

Heparin dialysis buffer

Composition	Per 1 L
50 mM Tris·HCl, pH 8 (Moore, 1996)	50 ml of 1 M
100 mM NaCl	20 ml of 5 M
0.05% NP-40	5 ml of 10% (v/v)
10% glycerol	100 ml
5 mM β-mercaptoethanol	360 µl
1 mM benzamidine	1 ml of 1 M
Water	To 1 L
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

Heparin buffer A/B

Composition	Per 1 L
50 mM Tris·Cl, pH 8 (Moore, 1996)	50 ml of 1 M
0.05% NP-40	5 ml of 10% (v/v)
10% glycerol	100 ml
5 mM β-mercaptoethanol	360 µl
Water	To 1 L
Split into 2 × 500 ml, which will be buffer A and buffer B	
Add 2.92 g NaCl to 500 ml for buffer A (final NaCl concentration 100 mM)	
Add 29.2 g NaCl to 500 ml for buffer B (final NaCl concentration 1 M)	
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

Taq crosslinking buffer

Composition	Per 2 L
50 mM HEPES, pH 8	100 ml of 1 M
50 mM KCl	7.45 g solid
0.1% NP-40	20 ml of 10% (v/v)
0.1% Tween 20	20 ml of 10% (v/v)
Water	To 2 L
Store up to 1 week or longer at 4°C	

Taq post-crosslinking dialysis buffer

Composition	Per 2 L
20 mM Tris·HCl, pH 7.5 (Moore, 1996)	40 ml of 1 M
200 mM KCl	29.8 g
1 mM EDTA	4 ml
Water	To 2 L
Store up to 1 week or longer at 4°C	

Taq storage buffer

Composition	Per 1 L
50 mM Tris·HCl pH 8	50 ml 1 M
100 mM NaCl	25 ml 5 M
0.1 mM EDTA	0.2 ml 0.5 M
50% glycerol	500 ml
3 mM DTT	Add 231 mg of DTT per 500 ml immediately before use
Water	To 1 L
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

Buffers for M-MLV reverse transcriptase purification

M-MLV RT lysis buffer

Composition	Per 100 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	5 ml of 1 M
100 mM NaCl	2 ml of 5 M
10 mM imidazole	0.5 ml of 2 M (pH 8)
1 mM DTT	200 µl of 0.5 M
0.1% Triton X-100	1 ml of 10% (v/v)
Water	To 100 ml
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

M-MLV RT buffer W500

Composition	Per 100 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	5 ml of 1 M
500 mM NaCl	10 ml of 5 M
10 mM imidazole	500 µl of 2 M (pH 8)
1 mM DTT	200 µl of 0.5 M
0.1% Triton X-100	1 ml of 10% (v/v)
Water	To 100 ml
Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.	

M-MLV RT elution buffer

Composition	Per 100 ml
50 mM Tris·HCl, pH 8 (Moore, 1996)	5 ml of 1 M
100 mM NaCl	2 ml of 5 M
250 mM imidazole	12.5 ml of 2 M (pH 8)
1 mM DTT	200 µl of 0.5 M
0.1% Triton X-100	1 ml of 10% (v/v)
Water	To 100 ml

Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.

SP buffer A

Composition	1 L	2 L
50 mM Tris·HCl, pH 8 (Moore, 1996)	50 ml of 1 M	100 ml of 1 M
100 mM NaCl	20 ml of 5 M	40 ml of 5 M
0.1 mM EDTA	200 µl of 0.5 M	400 µl of 0.5 M
5 mM DTT	770 mg of powder	1540 mg
0.1% Triton X-100	10 ml of 10% (v/v)	20 ml of 10% (v/v)
Water	To 1 L	To 2 L

Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.

SP buffer B

Supplement 500 ml of SP buffer A (see recipe) with 26.3 g of NaCl to give a final concentration of 1 M NaCl.

M-MLV RT storage buffer

Composition	1 L
50 mM Tris·HCl, pH 8 (Moore, 1996)	50 ml of 1 M
100 mM NaCl	20 ml of 5 M
0.1 mM EDTA	200 µl of 0.5 M
5 mM DTT	Add 385 mg of DTT to 500 ml immediately before use
0.1% Triton X-100	10 ml of 10% (v/v)
50% glycerol	500 ml
Water	To 1 L

Store up to 1 week or longer at 4°C. However, reducing agents (DTT or BME) and protease inhibitor (benzamidine) should be added immediately before use.

LB agar

Combine the ingredients listed for LB liquid medium (see recipe) plus 7.5 g of agar (e.g., Thermo Fisher, AAJ10654P2) per L. Sterilize by autoclaving on a liquid cycle (20 min at 15 psi). Allow the solution to cool to approximately 55°C, add antibiotic to the desired final concentration, and pour a thin layer of solution into plastic Petri dishes (~20 ml in each), being careful to avoid introducing bubbles. Allow the plates to solidify at room temperature before transferring to 4°C for storage of up to ~1-2 months.

LB liquid medium

Composition	1 L
1% (w/v) Bacto tryptone (e.g., Thermo Fisher, 211705)	10 g
1% (w/v) sodium chloride	10 g
0.5% (w/v) yeast extract	5 g
Water	To 1 L

Mix until the ingredients are dissolved, and autoclave on a liquid cycle (20 min at 15 psi) to sterilize

Store up to 1 year or longer at room temperature

Phosphate-buffered saline (PBS)

Composition	1 L
137 mM NaCl	8 g of solid NaCl
2.7 mM KCl	0.2 g of solid KCl
10 mM Na_2HPO_4	1.44 g of solid Na_2HPO_4
1.8 mM KH_2PO_4	0.24 g of solid KH_2PO_4

Adjust the pH to 7.4 with HCl, and add distilled water to a total volume of 1 L.

Store up to 1 year or longer at room temperature

SDS sample buffer

Composition	10 ml
200 mM Tris·HCl, pH 6.8 (Moore, 1996)	2 ml of 1 M stock
8% sodium dodecylsulfate	0.8 g
40% glycerol	4 ml
4% (w/v) β-mercaptoethanol	0.4 ml
50 mM EDTA	1.0 ml of 0.5 M, pH 7.5 stock
0.08% (w/v) bromophenol blue	8 mg

Store up to 1 year or longer at −20°C

Sodium acetate solution, 3 M, pH 5.2

Add 24.6 g of sodium acetate powder per 100 ml desired final volume of solution, adjust the pH to 5.2 with glacial acetic acid, and bring up to the desired final volume with water.

COMMENTARY

Background Information

RT-qPCR is a conceptually simple, well established, and sensitive method for RNA detection and quantification, the fundamental principles of which have been reviewed in detail elsewhere (Bustin et al., 2009; Heid et al., 1996; Higuchi et al., 1993; Rasmussen, 2001; Wittwer et al., 1997; Wong & Medrano, 2005). The advantages of RT-qPCR for clinical detection of viruses were realized early on (Beuret, 2004; Bustin & Mueller, 2005; Ishiguro et al., 1995; Monpoeho et al., 2002; Ozoemena, Minor, & Afzal, 2004), and the usefulness of this approach was demonstrated, in particular, during the first outbreak of SARS coronavirus (Hui et al., 2004; Jiang et al., 2004; Lau et al., 2003; Ng et al., 2003; Peiris et al., 2003; Poon et al., 2003; Poon et al., 2004). While other methods for rapid viral RNA detection have recently shown promise (Arizti-Sanz et al., 2020; Bloom et al., 2020; Dao Thi et al., 2020; Vonesch et al., 2020; Zhang et al., 2020), RT-qPCR has remained the state of the art for clinical diagnostics and has been the primary workhorse for SARS-CoV-2 testing.

Efforts to slow the spread of SARS-CoV-2 in the current pandemic were unfortunately impeded by a lack of diagnostic testing. This problem was exacerbated by supply shortages of the Thermo Fisher TaqPath RT-qPCR master mix, which the United States Centers for Disease Control (CDC) selected for its testing protocol (Centers for Disease Control and Prevention, 2020). During the peak of infections in New York City in spring 2020, the director of the city's public-health laboratory lamented that "there are labs that are running out [of

TaqPath] and begging other labs for a single tube so that they can get another day's worth of testing done" (Baird, 2020). In response to testing shortages, the Food and Drug Administration (F.D.A.) issued Emergency Use Authorizations to streamline approval of new laboratory-derived tests. Despite this, *The New Yorker* reported on March 24, 2020 that:

"The current trouble is a critical shortage of the physical components needed to carry out tests of any variety. Among these components are so-called viral transport media, which are used to stabilize a specimen as it travels from patient to lab; extraction kits, which isolate viral RNA from specimens once they reach the lab; and the reagents that do the actual work of determining whether the coronavirus that causes COVID-19 is present in the sample" (Baird, 2020).

Shortages like these could possibly be prevented in the future by empowering university labs to produce reagents themselves, following standardized recipes, for use in local "pop-up" testing centers (Aitken et al., 2020; Giadone et al., 2020; IGI Testing Consortium, 2020; Mascuch et al., 2020).

Although homemade PCR master mix recipes are widely used (Karsai, Müller, Platz, & Hauser, 2002; Pellissier, Glogowski, Heinemann, Ballivet, & Ossipow, 2006), published recipes for one-step RT-qPCR master mixes are rare (Bhadra, Maranhao, & Ellington, 2020; Mascuch et al., 2020). The bare-bones recipe presented in this article could potentially be improved by including features found in commercial master mixes, for instance, mutationally enhanced reverse transcriptases, more sophisticated variants of hot-start *Taq* polymerase, or a mixture of uracil N-glycosylase and dUTP to prevent contamination by the amplification products of previous reactions. Other thermostable DNA polymerases may, in principle, be substituted for *Taq* DNA polymerase in our master mix, but it is important to note that TaqMan reactions rely on the 5′-3′ exonuclease activity of the polymerase; polymerases lacking this activity will not work on their own for TaqMan-based RT-qPCR and need to be supplemented with a polymerase that has 5′-3′ exonuclease activity (Bhadra et al., 2020). Further refinements of the BEARmix RT-qPCR formulation will be posted on our GitLab page (*https://gitlab.com/tjian-darzacq-lab/bearmix*). We hope that continued development and dissemination of open-source RT-qPCR methods will help reduce the dependence of clinical testing centers and research labs on black-box commercial products. Hopeful signs are recent publications describing other testing methods based on homemade enzymes (Bhadra et al., 2020; Mascuch et al., 2020; Vonesch et al., 2020) and growing online resources for open-source molecular biology (OpenWetWare; Open Enzyme Collection; Pipette Jockey; see Internet Resources).

Although we have tested BEARmix with a single TaqMan primer/probe set, it should be possible to combine several primer pairs flanking TaqMan probes labeled with distinct fluorophores. A single reaction could, for instance, include multiple SARS-CoV-2 targets as well as a human RNA target to control for overall RNA extraction and recovery.

Direct addition of swab samples to RT-PCR reactions bypasses an RNA purification step, saving time and money and simplifying the testing workflow. A major disadvantage is that RNA is not concentrated, limiting the amount of sample RNA that can be added and, hence, the detection sensitivity. Unfortunately, commonly used swab-collection saline solutions such as UTM and V-C-M inhibit RT-PCR when at high concentrations, which restricts the amount of sample that can be added per reaction (Graham et al., 2021). Collection of samples in a low-salt proteinase K solution, followed by heat-inactivation (Basic Protocol 2), allows a much greater quantity of sample to be added per reaction, potentially providing a several-fold increase in sensitivity (Graham et al., 2021).

Critical Parameters

Protein purification is a multi-stage process involving several different buffers. It is helpful to prepare these buffers in advance. Reducing agents (β-mercaptoethanol and DTT) and the protease inhibitor benzamidine, however, should be added immediately before use, as these components can break down or oxidize over time.

When sonicating the resuspended bacteria during purification of M-MLV reverse transcriptase, be sure to submerge the tip completely to avoid "frothing" of the mixture, which can cause protein denaturation.

If using a vacuum aspirator to pull liquid through the Ni-NTA agarose gravity column, be careful not to pull air into the resin bed, as air bubbles will disrupt the flow of buffer over the resin. Likewise, be careful when using an FPLC to avoid introducing air bubbles into the system, which could get lodged in the column.

Because the specific activity of an enzyme may vary between different protein preps, it

may be helpful to adjust the enzyme concentrations recommended in Basic Protocol 1. For each new enzyme prep, it is a good idea to perform test reactions with various enzyme concentrations and choose a concentration that gives reliable amplification at low RNA concentrations.

Isopropanol precipitation (Alternate Protocol 1) may be used as a crude yet effective means of concentrating and partially purifying RNA for use in RT-PCR. A pitfall of this method is that RNA may be lost due to accidental aspiration of the pellet. While an experienced person can usually avoid this, it is essential to include a recovery control, such as a human RNA endogenously present in the sample or exogenous MS2 bacteriophage genomic RNA added to the sample prior to isopropanol precipitation, to check that RNA has been successfully recovered for each sample.

Successful RT-qPCR requires efficiently amplifying the correct target sequence while minimizing nonspecific amplification. Optimal primer design is crucial (Dugast-Darzacq & Grange, 2009), and online apps are available to assist with primer selection (e.g. PrimerQuest, from IDT *https://www.idtdna.com/Primerquest/Home/Index*). When initially establishing a testing protocol, it is best to evaluate multiple primer sets. For diagnostic testing, it is essential to choose primer sets that do not amplify nucleic acids from human cells or other pathogens. Demonstrating specificity in this way is a strict requirement for FDA approval of laboratory-derived tests (FDA Molecular Diagnostic Template for Laboratories, 2020). We obtained good results with the SARS-CoV-2 N1 and N2 primers recommended by the United States Centers for Disease Control (CDC), and various other well-validated primer sets for SARS-CoV-2 are available (Vogels et al., 2020). Raising the annealing temperature may, in some cases, improve the specificity of primer annealing.

Additionally, it is critical to avoid off-target amplification of "primer dimers," which occur when primer pairs briefly anneal to each other at short regions of complementarity and are extended by *Taq* polymerase. These side products can inhibit PCR amplification of the desired amplicon by competing for primers and polymerase, and will result in nonspecific fluorescence signal in SYBR-based assays. Primer-dimer formation may be prevented in several ways. First, primers should be designed with as little complementarity as possible, to minimize the potential for annealing to other primers. Second, it is critical that non-hot-start reactions be kept cold after the addition of primers and polymerase. It is easiest to do this by placing tubes and multiwell plates in metal blocks on ice. To minimize the time spent at room temperature, reactions should be transferred directly from the ice to a PCR block pre-heated to 50°C. Keeping reactions cold is more critical for some primer pairs than for others. In particular, we found that the SARS-CoV-2 N1 primer set was highly sensitive to room temperature incubation, while N2 reactions could be incubated at room temperature for 1 hr with no loss of amplification (Graham et al., 2021). Finally, primer-dimer formation may be avoided by using a hot-start polymerase that becomes active only after incubation at high temperature. Formaldehyde treatment is a cheap and easy way to produce hot-start versions of thermostable enzymes (Basic Protocol 1), although it has the disadvantage that enzyme activity may not be completely restored after high-temperature de-crosslinking. We have not thoroughly optimized the crosslinking step in Basic Protocol 1, and it may be possible to improve the performance of the hot-start enzyme by more extensively evaluating the effect of different parameters such as the formaldehyde concentration and crosslinking time.

TaqMan detection provides an extra layer of specificity by requiring that a hydrolysis probe oligonucleotide anneal within the target PCR amplicon. Dye-based reactions often show amplification at late cycles (cycle number > 30) due to accumulation of primer-dimer or other nonspecific products, and it is crucial to include negative control reactions in every experiment to measure this background level of nonspecific amplification. In TaqMan reactions, nonspecific amplification can cause "flattening" of qPCR curves, as nonspecific amplicons compete with specific amplicons for limiting polymerase molecules, primers, and dNTPs (see Troubleshooting).

Troubleshooting

Table 2 lists common problems that users may encounter when following the protocols, their causes, and potential solutions.

Understanding Results

The goal of diagnostic RT-qPCR is to classify samples as positive or negative for viral RNA. Samples are typically classified as positive if their Cq value is below a pre-defined threshold, and negative otherwise. The chosen threshold should distinguish between known positive and known negative samples. To

Table 2 Troubleshooting Guide for the Protocols in this Article

Problem	Possible cause	Recommended solutions
Low expression of *Taq* polymerase or M-MLV RT enzymes	• Use of an inappropriate *E. coli* strain	• Check that you are using an *E. coli* strain optimized for protein production based on transcription with T7 RNA polymerase
	• IPTG or antibiotic not added	• Be sure that the correct antibiotics have been added to the medium
	• Culture induced at non-optimal optical density	• Be sure to add IPTG to a final concentration of 1 mM when the culture reaches the optical density specified in the protocol
Precipitation of protein during dialysis steps	• Use of a buffer with the incorrect pH or ionic strength may cause protein aggregation	• Use a pH meter or pH test paper to check that the dialysis buffer is of the correct pH • Retry the protocol with freshly made dialysis buffer, being very careful to add the correct amount of NaCl
Protein does not bind to the heparin or SP Sepharose column	• Incorrect composition of buffers A and B	• Double-check that the dialysis buffer and buffers A and B contain the correct quantities of NaCl and are at the correct pH
	• Buffers A and B connected to wrong inlets	• Double-check that buffers A and B are connected to the correct inlets of the FPLC
	• Gradient elution incorrectly programmed	• Double-check that the FPLC has been correctly programmed to produce a linear gradient between an initial composition of 100% buffer A, 0% buffer B, and a final composition of 0% buffer A, 100% buffer B. Refer to the section of your FPLC manufacturer's manual that describes gradient elution
Variable recovery in isopropanol precipitation	• Accidental aspiration of pellets may lead to a complete loss of the sample	• Be careful to avoid aspirating at the very bottom of the tube on the side of the tube facing outward in the centrifuge. Use fine gel loading tips and ensure adequate lighting when aspirating, to avoid losing pellets.
	• Insufficient removal of isopropanol may make pellets impossible to redissolve.	• Carefully but thoroughly aspirate all traces of isopropanol, and allow pellets to air-dry at room temperature for a few minutes to ensure that all isopropanol has evaporated
No amplification observed in positive control reactions	• In vitro transcribed RNA may be degraded or not at the correct concentration	• Validate the primers and positive control RNA using a commercial one-step RT-qPCR master mix or a two-step reaction
	• Primers may be poorly designed	• Test the activity of *Taq* polymerase by performing PCR using a DNA template and primers that have been shown to work previously

(*Continued*)

Table 2 Troubleshooting Guide for the Protocols in this Article, *continued*

Problem	Possible cause	Recommended solutions
	• M-MLV reverse transcriptase or *Taq* polymerase may be defective or at the wrong concentration	• Retry the enzyme and master mix preps, and repeat the RT-qPCR with these new preps • Vary the concentration of enzymes added around the recommended values
Amplification observed in negative control for dye-based reactions	• Amplification of non-target sequences (e.g., from human DNA or RNA)	• Try using different primer pairs
	• Amplification of primer dimers	• Be especially careful to keep reactions on ice during setup, and transfer them directly to a pre-heated PCR block to avoid mis-annealing and primer dimer formation
	• Contamination of one or more reagents with in vitro transcribed RNA or the products of previous rounds of PCR	• Prepare fresh stocks of each reagent, and test whether this eliminates amplification in negative controls. Always work with concentrated in vitro−transcribed RNA or PCR amplicons as far away as possible from where RT-qPCR reactions are prepared. Use different sets of pipettes to set up RT-qPCR reactions and to work with concentrated in vitro transcribed RNA or amplified PCR products. Avoid opening finished RT-qPCR plates unless it is essential (e.g., to validate amplicons of new primer pairs by sequencing). • Note that for dye-based detection, there is almost always some nonspecific background amplification at late (> 30) cycles. This background amplification is not necessarily a problem if it is clearly distinguishable from specific amplification based on Cq value.
"Flat" amplification curves in hydrolysis probe reactions	• Depletion of primers and dNTPs by nonspecific amplification may produce curves that have an unusually low slope and a non-sigmoidal appearance	• Test for nonspecific amplification by including, in the same reaction, a dsDNA binding dye that is spectrally distinct from the hydrolysis probe (e.g., SYTOX Orange with a FAM-labeled probe). Nonspecific amplification is indicated by the appearance of dye-based signal in the absence of hydrolysis probe signal • Follow the recommendations given above to avoid primer dimers, and if possible try redesigning your primers • Increase the annealing temperature of the qPCR cycle by 1°-2°C to increase the specificity of annealing

(*Continued*)

Table 2 Troubleshooting Guide for the Protocols in this Article, *continued*

Problem	Possible cause	Recommended solutions
Upward drift of baseline fluorescence over the course of the qPCR in TaqMan reactions	• Exonuclease activity in one or both purified enzymes leads to slow hydrolysis of the TaqMan probe in the absence of amplification	• To account for baseline drift, perform baseline subtraction on the curves or use the second-derivative method to determine Cq values • If baseline drift is so severe that it interferes with quantification, retry the enzyme and master mix preps, and repeat the RT-qPCR with these new preps. Combine only the purest FPLC fractions in the final purification step, based on the appearance of a single dominant band in SDS-PAGE.

ensure that these results are reliable, it is necessary to perform careful control experiments with both a known viral RNA standard and clinical specimens.

First, it is necessary to know the minimum number of viral RNA molecules that must be present per reaction to detect amplification, also known as the limit of detection (LOD). FDA guidelines recommend preparing a series of 2-3 fold dilutions of a known viral RNA standard and performing several RT-qPCR replicates of each to determine the lowest concentration at which amplification is consistently observed (FDA Molecular Diagnostic Template for Laboratories, 2020). Dilution of known quantities of inactivated virus in a real "clinical matrix" (e.g., negative swab sample) is considered the gold standard for determining the LOD. This preliminary LOD is then verified by testing 20 replicates at the LOD concentration to check that at least 19/20 are positive. Clinically acceptable LODs are around 10^3-10^4 viral genomes per ml of swab sample (IGI Testing Consortium, 2020).

After determining the LOD, the assay must be validated using actual clinical specimens. At least 30 known positive and 30 known negative samples that have been tested using another trusted method are re-tested using the new assay. The results are considered reliable if there is at least 95% concordance between the two assays (FDA Molecular Diagnostic Template for Laboratories, 2020).

Additional controls are required within each assay. RNA recovery controls such as human RNA or spiked-in MS2 bacteriophage genomic RNA are used to ensure that RNA was successfully recovered during the RNA extraction or purification step. It is best to perform reactions in duplicate, if possible. If there is a lack of concordance between duplicates or between different SARS-CoV-2 probes, then, it is best to re-test those samples.

For research purposes, RT-qPCR is typically used not just to determine the presence or absence of an RNA but to measure its concentration. In this case, a calibration curve should be included in every experiment, consisting of at least 6 serial dilutions of an in vitro–transcribed RNA standard. The starting concentration and fold dilution of these standards should be adjusted such that the range of Cq values observed for the test samples falls between the highest and lowest Cq values in the calibration curve. As an additional internal standard, RT-qPCR should also be performed for reference RNAs whose expression level is not expected to differ between conditions. Additional guidelines for performing RT-qPCR and reporting data can be found in Bustin et al. (2009).

Time Considerations

Preparation of *Taq* polymerase and M-MLV reverse transcriptase (Basic Protocol 1) is most conveniently spread over 6 days. Bacterial transformation on day 1 requires about 1 hr. Picking colonies on day 2 requires about 5 min. Growing, inducing, and collecting large-scale cultures on day 3 requires 5-7 hr, with long waiting periods. It is convenient to prepare in vitro–transcribed RNA standards (Support Protocol) or buffers for subsequent steps during these waiting periods. Ni-NTA purification on day 4 requires about 6 hr, followed by an overnight dialysis step. FPLC purification and analysis of fractions by gel electrophoresis on day 5 requires about

3-4 hr, followed by a second overnight dialysis step. Dialysis and aliquoting of protein on day 6 requires about 2.5-3.5 hr. Hot-start *Taq* preparation by formaldehyde crosslinking adds an additional day to the protocol, with about 4 hr of incubation time and an overnight dialysis step. For the sake of efficiency, buffers for protein purification can be prepared a few days in advance, but mercaptoethanol, DTT, and protease inhibitors should only be added immediately before use.

Sample preparation for direct RT-qPCR (Basic Protocol 2) involves two 30-min incubation steps and can be completed in about 1 hr. Isopropanol precipitation (Alternate Protocol 1) requires about 2 min of total hands-on time per sample in addition to 1 hr of incubation and centrifugation steps. Setup of RT-qPCR reactions in Basic Protocol 3 typically requires about 20-30 min for a 96-well plate if RNA samples are pre-arrayed in a second 96-well plate and a multichannel pipette is used to transfer samples and master mix. This is strongly recommended, as pipetting individual samples is substantially more time consuming and error-prone. The RT-qPCR program requires about 1.5 hr. Imaging plates for endpoint detection (Alternate Protocol 2) requires 2-3 min.

Preparation of in vitro−transcribed RNA (Support Protocol) requires about 2 hr to perform the PCR, 1-1.5 hr to gel purify the product, and 5 min to set up an in vitro transcription reaction, which is allowed to incubate overnight. RNA purification the next day takes around 30 min to 1 hr.

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Thomas G.W. Graham: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing, Claire Dugast-Darzacq: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing, Gina M. Dailey: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing, Xavier Darzacq: Conceptualization, Funding acquisition, Resources, Supervision, Robert Tjian: Conceptualization, Funding acquisition, Resources, Supervision

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study. However, further potential refinements of the BEARmix RT-qPCR formulation will be posted on our GitLab page (*https://gitlab.com/tjian-darzacq-lb/bearmix*).

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Internet Resources

https://gitlab.com/tjian-darzacq-lab/bearmix

More details on the protocols in this article can be found at this URL.

https://openbioeconomy.org/projects/open-enzyme-collections/

Open Enzyme Collection.

https://openwetware.org/wiki/Main_Page

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PrimerQuest Tool.