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Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE updated

Forked from Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE

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1 Works for me dx.doi.org/10.17504/protocols.io.bqzrmx56

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ABSTRACT

The Stanford University and Purigen Biosystems team's SARS-CoV-2 assay leverages on-chip microfluidics to eliminate laborious and time consuming steps associated with standard molecular diagnostics such as solid phase spin-column extraction and PCR amplification. Purification of nucleic acids from a variety of biological sources is achieved in a one-step, automated fashion using on-chip isotachophoresis (ITP). The purified nucleic acids are then amplified using reverse transcription (RT) loop-mediated isothermal amplification (LAMP) in 30 minutes, less than half of the time associated with standard qPCR. We then use CRISPR-Cas12 fluorescent detection to identify amplicons associated with the SARS-CoV-2 genome for enhanced specificity.

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FORK NOTE

FORK FROM

Forked from Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE, ashwinrc

KEYWORDS

CRISPR-diagnostics, microfluidics, SARS-CoV-2, RNA, Nucleic acid test, Isotachophoresis, Stanford, Purigen, RT-LAMP, COVID-19, CRISPR-Cas12

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MATERIALS TEXT

Materials for Purigen Ionic Install:

Required Materials

Table2: Contents provided in the Virtual Installation and Training Kit

Item Name	Description
Ionic System Cleaning Chip	1 plastic cleaning chip
Leveling Kit	1 Leveling tool and charging cable
Web Camera	1 Adjustable web camera on stand
USB Flash Drive	1 64 G USB flash drive

Table 3: Contents provided in the Ionic System Installation and Training Kit

Item Name	Description
System Validation Kit	2 fluidic chips, 2 reservoirs, and reagents
Reference Samples	16 cell or FFPE tissue reference samples
Reagent Reservoirs	10 reagent reservoirs
Training Purification Kit	3 fluidic chips and reagents for the following protocols: • Viral RNA - PBS • Viral RNA - Saliva • Viral RNA - Water 3 fluidic chips for pipetting practice only 50, 1.5 mL Eppendorf LoBind DNA Tubes

Equipment provided by Purigen

Citation: jnesvet (01/07/2021). Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE updated. https://dx.doi.org/10.17504/protocols.io.bqzrmx56

Table 4: Equipment Provided by the user

User-Supplied Equipment	Installation	Training
Scissors	✓	
Box Cutter	✓	
P200 Multichannel Pipette	✓	✓
P200 Single Channel Pipette	✓	✓
P1000 Single Channel Pipette	✓	✓
P20 Single Channel Pipette	✓	✓
Pipette Tips	✓	✓
Microcentrifuge	✓	✓
Programmable Thermomixer	✓	✓
Vortex Mixer (adjustable speed)	✓	✓
15 mL centrifuge tube		✓

User Provided Laptop

Laptop or PC for RingCentral Webconference

1. Operating System
   - Windows 10
2. Connecting Port Type
   - USB2.0, or 3.0 High Speed
3. Network
   - Internet connection required
4. Audio
   - Must have functioning speakers and microphone. Use of a headset is not recommended but can be used if it provides enough flexibility to manipulate the instrument and perform basic laboratory tasks.
5. RingCentral App
   - Install RingCentral app: https://app.ringcentral.com/unified-login

⚠ Please **test the compatibility of your laptop or PC** with RingCentral prior to your scheduled installation and training

Equipment provided by the user for the Purigen Install

Materials for Purigen Ionic Extraction:

Citation: jnesvet (01/07/2021). Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE updated. https://dx.doi.org/10.17504/protocols.io.bqzrmx56

Kit Contents

Table1: Contents provided in the Viral RNA Kit

Container	Item Name	Description	Volume	Quantity per Kit
-20ºC Reagents Box	Lysis 1	Lysis Buffer 1	1.4 mL	6
-20ºC Reagents Box	Proteinase K	Proteinase K Reagent	1.0 mL	1
RT Reagents Box	Lysis 2	Lysis Buffer 2	4.0 mL	1
RT Reagents Box	1 - Extraction Buffer	Extraction Buffer	12.0 mL	1
RT Reagents Box	2 - Anodic Buffer	Anodic Buffer	12.0 mL	1
RT Reagents Box	3 - Separation Buffer	Separation Buffer	2.5 mL	6
RT Reagents Box	4 - Neutralization Buffer	Neutralization Buffer	12.0 mL	1
RT Reagents Box	5 - Cathodic Buffer	Cathodic Buffer	12.0 mL	1
Fluidic Chip Set Box	Purigen Ionic Fluidic Chip	Fluidic Chips	N/A	6

Table 2: Reagents Provided by the User

User-Supplied Reagents	For Lysate Preparation	For Purification
Viral Samples in PBS, Saliva or Water	✓	

Table 3: Equipment Provided by the User

User-Supplied Equipment	For Lysate Preparation	For Purification
12-column Reservoir (Agilent 204365-100)		✓
P200 Multichannel Pipette		✓
P200 Single Channel Pipette	✓	
P1000 Single Channel Pipette	✓	✓
P20 Single Channel Pipette		✓
Microcentrifuge	✓	
Programmable ThermoMixer	✓	
Vortex Mixer (adjustable speed)	✓	✓
15 mL centrifuge tube (or larger if preparing for more than 6 chips)	✓	

Table 4: Labware Provided by the User

User-Supplied Equipment	For Lysate Preparation	For Extract Collection
DNA LoBind Tube, 1.5 mL (Eppendorf 22431021)	✓	✓
Optional DNA LoBind Tube, 2.0 mL (Eppendorf 22431048)	✓	
Optional DNA LoBind Plate, 96 well (Eppendorf 951032000)		✓

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Kit contents and Equipment provided by the user for the Purigen Extraction

Materials list for LAMP and CRISPR reaction steps:

Citation: jnesvet (01/07/2021). Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE updated. https://dx.doi.org/10.17504/protocols.io.bqzrmx56

A	B	C
Reagent or Consumable	**Supplier**	**Catalog #**
10x LAMP reaction buffer	Custom	N/A
25x primers	Custom	N/A
WarmStart Bst2.0 DNA Polymerase	New England Biolabs	M0538L
WarmStart RTx Reverse Transcriptase	New England Biolabs	M0380L
RNP mix	Custom	N/A
Reporters	Custom	N/A
LAMP Positive control (PC)	Custom	N/A
Nuclease free water	Thermo Fisher	10977015

Equipment and consumables:

- Pipette (P10, P20 and P200)
- Pipette tips (10 µL, 20 µL and 200 µL)
- 8-tube strips or 96 well plates
- 1.5 mL Eppendorf tubes
- Thermal cycler or Plate reader (any company's product capable of fluorescence readout)

Primers and guide RNA sequences used in this assay:

A	B
LAMP primer	**Sequence (5'-3')**
E-gene F3	CCG ACG ACG ACT ACT AGC
E-gene B3	AGA GTA AAC GTA AAA AGA AGG TT
E-gene FIP	ACC TGT CTC TTC CGA AAC GAA TTT GTA AGC ACA AGC TGA TG
E-gene BIP	CTA GCC ATC CTT ACT GCG CTA CTC ACG TTA ACA ATA TTG CA
E-gene LF	TCG ATT GTG TGC GTA CTG C
E-gene LB	TGA GTA CAT AAG TTC GTA C
Guide RNA and reporter sequences	Sequence (5'-3')
E gene guide RNA	UAA UUU CUA CUA AGU GUA GAU GUG GUA UUC UUG CUA GUU AC
ssDNA reporter	/56-FAM/TTATT/3IABkFQ/

Note that the LAMP primers and reporters are already part of the "**Materials list for LAMP and CRISPR reaction steps**", and the sequences here are provided only for reference

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ABSTRACT

The Stanford University and Purigen Biosystems team's SARS-CoV-2 assay leverages on-chip microfluidics to eliminate laborious and time consuming steps associated with standard molecular diagnostics such as solid phase spin-column extraction and PCR amplification. Purification of nucleic acids from a variety of biological sources is achieved in a one-step, automated fashion using on-chip isotachophoresis (ITP). The purified nucleic acids are then amplified using reverse transcription (RT) loop-mediated isothermal amplification (LAMP) in 30 minutes, less than half of the time associated with standard qPCR. We then use CRISPR-Cas12 fluorescent detection to identify amplicons associated with the SARS-CoV-2 genome for enhanced specificity.

BEFORE STARTING

Wear appropriate PPE including gloves, lab coat, goggles.
Raw NP swab samples must be handled according to BSL-2 safety level or higher.

Preparation for Purigen Ionic Instrument

1 Prepare your laboratory for the receipt of the Purigen Ionic Instrument.

Ionic™ Purification System Site Prep Guide - Version 3.0.pdf

Note: When handling nucleic acids, it is critical to reduce contamination. Therefore, we suggest 3 separate preparation areas: 1) a "patient sample preparation area" for viral RNA extraction with the Purigen Ionic Instrument and dispensing sample into LAMP reaction 8-strip tubes or 96 well plates, 2) a "LAMP reagent preparation area" for preparing the LAMP mastermix and dispensing it into 8-strip tubes or 96 well plates prior to viral RNA addition, and 3) a "CRISPR/amplicon preparation area" for preparing the CRISPR reaction mix and performing the CRISPR reactions.

To aid in reducing contamination, spray surfaces, vortex, microcentrifuge, pipettes, and all reagent tubes with RNase Away or RNase Zap prior to use. Change gloves when necessary.

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2 Complete Purigen Ionic installation and training with Purigen's Field Application Support.

Ionic System Virtual Installation and Training Guide 12202020.pdf

During the training run with the Field Application Support, you will process positive and negative control samples. The eluates can be stored at **-80 °C** for future downstream processing with LAMP and CRISPR, or stored on ice for same day downstream LAMP and CRISPR processing to ensure the positive and negative controls are validated.

Viral RNA Extraction from Patient Samples

3 Purify viral RNA using the Purigen Ionic Viral RNA kit.

Ionic Viral RNA Kit User Guide 20200106.pdf

This is a safe stopping point. If you are not proceeding directly to LAMP/CRISPR, store the resulting viral RNA samples on ice for same-day use, at -80 for long-terms storage.

Program thermal cycler for LAMP 30m

4 30m

LAMP can be performed on any thermal cycler. Here, we show the program on the Bio-Rad CFX Maestro software, but users can adjust the programs in their own instrumentation accordingly.

Program the thermal cycler to hold **62 °C** for **00:30:00** . Save the protocol as "Stanford Purigen LAMP protocol" for future use.

4.1 If applicable, set the lid heater to a value greater than **62 °C** . We recommend **100 °C** .

4.2 No fluorescence readouts are required for the LAMP amplification step. Adjust the program accordingly, if necessary.

Preparation of LAMP Mastermix

5

To reduce cross-contamination, prepare the LAMP mastermix and dispense in 8-tube strips or 96 well plates in an area separate from the patient sample preparation area and the CRISPR/amplicon preparation area.

Decontaminate LAMP reagent preparation area with RNase Away or RNase Zap spray, including all surfaces and

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pipettes. Thaw out 10x LAMP reaction buffer and 25x primers on ice, keep Bst2.0 WarmStart DNA Polymerase and WarmStart RTx Reverse Transcriptase on ice.

6 After thawing, briefly vortex 10x LAMP reaction buffer and 25x primers, while inverting Bst2.0 WarmStart DNA Polymerase and WarmStart RTx Reverse Transcriptase to mix. Collect reagents at the bottom of the tubes with a brief centrifuge on benchtop microcentrifuge. Keep all reagents on ice, decontaminate all reagent tubes with RNase Away or RNase Zap prior to use.

7 Determine number of reactions (*N*) to prepare for each batch of LAMP amplifications. Each patient sample should have duplicate LAMP reactions performed. Additionally, be sure to include one LAMP Positive Control (PC) and one No Template Control (NTC) in each batch. Mastermix should be prepared in slight excess to account for pipette error, therefore the total number of reactions (*N*) = (2.2 x [# patient samples]) + 1 PC + 1 NTC. So for example, if you were to amplify all 8 patient samples from a single chip, *N* = (2.2 x 8) + 1PC + 1 NTC = 19.6

7.1 Calculate the volume of each reagent required for N total reactions according to the table below.

Reagent	Mastermix Reagent Volume
10x Isothermal Amplification Buffer	N x 2.5 uL
25x primers	N x 1.0 uL
WarmStart Bst2.0 DNA Polymerase	N x 1.0 uL
WarmStart RTx Reverse Transcriptase	N x 0.5 uL
dNTP mix	N x 3.5 uL
Total Mastermix Volume	N x 8.5 uL

8 Organize the 8-tube strips or 96 well plate for the given *N* number of reactions. As an example, Patient Samples 1-8 (P1-P8) duplicates plus a PC and NTC are shown below. Please scale accordingly. Label the strips with the corresponding patient sample number.

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1 A	P1 B	PC									
B	P2 A	P2 B	NTC									
C	P3 A	P3 B										
D	P4 A	P4 B										
E	P5 A	P5 B										
F	P6 A	P6 B										
G	P7 A	P7 B										
H	P8 A	P8 B										

*Note that because no fluorescent data is collected in this step, the top of the tube can be labeled.

9 Dispense mastermix reagents in a clean 1.5 mL microcentrifuge tube. Vortex to thoroughly mix mastermix. Briefly centrifuge to collect reagents at bottom of tube.

10 Set up 8-tube strips or 96 well plate in a 96-well cooler rack or over ice.

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10.1 Dispense **8.5 µl** of mastermix into each tube or well.

11 Prepare NTC reactions in reagent preparation area. Decontaminate Nuclease Free Water (Nuc-free H2O) with RNase Away or RNase Zap, then pipette **16.5 µl** into appropriately labeled tubes and securely cap them. Now, you can move the strips loaded with the mastermix to the patient sample preparation area.

Patient Sample Template Addition

12 Briefly vortex viral RNA eluates collected from the Purigen Ionic. Centrifuge to collect contents at bottom of the tube. Keep patient samples in cold rack or on ice.

13 Carefully dispense **16.5 µl** of patient sample eluates into the corresponding wells containing the **8.5 µl** of mastermix . Pipette the sample up and down 5 times to mix, *DO NOT VORTEX.* Change tips after each addition to reduce contamination

14 Carefully dispense **16.5 µl** of the LAMP positive control (LAMP Pos Ctrl) to appropriate tube or well.

15 Securely cap wells after addition, briefly centrifuge 8-tube strips or 96 well plates to collect samples at bottom.

Perform 30 Minute LAMP Reaction on Thermal Cycler 30m

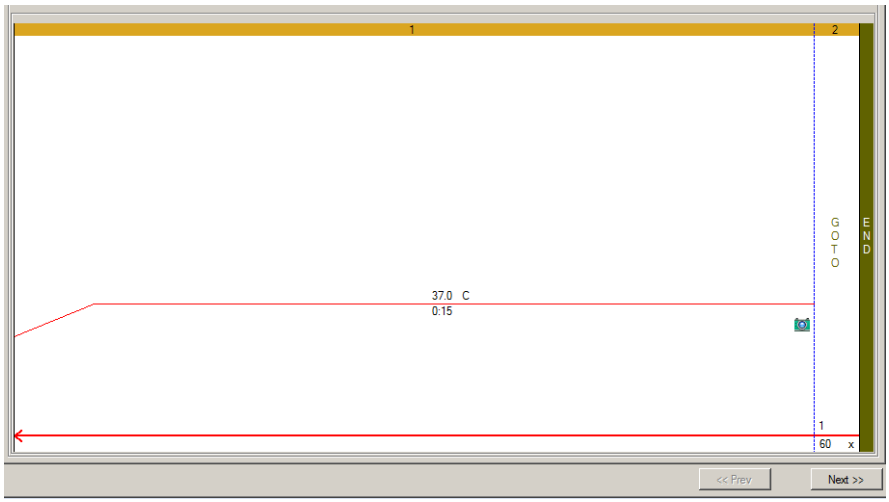
16 Load "Stanford Purigen LAMP protocol" on thermal cycler. Add sealed tubes or 96 well plate to thermal cycler and perform LAMP for **00:30:00** . 30m

17 After LAMP reaction is complete, briefly centrifuge tubes or well plate and keep on ice in CRISPR/amplicon handling area.

Program thermal cycler for CRISPR Reaction 30m

18 The CRISPR reaction should be performed at **37 °C** . Program the thermal cycler for a 15 second period with a fluorescent reading, repeated for 60 cycles. An example is shown below. Save the protocol as "Stanford Purigen CRISPR protocol" for future use.

Citation: jnesvet (01/07/2021). Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE updated. https://dx.doi.org/10.17504/protocols.io.bqzrmx56



Note the reporters are FAM labeled, so ensure the reading is either in the FAM or SYBR green fluorescent channel.

18.1 If applicable, set lid heater to **45 °C**

Preparation of CRISPR Reaction Mix

19 Using the same number of *N* determined for LAMP reactions in Step 7, prepare the CRISPR reaction mix

Note that because tubes containing amplicon will be handled, all steps in this section should be performed in an area dedicated for amplicon handling, separate from both the reagent preparation and patient sample preparation area. Decontaminate surfaces and pipettes with RNase Away or RNase Zap.

Note that the CRISPR reaction proceeds immediately after LAMP amplicons are added. Therefore, this reaction is very time sensitive and the order of reagent addition is extremely important. Because of the time sensitive nature, each laboratory personnel should perform no more than 48 CRISPR reactions (6 tube strips) per run.

20 Calculate the volume of each reagent required for N total reactions according to the table below.

Reagent	CRISPR Reaction Mix Volume
RNP mix	N x 17 uL
reporters	N x 1 uL
Total volume	N x 18 uL

Organize clean 8-tube strips or 96 well plate for the given *N* number of reactions in the same format as in Step 8 for the

Citation: jnesvet (01/07/2021). Stanford and Purigen Biosystems Microfluidics Team protocol XPRIZE updated. https://dx.doi.org/10.17504/protocols.io.bqzrmx56

21 CRISPR reaction.

	1	2	3	4	5	6	7	8	9	10	11	12
A	P1 A	P1 B	PC									
B	P2 A	P2 B	NTC									
C	P3 A	P3 B										
D	P4 A	P4 B										
E	P5 A	P5 B										
F	P6 A	P6 B										
G	P7 A	P7 B										
H	P8 A	P8 B										

Note that fluorescent measurements will be taken during CRISPR reaction, so do not label the top of the tubes or wells.

22 Dispense reagents in a clean 1.5 mL microcentrifuge tube to prepare CRISPR reaction mix. Vortex to thoroughly mix. Briefly centrifuge to collect reagents at bottom of tube, then keep on ice.

23 ⚠

Add **2 µl** from LAMP reaction tube into the new corresponding tube or 96 well plate. Store the remaining solution in the freezer.

Note that the amplicons must be added prior to the CRISPR reaction mix to ensure speedy pipetting in the following step.

24 ⚠

Quickly add **18 µl** of the CRISPR reaction mix to each tube, changing pipette tips after each addition. It is important to add the CRISPR reaction mix to the PC and NTC tubes prior to the patient samples to ensure the timing is accurate.

Note: do not pipette-mix or vortex for this step. At most, 48 CRISPR reactions should be performed at a time, and it should take approximately 3 minutes to fully dispense the CRISPR reaction mix into the 48 individual tubes.

25 Load the "Stanford Purigen CRISPR protocol" on the thermal cycler. Add tube strips or 96 well plate and run CRISPR reaction for 15 minutes.

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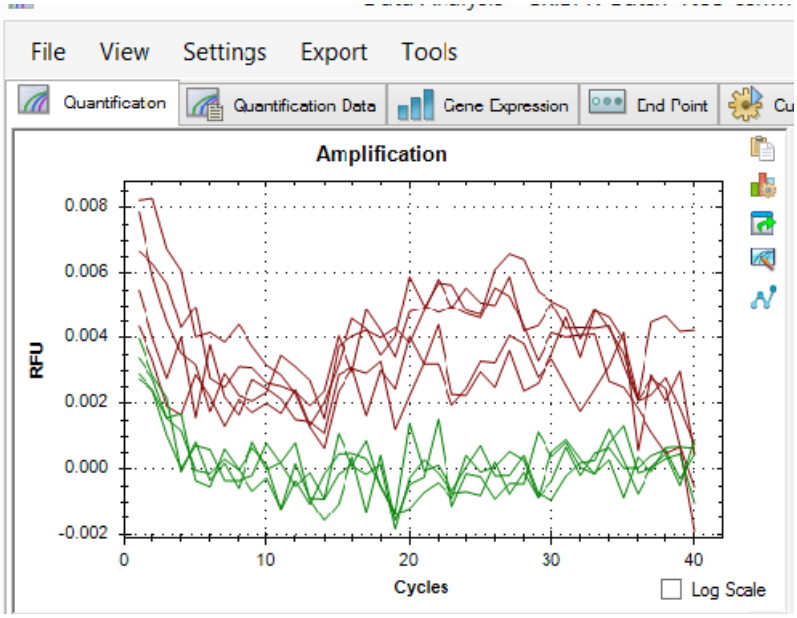
CRISPR Data Analysis

26 After the CRISPR run has completed, the data analysis will be dependent on the software. We give as two examples the Bio-Rad CFX Maestro or the ABI 7500 software.

For other pieces of software not described below, users should view the raw fluorescence data (no baseline subtraction) on a linear scale.

27 The Bio-Rad CFX Maestro software automatically performs a baseline correction which hinders data analysis (as shown below). Therefore it is necessary to view the raw fluorescence data and signal from the CRISPR reaction.



Baseline-subtracted CRISPR data for positive (red) and negative (green) samples are indistinguishable due to baseline subtraction data processing. Raw fluorescence data should be used for data analysis.

27.1 To remove the baseline subtraction, go to the Settings tab --> Baseline Setting. The default option is "Baseline Subtracted Curve Fit" checked, which should be changed to "No Baseline Subtraction."

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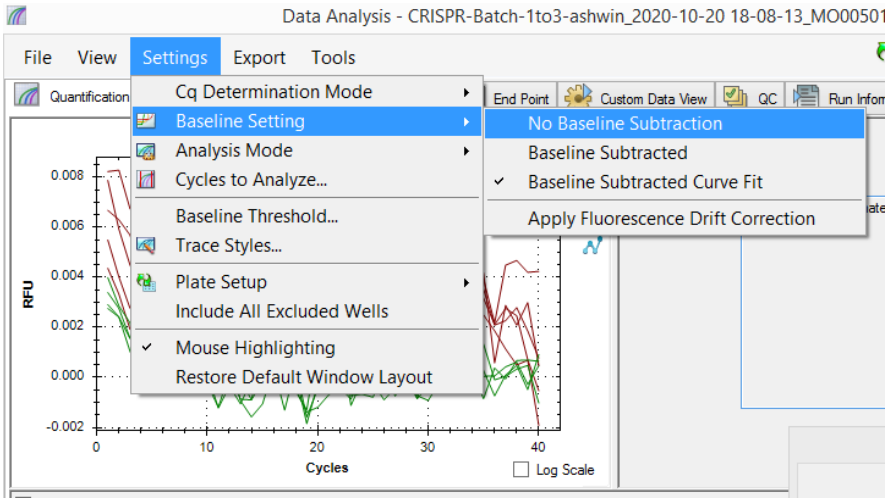
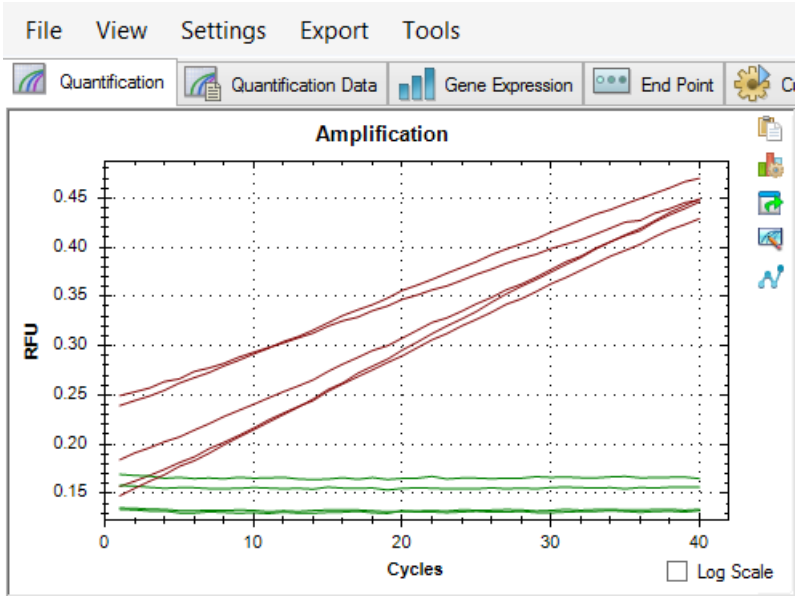


Change data analysis to "No Baseline Subtraction."

27.2



The raw data without baseline subtraction now shows clear distinguishing between positive (red) and negative (green) patient samples.

28 For the ABI 7500 Software, go to the "Amplification Plot" tab. Under "Plot Settings," Select Rn vs. Cycle for the Plot Type, and "Linear" for the Graph Type.

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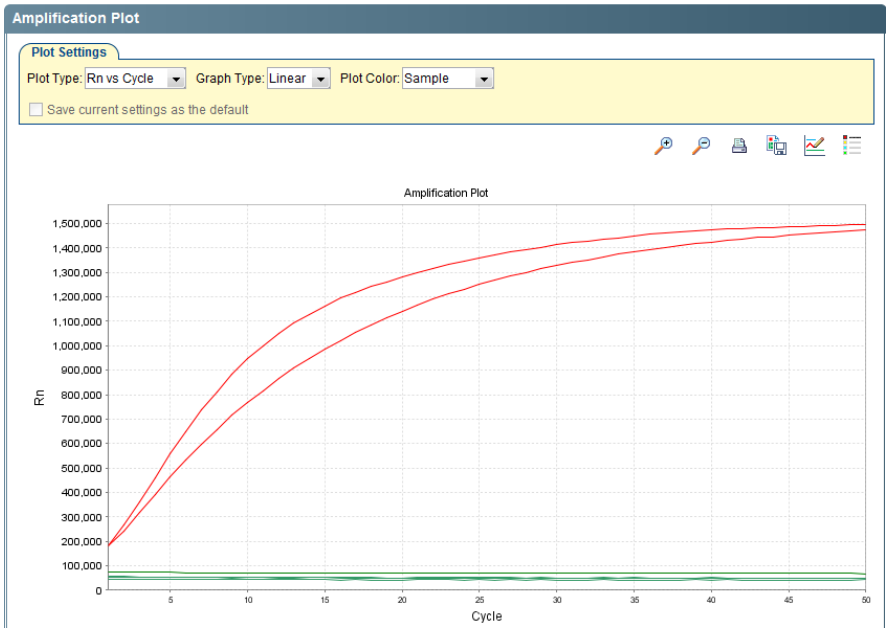
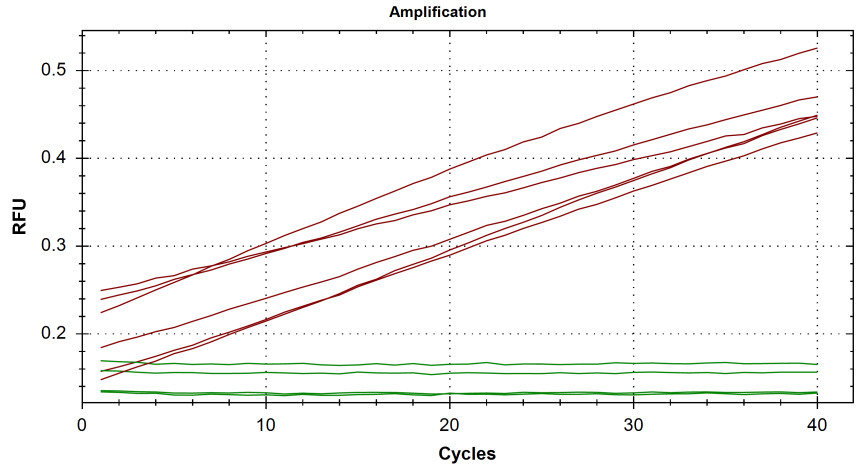


Interpretation of Results

29 The CRISPR assay interpretation is qualitative. A few examples of CRISPR fluorescent readout is shown below. A positive CRISPR result will show an increase in fluorescence over time. Note the slopes of positive samples (shown in red) can vary, while negative samples (shown in green) remain flat throughout the CRISPR readout.

Ensure that the raw fluorescence data is being analyzed.

Note the absolute values of the fluorescence will vary from run to run and instrument to instrument. Therefore, it should not be expected that the initial fluorescence reading is zero.



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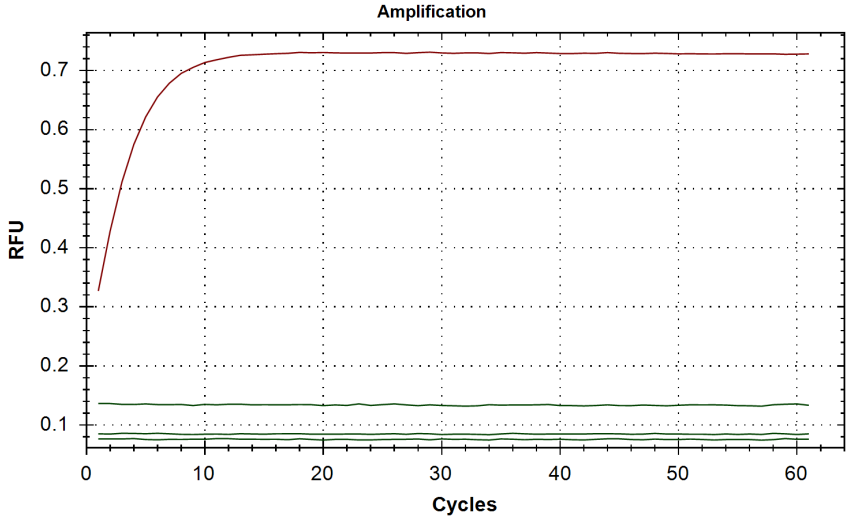

30 Ensure that the NTC sample does not show a positive slope in the CRISPR readout. If the NTC sample does appear positive, this is likely due to contamination from the LAMP reaction setup and the results are invalid. If possible, repeat the protocol from the sample extraction on remaining raw sample using the Purigen system (Step 3).

Ensure that the LAMP Positive Control sample does show a positive slope in the CRISPR readout. If the sample does not appear positive, this could be due to the time sensitive nature of CRISPR, and it may have taken too long to add the CRISPR reaction mix to all 48 samples. To troubleshoot, try running another CRISPR reaction on just the LAMP PC. If it now appears positive, it likely took to long to load the CRISPR reaction mix into the 48 samples. Redo the CRISPR reaction starting from Step 23, instead doing 16 samples at a time. If this troubleshooting did not solve the issue, repeat the protocol from the sample extraction on remaining raw sample using the Purigen system (Step 3).

31 Once the results have been validated by the controls, the patient samples can be interpreted as follows:

If 2/2 or 1/2 of the patient sample replicates shows a positive CRISPR signal, this can be interpreted as positive for SARS-CoV-2.

If 0/2 of the patient sample replicates shows a positive CRISPR signal, this can be interpreted as negative for SARS-CoV-2.

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