

Detection of RNA from SARS-CoV-2 using fragment analysis

Overview

This protocol describes a method for detecting RNA fragments from SARS-CoV-2 (the coronavirus that causes COVID-19) by capillary electrophoresis (CE). Briefly, purified nucleic acid is mixed with a cocktail of fluorescent primers that are specific to the nucleoprotein (N protein), spike protein (S protein), and *orf-1ab* sequences in the SARS-CoV-2 genome. Primer pairs in the cocktail have been designed to generate well-separated, gene-specific amplicons. The sample–primer mixture is reverse transcribed and subjected to endpoint PCR in a single reaction. The resulting fragments are separated by size and detected using an Applied Biosystems™ genetic analyzer. This method provides a simple workflow for researchers to detect RNA from SARS-CoV-2.

This protocol and the reagents described within are for Research Use Only. Not for use in diagnostic procedures.

Materials

- Micropipettors
- Vortex mixer
- Applied Biosystems™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit
- Applied Biosystems™ TaqMan® Fast Virus 1-Step Master Mix
- Applied Biosystems™ MicroAmp™ Clear Adhesive Film
- 5′-labeled/unlabeled primer pairs (see below for sequences)
- Tris-EDTA (TE) buffer
- Applied Biosystems™ VetMAX™ Xeno™ Internal Positive Control RNA
- Applied Biosystems™ 96-well PCR plate
- Plate centrifuge for 96-well plates
- Applied Biosystems™ Veriti™ 96-Well Fast Thermal Cycler or equivalent
- Applied Biosystems™ Hi-Di™ Formamide (stored at –20°C)
- Applied Biosystems™ DS-33 Matrix Standard Kit (Dye Set G5)
- Applied Biosystems™ GeneScan™ 600 LIZ™ Dye Size Standard v2.0
- Applied Biosystems™ SeqStudio™ or 3500 Series Genetic Analyzer
- Applied Biosystems™ GeneMapper™ software or Peak Scanner app (apps.thermofisher.com)

Sample purification

This protocol utilizes nucleic acids purified from respiratory samples. Many different nucleic acid extraction instruments and kits are available. Follow the manufacturer's instructions and use the appropriate reagents for extracting viral RNA. If you are not using the purified nucleic acids immediately, store them according to the conditions suggested in the manufacturer's protocol.

Fragment analysis

1. 10X PCR primer master mix

- Sequences for the primers listed below can be found in the "Sequences" section.
- Combine:

Component	Quantity
<i>orf-1ab</i> 5'-labeled forward primer (80 µM stock)	5 µL
<i>orf-1ab</i> reverse primer (80 µM stock)	5 µL
S protein gene 5'-labeled forward primer (80 µM stock)	5 µL
S protein gene reverse primer (80 µM stock)	5 µL
N protein gene 5'-labeled forward primer (80 µM stock)	5 µL
N protein gene reverse primer (80 µM stock)	5 µL
Xeno RNA 5'-labeled forward primer (80 µM stock)	5 µL
Xeno RNA reverse primer (80 µM stock)	5 µL
TE buffer	460 µL
Total volume	500 µL

2. Reverse transcription and PCR amplification

- Thaw all reagents listed below on ice.
- Vortex assays briefly to mix, then centrifuge.
- If previously frozen, mix thawed nucleic acid samples by gentle inversion or flicking 3–5 times, then centrifuge briefly.
- Prepare a master mix using the amounts of components shown in the table below:

IMPORTANT: Change the pipette tip after the transfer of each component.

Note: The TaqMan Fast Virus 1-Step Master Mix is a 4X formulation and is viscous. Use appropriate caution when drawing the mix into the pipette.

Component	Quantity per reaction	Quantity in master mix for one 96-well plate*
TaqMan Fast Virus 1-Step Master Mix (4X)	2.5 µL	265 µL
10X PCR primer master mix	1.0 µL	106 µL
Genomic DNA (optional), 10 ng/µL**	1.0 µL	106 µL
Xeno RNA spike-in control	0.5 µL	53 µL
Sample + DNase/RNase-free water†	5.0 µL	
Total volume of one reaction	10 µL	
Total volume of master mix		530 µL

* The volumes shown for a 96-well plate include 10% extra volume (96 x 110% = 106) to compensate for the volume loss that occurs during pipetting. If setting up fewer than 96 reactions, adjust accordingly.

** Adding nonspecific genomic DNA may help prevent losses with very dilute samples, but inclusion is optional.

† Depending on the quantity of sample that is available, either 5 µL of undiluted sample can be used, or a smaller amount can be brought up to a total volume of 5 µL with DNase/RNase-free water.

- e. Label a 96-well plate “PCR plate” and add 5 μL of master mix to each plate well.
- f. Add samples to the master mix in the wells.
- g. Seal the plate with MicroAmp Clear Adhesive Film.
- h. Vortex plate to mix. Be sure all components are thoroughly mixed in all the wells before proceeding.
- i. Centrifuge at 2,000 rpm for 1 min in a plate centrifuge to collect the contents and reduce air bubbles.
- j. Run the PCR using the following program:

	Stage/step					
	Reverse transcription	Polymerase inactivation	Cycling (40 cycles)		Final extension	Hold
			Denature	Anneal and extend		
Temperature	50°C	95°C	95°C	60°C	72°C	4°C
Time	15 min	2 min	3 sec	45 sec	7 min	Indefinite

- k. Place the plate on ice or store the plate at 4°C until ready for electrophoresis. For longer-term storage, place plates at -25°C to -15°C .

3. Prepare samples for electrophoresis

- a. Remove a tube of Hi-Di Formamide from the freezer and thaw at room temperature.
IMPORTANT: Avoid excessive freeze-thaw cycles of Hi-Di Formamide. If necessary, aliquot and freeze 500 μL in separate tubes.
- b. Prepare the running solution using the amounts shown:

Component	Quantity per sample	Quantity in running solution for one 96-well plate*
Hi-Di Formamide	8.5 μL	901 μL
GeneScan 600 LIZ Size Standard	0.5 μL	53 μL
Completed PCR reaction	1.0 μL	
Total volume of one sample	10 μL	
Total volume of running solution		954 μL

* The volumes shown for a 96-well plate include 10% extra volume ($96 \times 110\% \approx 106$) to compensate for the volume loss that occurs during pipetting. If setting up fewer than 96 reactions, adjust accordingly.

- c. Dispense 9 μL of running solution into wells of a 96-well plate.
- d. Add 1 μL of completed PCR reaction to each well.
- e. Seal the plate with MicroAmp Clear Adhesive Film, vortex for 2–3 sec, and centrifuge at 2,000 rpm for 1 min.
- f. Set the thermal cycler to 95°C and heat the plate for 3 min to denature fragments. Then place the plate at 4°C or on ice until loaded onto the CE instrument. Denatured samples can be stored at 4°C for several days if they won't be run immediately on the CE instrument, or frozen at -20°C for longer-term storage.

Note: Protect the plate from light until ready to run.

4. Electrophoresis

- a. Confirm that the CE instrument has been calibrated with the correct dye matrix for the chosen fluorophores. Use the DS-33 Matrix Standard Kit (Dye Set G5) for 6-FAM™, VIC™, NED™, PET™, and TED™ dyes. Contact your local Applied Biosystems™ field service engineer (FSE) for assistance with calibration, if needed.
- b. Remove the MicroAmp Clear Adhesive Film and replace with a 96-well plate septum.
- c. Load the plate onto the genetic analyzer. For 3500 Series instruments, load the plate into the plate assembly. For the SeqStudio instrument, place the plate into the autosampler and close the lid.
- d. Select or create an appropriate run module according to your capillary length, number of capillaries, and polymer type. The recommended default run modules are listed below:
 - i. For 3500 Series instruments:
 1. FragmentAnalysis50_POP7_G5
 2. FragmentAnalysis50_POP6_G5
 3. FragmentAnalysis50_POP4_G5
 - ii. For the SeqStudio instrument:
 1. FragAnalysis; choose G5 dye set

Note: Be sure to select the run module that corresponds to the size of your array.

5. Analyze using GeneMapper software or the Peak Scanner cloud app

- a. Fragments generated by VIC dye-labeled primers should be approximately the following lengths:
 - i. Xeno RNA control 327 nucleotides
 - ii. N protein 396 nucleotides
 - iii. *orf-1ab* 425 nucleotides
 - iv. S protein 437 nucleotides

Note: Panels, bins, and analysis methods for use in GeneMapper software or the Peak Scanner app can be downloaded at http://resource.thermofisher.com/page/WE28396_1/

Note: Different fluorophores might produce slight differences in fragment migration lengths.

- b. Guidelines for interpreting results:
 - i. If the Xeno RNA peak is not present, then no call is possible since no cDNA was generated. Try repeating the sample, starting from the amplification step.
 - ii. If the Xeno RNA peak is present but no SARS-CoV-2 RNA peaks are present, the sample is negative.
 - iii. If the Xeno RNA peak is present and only one of the SARS-CoV-2 RNA peaks is present, the sample is indeterminate. Try running the sample again, starting from the amplification step and using more input material.
 - iv. If the Xeno RNA peak is present and two or three of the SARS-CoV-2 RNA peaks are present, the sample is positive.

c. An example experiment analyzed using GeneMapper software is shown below (Figure 1).

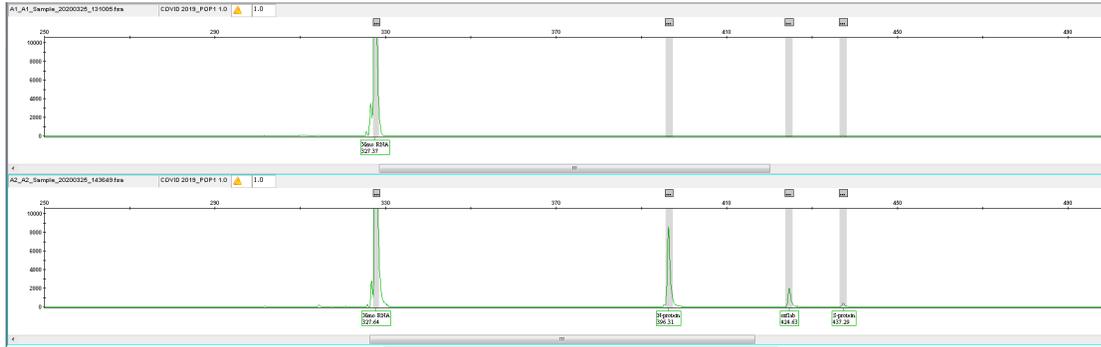


Figure 1. Example data generated using this protocol. The top panel shows a negative result, with the Xeno RNA peak but no peaks specific to SARS-CoV-2. The bottom panel shows a positive result, from a sample containing 50 copies of the viral genome in a reaction, with the Xeno RNA peak and the three SARS-CoV-2 peaks. Differences in peak heights reflect the different amounts of the target sequence in that sample.

Sequences

Oligonucleotide primers

Oligonucleotide primers can be ordered at <https://www.thermofisher.com/order/custom-oligo/fluorescent-labeled-primer-pairs>. Ensure that the forward primers are labeled with a fluorescent dye, and the reverse primers are unlabeled. Oligos can be ordered desalted and dried; resuspend each dried oligo to a final concentration of 80 μ M in TE buffer.

Note: The protocol was developed using VIC dye-labeled primers. Other fluorophores in the G5 dye set (e.g., 6-FAM, NED, or PET) can be used but may demonstrate slight differences in performance.

<i>orf-1ab</i> forward	5'-TGCCTGGAATATTGGTGAA-3'
<i>orf-1ab</i> reverse	5'-ACAATTTCAACAAGCACAGGTTGAG-3'
S protein forward	5'-TCGAAGACCCAGTCCCTACTTATT-3'
S protein reverse	5'-CTGAAGAAGAATCACCAGGAGTCAA-3'
N protein forward	5'-GGACCAGGAAC TAATCAGACAAGGA-3'
N protein reverse	5'-TTAGGCCTGAGTTGAGTCAGC-3'
Xeno RNA control forward	5'-GCTGACTCCAGTGGTCAAAC-3'
Xeno RNA control reverse	5'-ACCCTTGCTAGTAGGTGTAGATTCTC-3'

DNA control sequences

DNA control fragments can be ordered through the Invitrogen™ GeneArt™ Instant Designer Gene Synthesis Portal. Resuspend individual DNA fragments in TE (aliquots can be combined into a single control sample mix). Be careful to avoid contaminating other reagents used in the detection reaction with the DNA templates.

orf-1ab

TGCCTGGAATATTGGTGAACAGAAATCAATACTGAGTCCTCTTTATGCATTTGCATCAGAGGCTGCTCGTGTGTAC
GATCAATTTTTCTCCCGCACTCTTGAACTGCTCAAAATTTCTGTGCGTGTTTTACAGAAGGCCGCTATAACAATACTA
GATGGAATTTTACAGTATTTCACTGAGACTCATTGATGCTATGATGTTTACATCTGATTTGGCTACTAACAATCTAGT
TGTAATGGCCTACATTACAGGTGGTGTGTTTTCAGTTGACTTCGCAGTGGCTAACTAACATCTTTGGCACTGTTTATG
AAAACTCAAACCCGTCCTTGATTGGCTTGAAGAGAAGTTTAAGGAAGGTGTAGAGTTTCTTAGAGACGGTTGGGAA
ATTGTTAAATTTATCTCAACCTGTGCTTGTGAAATTGT

S protein

TCGAAGACCCAGTCCCTACTTATTGTTAATAACGCTACTAATGTTGTTATTTAAAGTCTGTGAATTTCAATTTTGTA
TGATCCATTTTTGGGTGTTTATTACCACAAAAACAACAAAAGTTGGATGGAAAGTGAGTTCAGAGTTTATTCTAGTG
CGAATAATTGCACTTTTGAATATGTCTCTCAGCCTTTTTCTTATGGACCTTGAAGGAAAACAGGGTAATTTCAAAAAT
CTTAGGGAATTTGTGTTTAAAGAATATTGATGGTATTTTTAAAATATATTTAAGCACACGCCTATTAATTTAGTGCG
TGATCTCCCTCAGGGTTTTTCGGCTTTAGAACCATTGGTAGATTTGCCAATAGGTATTAACATCACTAGGTTTCAA
CTTTACTTGCTTTACATAGAAGTTATTTGACTCCTGGTATTCTTCTTCAG

N protein

GGACCAGGAACATAATCAGACAAGGAAGTATTACAAACATTGGCCGCAAATTTGCACAATTTGCCCCAGCGCTTCAG
CGTTCTTCGGAATGTGCGCATTGGCATGGAAGTACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAA
TTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGTGTAATAAGCATATTGACGCATACAAAACATTCCC
ACCAACAGAGCCTAAAAGGACAAAAGAAGAAGGCTGATGAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGC
AAACTGTGACTCTTCTTCTGCTGCAGATTTGGATGATTTCTCAAACAATTGCAACAATCCATGAGCAGTGCTGAC
TCAACTCAGGCCTAA

Ordering information

Product	Quantity	Cat. No.
MagMAX Viral/Pathogen Nucleic Acid Isolation Kit	1 kit	A42352
TaqMan Fast Virus 1-Step Master Mix	1 mL	4444432
MicroAmp Clear Adhesive Film	100 films	4306311
5'-labeled/unlabeled primer pairs	10 nmol	450056
VetMAX Xeno Internal Positive Control RNA	100 reactions	A29763
Veriti 96-Well Fast Thermal Cycler	1 instrument	4375305
Hi-Di Formamide	25 mL	4311320
DS-33 Matrix Standard Kit (Dye Set G5)	8 runs	4345833
GeneScan 600 LIZ Dye Size Standard v2.0	800 reactions	4408399
SeqStudio Genetic Analyzer	1 system	A35644
3500 Genetic Analyzer for Fragment Analysis	1 system	A30468
GeneMapper Software 6, full installation	1 license	4475073

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