Reverse Transcription-PCR (RT-PCR) of the SARS-CoV-2 S-gene for Sequencing

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1.0 Purpose

1.1 The purpose of this procedure is to describe a fast double-reaction amplification of the SARS-CoV-2 S-gene for subsequent sequencing. Full coverage of the S-gene requires two overlapping amplicons to be produced via separate RT-PCR reactions.

2.0 Definitions

2.1 RT-PCR: Reverse Transcription Polymerase Chain Reaction

2.2 Room temperature: 15-25°C

3.0 Equipment (Use Examples or Equivalent)

3.1 Sterile, nuclease-free 1.5 mL micro-centrifuge tubes

3.2 0.2 mL PCR reaction tube strips or plates

3.2.1 PCR 8-tube strips (Brand Tech Scientific Inc. Catalog. No. 781332)

3.2.2 PCR Plate, 96-well, semi-skirted, flat deck (Life Technologies, Catalog No. AB-1400)

3.2.3 TempPlate pierceable sealing foil, sterile (USA Scientific: Catalog No. 2923-0110)

3.2.4 Sealing Roller (BIO-RAD: Catalog No. MSR-0001)

3.2.5 Silicone compression mat (Sigma Aldrich: Catalog No. AXYCMFLAT)

3.3 Vortex

3.4 1.5 mL tube, 0.2 mL strip tube, and 96 well plate compatible centrifuge

3.5 Cold blocks for 0.2 mL and 1.5 mL PCR reaction tubes (ISC BioExpress)

3.6 Pipettes (10 µL, 20 µL, 200 µL, and 1000 µL)

3.6.1 Multichannel pipettes also recommended (20 µL, and 200 µL)

3.6.2 Corresponding aerosol barrier pipette tips

3.7 Disposable reagent reservoir, sterile (Axygen: Catalog No. RES-V-25-S)

3.8 96-well format PCR Thermocycler System (BioRad: T100)

3.9 1.5 mL tube, 8-tube strip, or 96-well format magnetic separation rack (Life Technologies DynaMag-2: Catalog No. 12321D, Alpaqua Magnum FLX: Catalog No. A000400)

3.10 DNA electrophoresis and visualization equipment (QIAxcel advanced: Catalog No. 9001941)

4.0 Reagents

4.1 Nuclease-free water

4.2 SuperScript™ IV One-Step RT-PCR System (Invitrogen: Catalog. No. 12594100 – 100 reactions)

4.3 SPRI beads (Beckman Coulter: Catalog No. A63880, A63881, A63882, or equivalent)

4.4 Molecular biology grade absolute ethanol

5.0 Primers

5.1 SARS-CoV-2 S-gene primers listed in Table 1 are from Integrated DNA Technologies Inc. (IDT) https://www.idtdna.com (or equivalent). Primers must be RNase Free HPLC purified.

5.1.1 Prepare 10 µM stocks of each individual primer.

5.1.2 Pool the 10 µM forward and reverse primers in a 1:1 ratio for both the S1 and S2 primer pools.

5.2 CDC provided primers are premixed at 1x strength and dried.

5.2.1 Reconstitute in 1 mL nuclease-free water.

Table 1: S1 and S2 primer pools

S1 primer pool			
Oligo	**# Bases**	**Sequence 5'-3'**	**µM in pool**
S1F_21358	29	ACAAATCCAATTCAGTTGTCTTCCTATTC	5
S1R_23813	22	TGCTGCATTCAGTTGAATCACC	5
S2 primer pool			
Oligo	**# Bases**	**Sequence 5'-3'**	**µM in pool**
S2F_23288	21	GTCCGTGATCCACAGACACTT	5
S2R_25460	24	GCATCCTTGATTTCACCTTGCTTC	5

6.0 Samples and Controls

6.1 Samples

6.1.1 Specimens collected from individuals with a positive COVID-19 diagnostic test that have been inactivated and extracted via filter or bead-based RNA extraction platform.

6.1.2 Propagated isolate derived from such clinical specimens and similarly inactivated and extracted.

6.2 Controls

Table 2: Positive and Negative Controls

Control	Material	Frequency	Expected Outcome
Positive	Twist Bioscience Custom NGS Spike Control e.g. Delta REF: 103885; Omicron REF: 103885 Diluted to 20K copies/µL Or Previously sequenced RNA from propagated isolate of currently circulating SARS-CoV-2 variant, Ct <20 Or Previously sequenced RNA from clinical sample of currently circulating SARS-CoV-2 variant, Ct <20	Every run	Amplicons detectable by electrophoresis
Negative	Water	Every run	Amplicons not detectable by electrophoresis

7.0 Safety Precautions

7.1 Adhere to the safety guidelines provided in the Biosafety in Microbiological and Biomedical Laboratories and follow all established site-specific safety procedures, including wearing proper personal protective equipment (PPE).

8.0 Comments and Questions

8.1 Please send comments and questions by email to CDC Influenza Division Technical Support: idseqsupport@cdc.gov.

9.0 Gather Reagents

9.1 Thaw and store at the indicated temperature during the procedure.

9.2 Flick/invert the reagent tubes to ensure they are well mixed, and spin down before opening.

9.3 **Keep enzymes at in a freezer or on a cold block (approximately -20°C)**

9.3.1 SuperScript IV RT Mix

9.4 **Keep in a refrigerator, cold block, or ice bucket (approximately 4°C)**

9.4.1 SSIV 2X Reaction Mix

9.4.2 S1 and S2 Primer Pool

9.4.3 Samples and Controls

9.5 **Room temperature (15-25°C).**

9.5.1 Nuclease-free water

9.5.2 SPRI beads (solid-phase reversible immobilization beads)

9.5.2.1 Vortex immediately before use

9.5.3 Freshly prepared 80% ethanol

10.0 RT-PCR Procedure

10.1 Combine the components of Table 3 to prepare two reaction master mixes, one for each S1 and S2 reaction, sufficient for all samples and controls.

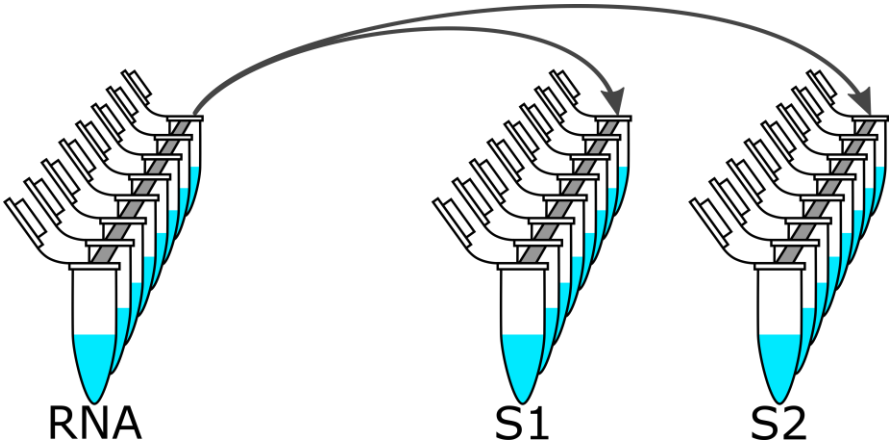
Table 3: RT-PCR master mix for each SARS-CoV-2 amplicon

Reagent	Volume (µL) Per Reaction	µL Per Master Mix
Nuclease-free Water	4.25	
SSIV 2X Reaction Mix	12.5	
SuperScript IV RT Mix	0.25	
S1 or S2 Primer Pool	5	
Subtotal	**22**	

10.1.1 Aliquot 22 µL of each reaction mix into respective wells of a 96-well PCR plate or into 0.2 mL PCR tubes.

10.1.2 For each sample, positive control, and negative control add 3 µL of RNA or water to an S1 master mix containing well and a corresponding S2 master mix containing well.

Figure 1:



10.2 Seal, gently mix, centrifuge, and incubate.

10.2.1 Securely seal to ensure no evaporation occurs.

Table 4: Cycling conditions		
Step	**Temperature (°C)**	**Time (mm:ss)**
1	50	10:00
2	98	2:00
3	98	0:10
4	60	0:10
5	72	1:15
6	Repeat steps 3-5 for 40 total cycles	
7	72	5:00
8	4	Hold

11.0 Amplicon QC

11.1 QC amplicons via electrophoresis.

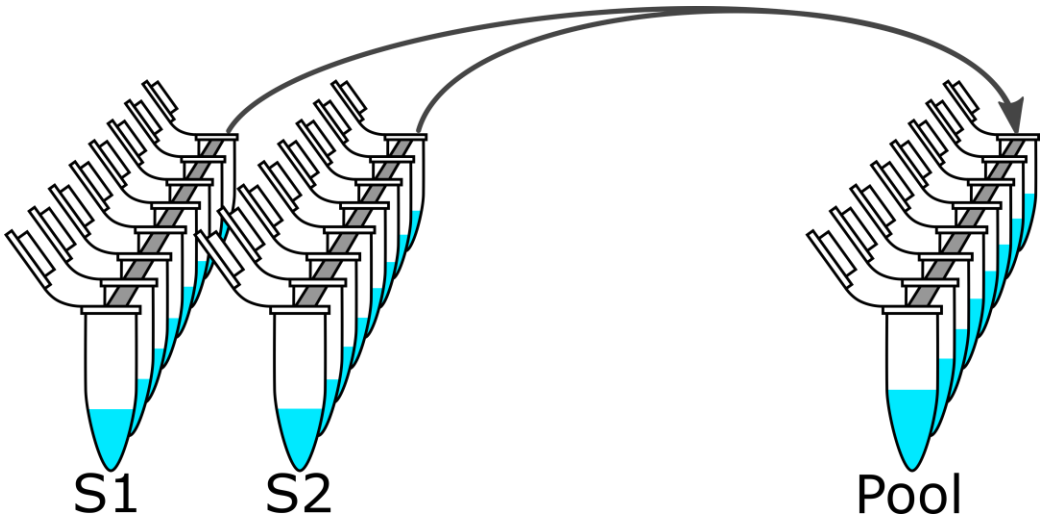
11.1.1 S1: 2.5 kb

11.1.2 S2: 2.2 kb

12.0 Combining S1 and S2 Amplicons

12.1 Combine corresponding S1 and S2 reactions into 50 µL amplicon pools.

Figure 2:



13.0 SPRI Bead Clean Up with Ethanol Wash and Water Elution

13.1 Add 1x (50 µL) of SPRI beads to each sample, mix gently, and incubate at room temperature for 5 minutes.

13.2 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

13.2.1 Remove and discard the supernatant.

13.3 With the samples on the magnet and without disturbing the pellet:

13.3.1 Add 200 µL of 80% ethanol to each sample.

13.3.2 Immediately remove and discard the ethanol.

13.3.2.1 Do not allow the beads to dry to the point of cracking.

13.3.2.2 Proceed immediately to the next step.

13.4 With the sample on the magnet and without disturbing the pellet:

13.4.1 Add 200 µL of 80% ethanol to each sample.

13.4.2 Immediately remove and discard the ethanol.

13.4.2.1 Do not allow the beads to dry to the point of cracking.

13.4.2.2 Proceed immediately to the next step.

13.5 Spin down the samples, pellet on a magnet for 10 seconds or until the beads collect to one side.

13.5.1 Remove and discard any residual ethanol.

13.5.1.1 Do not allow the beads to dry to the point of cracking.

13.5.1.2 Proceed immediately to the next step.

13.6 Remove from the magnet, add 15 µL of water to each sample, gently resuspend, and incubate at room temperature for 10 minutes.

13.7 Spin down the sample, pellet on a magnet for 2 minutes or until the supernatant is clear.

13.5.2 Remove and retain the supernatant (cleaned amplicons) in a new plate.

14.0 Proceed to Sequencing

14.1 The amplicons produced here are suitable for nanopore sequencing as described in LP-512 – Native Library Preparation and Nanopore Sequencing of Influenza A Virus and SARS-CoV-2 S-gene Amplicons.

14.2 The user may decide to sequence the amplicons produced here via other methods of library preparation and or other sequencing platforms that are suitable for 2.2 kb and 2.5 kb amplicons; however, it is the responsibility of that user to validate the chosen sequencing method. It is not recommended to use sequencing data that is of partial or low coverage or low quality.

15.0 Related Procedures

15.1 LP-512 – Native Library Preparation and Nanopore Sequencing of Influenza A Virus and SARS-CoV-2 S-gene Amplicons

16.0 References

16.1 User Guide: SuperScript™ IV One-Step RT-PCR System (Invitrogen)

16.2 Biosafety in Microbiological and Biomedical Laboratories (BMBL), current edition