Point-Of-Need One-Pot Multiplexed RT-LAMP Test For Detecting Three Common Respiratory Viruses In Saliva

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Contents

Materials and methods 2

Supplementary figures 3

Supplementary tables 6

Supplementary video 7

Materials and methods

Analyzer design and fabrication

The analyzer housing, adapters to mount Printed Circuit Boards (PCBs), and the machined aluminum heating block were designed using SolidWorks CAD software. Structural parts were fabricated using MakerBot MethodX 3D printer (Brooklyn, NY) with MakerBot ABS (acrylonitrile butadiene styrene) material, and the heating block was machined by Protolabs Network. The heating block uses four two-ohm power resistors (MP725-2.00) mounted using a thermally conductive adhesive paste (Arctic Alumina) and an MC65F103A 10 k-ohm thermistor (Amphenol Thermometrics, St. Mary's, PA) in a small recess for temperature feedback. PCBs were designed using AutoDesk Eagle CAD software and fabricated by OSH Park LLC (Lake Oswego, OR). The excitation LEDs (SK6812) were purchased from Adafruit Industries (New York, NY), whereas other electronic components such as the Raspberry Pi Zero, power resistors, thermistor, and spectral sensors (AS7341) were purchased from DigiKey.com. A cost estimate of the analyzer is provided in **Supplementary Table S3.**

Target RNAs

Heat-inactivated SARS-CoV-2 (ATCC VR-1986HK) RNA and Quantitative Genomic RNAs of Influenza A virus or Flu-A (H1N1) strain A/PR/8/34 (ATCC VR-95DQ) and Human Respiratory Syncytial Virus strain A2 (ATCC VR-1540DQ) were purchased from American Type Culture Collection (Manassas, VA).

RT-LAMP assay

The RT-LAMP reaction mix consists of 1x isothermal buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8), single-plex, duplex or triplex primers, 0.5 M Betain, 6 mM $MgSO_4$, 1.4 mM deoxyribonucleotide triphosphates (dNTPs), 0.5 U/µL Bst 2.0 DNA polymerase, 0.3 U/µL WarmStart Reverse Transcriptase, 1.5 µL purified RNA template (per target for multiplexed detection), 6 µM syto-9 (for applicable assay) and PCR grade water to bring total reaction volume to 25 µL. The LAMP assay was performed at a constant temperature of ~62°C. The single-plex RT-LAMP assay using syto-9 intercalating dye consists of the usual primer concentrations, 0.2 µM F3 and B3, 0.8, LF and LB, and 1.6 µM FIP and BIP; however, the DARQ assay substitutes 1.6 µM FIP and 6 µM syto-9 with 0.8 µM FIP and QFIP, and 0.65 µM Fd. For the multiplexed assays, the primer concentrations for all 6 primers for each target were reduced to 1/n of the concentration in single-plex version, such that n represents the number of primer sets or targets. Thus, for the duplex assay, the primer concentrations are 0.1 µM F3 and B3, 0.4 µM FIP, QFIP, LF, and LB, 0.325 µM Fd, and 0.8 µM BIP. The triplex assay, however, required optimizing the ratio of FIP:QFIP, such that FIP is in excess, resulting in the following primer concentrations, 0.067 µM F3 and B3, 0.4 µM FIP, 0.267 µM QFIP, LF and LB, 0.21 µM Fd and 0.53 µM BIP.

RT-PCR assay

The 20 µL RT-PCR reaction consists of 10 µL of qScript XLT One-Step RT-qPCR Tough Mix (2X), 1.5 µL of premixed primers, and a probe for detecting SARS-CoV-2 or 0.4 µM forward and reverse primers and 0.1 µM probe for detecting Flu-A and RSV, 3.5 µl of the RNA template, and 3.5 µl of H_2O. 2019-nCoV RUO Kit, catalog #10006713, for detecting SARS-CoV-2 and custom-designed primers and probes for Flu-A and RSV, detailed in **Supplementary Table S2**, were purchased from Integrated DNA Technologies, Coralville, USA. The RT-PCR involved cDNA synthesis at 50 °C for 10 min, initial denaturation at 95 °C for 1 min, followed by 45 cycles of a 5-second denaturation step at 95 °C and a 30-second annealing step at 55 °C in a Bio-Rad CFX96 Real-Time PCR system.

Data processing and analysis

The predicted fluorophore concentrations (C) were processed using baseline subtraction, similar to the method used by the CFX 96 benchtop thermal cycler. A linear fit was calculated for the values between 5 and 10 min, and the slope (m) and intercept (b) values were used to determine the subtraction value (SV):

$SV=t\times m+b$ for any time (t). The baseline-subtracted concentrations (C_{bs}) were then computed as $C_{bs}=C-SV$ and smoothed using an Exponential Moving Average (EMA) filter implemented in Python using the pandas library, with any negative values being set to zero to ensure non-negative fluorescence readings.

Supplementary figures

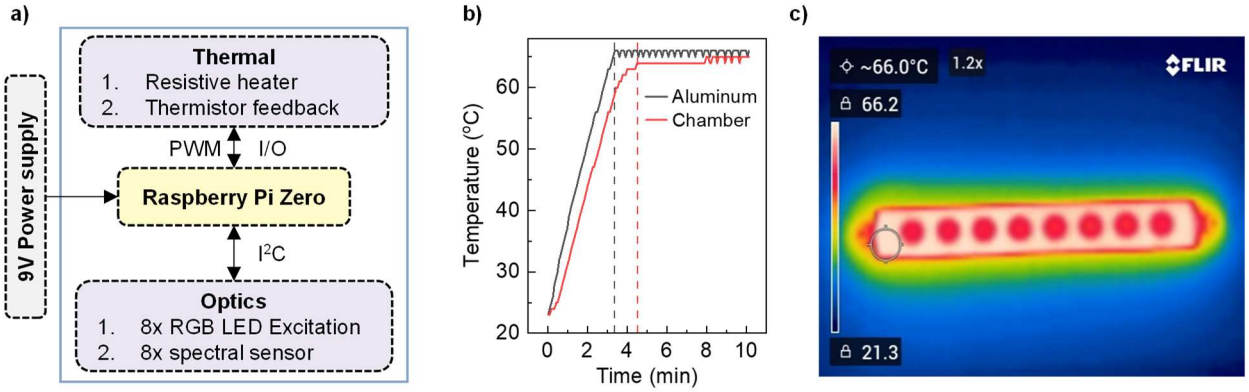


Figure S1. Additional information regarding the design and validation of the iNAAT instrument. (a) A brief overview of the electronic hardware design of the instrument: it consists of a Raspberry Pi Zero W, a thermal module that contains a resistive heater and thermistor feedback, and an optical module consisting of an RGB LED and a multi-channel spectral sensor. The Raspberry Pi Zero W microprocessor runs a Linux-based operating system and coordinates between the specific modules. The Zero W has built-in WiFi and Bluetooth Low Energy (BLE) submodules, enabling wireless communication, control, and over-the-air (OTA) software updates, if required. The high dimensional data is stored using the typical JSON format such that RFU at each detection channel for each excitation wavelength of each of the eight reaction chambers over time may be recorded and easily accessed for communicating with an external application such as a smartphone app using an API like the GATT server (Generic ATTribute Profile) for BLE. (b) shows characterization results for the thermal module while heating 25 µL water in each reaction chamber. The reaction temperature of ~62 °C is achieved within 5 minutes when the heating block is set to achieve and maintain ~65 °C. (c) The thermal image corroborates the measurement of an external sensor used in subfigure b.

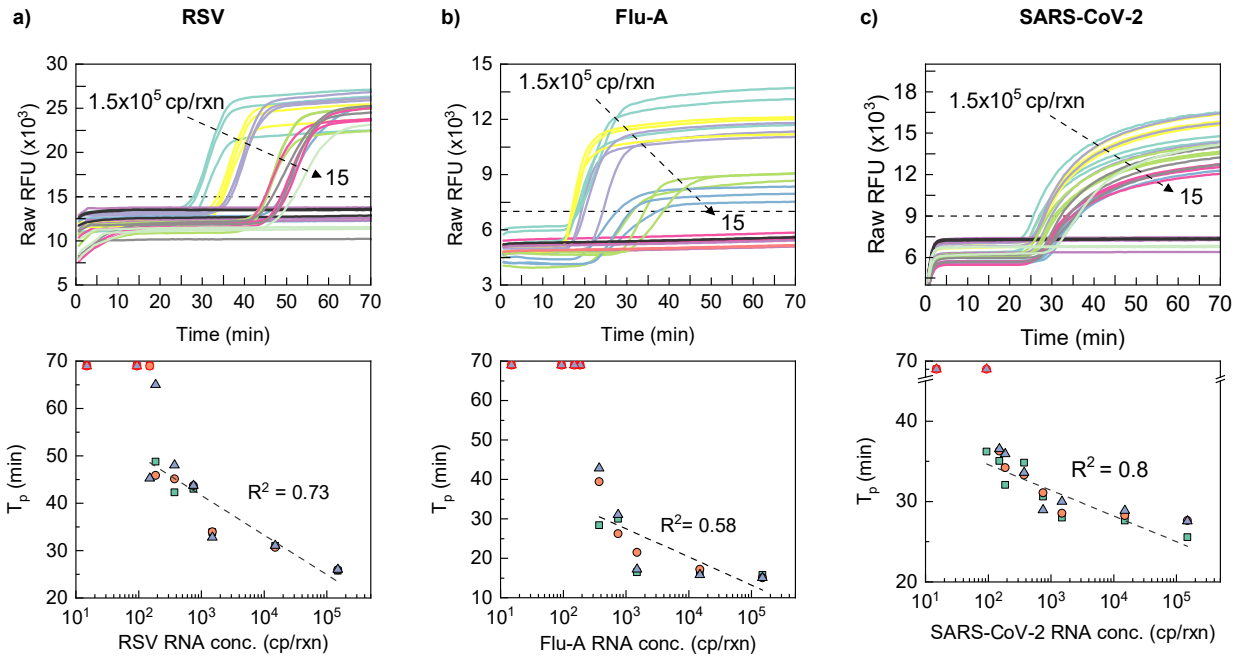


Figure S2. Results of the single-plex DARQ RT-LAMP reactions performed on a benchtop thermal cycler. (a) Real-time amplification curves (top) and summary of times to positive (bottom) for triplicates of the RSV assay on target RNAs in the range $1.5x10^5$ to 15 copies per reaction (cp/rxn) with 10x dilution and then in the range 750 to 93.75 cp/rxn with 2x dilution. (b) Real-time amplification curves (top) and summary of times to positive (bottom) for triplicates of the Flu-A assay on target RNAs in the same range. (c) Real-time amplification curves (top) and summary of times to positive (bottom) for triplicates of the SARS-CoV-2 assay on target RNAs in the same range. These results were used to benchmark the performance of the developed analyzer with a benchtop thermal cycler.

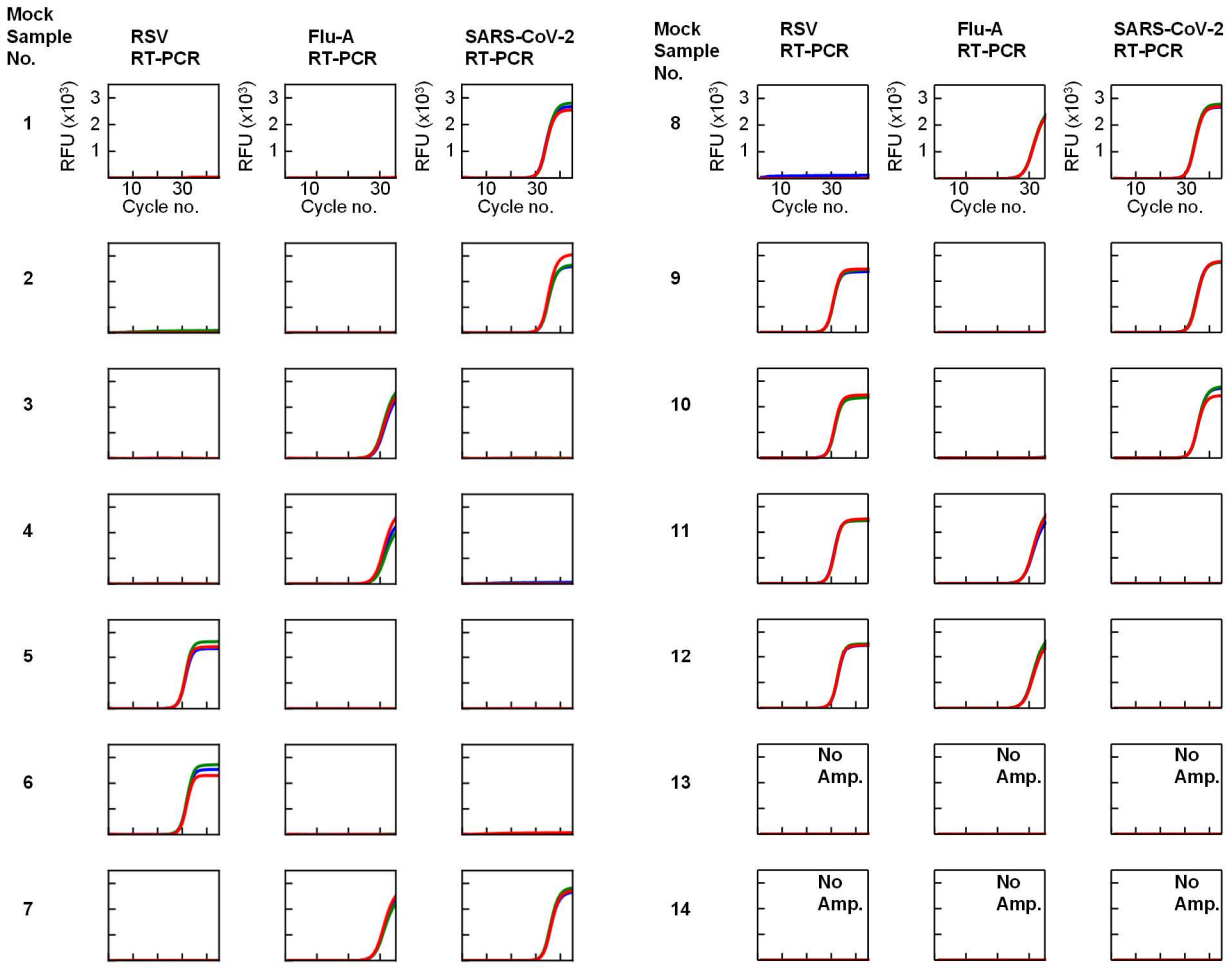


Figure S3. Amplification curves for single-plex RT-PCR tests for detecting RSV, Flu-A, and SARS-CoV-2 in mock saliva samples (tested in triplicates) to be used as ground truth.

Supplementary tables

Table S1. Multiplexed RT-LAMP assay primer sequences

Primer	Sequence 5' → 3'
RSV F3	GAGTTGAAGGGATTTTTGCA
RSV B3	TGGGTTGTTCAATATATGGTAGA
RSV FIP	TAACTGATTTTGCTAAGACCCCCGGATTGTTTATGAATGCCTATGG
RSV QFIP	/5IABKFQ/TAACTGATTTTGCTAAGACCCCCGGATTGTTTATGAATGCCTATGG
RSV Fd	GGGTCTTAGCAAAATCAGTTA/3FLOURT/
RSV BIP	CAAGCAGAAATGGAACAAGTTGTGCTGCTTCTCCACCCAATT
RSV LF	CACCGTAACATCACTTG
RSV LB	GAGGTGTATGAGTATGCTCAGA
Flu-A F3	GACTTGAAGATGTCTTTGC
Flu-A B3	TRTTATTTGGGTCTCCATT
Flu-A FIP	TTAGTCAGAGGTGACARRATTGCAGATCTTGAGGCTCTC
Flu-A QFIP	/5IABkFQ/TTAGTCAGAGGTGACARRATTGCAGATCTTGAGGCTCTC
Flu-A Fd	CAATYYTGTCACCTCTGACTAA/3ATTO550N/
Flu-A BIP	TTGTKTTCACGCTCACCGTGTTTGGACAAAGCGTCTACG
Flu-A LF	GTCTTGTCTTTAGCCA
Flu-A LB	CMAGTGAGCGAGGACTG
SARS-CoV-2 F3	AACACAAGCTTTCGGCAG
SARS-CoV-2 B3	GAAATTTGGATCTTTGTCATCC
SARS-CoV-2 FIP	CGCATTGGCATGGAAGTCACTTTGATGGCACCTGTGTAG
SARS-CoV-2 QFIP	/5IAbRQ/CGCATTGGCATGGAAGTCACTTTGATGGCACCTGTGTAG
SARS-CoV-2 Fd	AAGTGACTTCCATGCCAATGCG/3Cy5Sp/
SARS-CoV-2 BIP	TGCGGCCAATGTTTGTAATCAGCCAAGGAAATTTTGGGGAC
SARS-CoV-2 LF	TTCCTTGTCTGATTAGTTC
SARS-CoV-2 LB	ACCTTCGGGAACGTGGTT

Table S2. RT-PCR primers

Primer	Sequence 5' → 3'
RSV Forward	GCTCTTAGCAAAGTCAAGTTGAATGA
RSV Reverse	TGCTCCGTTGGATGGTGTATT
RSV Probe	/56-FAM/ACACTCAAC/ZEN/AAAGATCAACTTCTGTCATCCAGC/3IABKFQ/
Flu-A Forward	CAAGACCAATCYTGTCACCTCTGAC
Flu-A Reverse	GCATTYTGGACAAAVCGTCTACG
Flu-A Probe	/56-FAM/TGCAGTCCT/ZEN/CGCTCACTGGGCACG/3IABKFQ/
SARS-CoV-2 Forward	GACCCCAAAATCAGCGAAAT
SARS-CoV-2 Reverse	TCTGGTTACTGCCAGTTGAATCTG
SARS-CoV-2 Probe	/56-FAM/ACCCCGCAT/ZEN/TACGTTTGGTGGACC/3IABKFQ/

Table S3. Analyzer cost estimate

Vendor	Description	Function	Unit cost ($)	Quantity	Extended cost ($)
Adafruit	DC/DC step-down	Voltage converter	8.96	1	8.96
Adafruit	RGB led	Excitation source	0.5	8	4
Adafruit	0.1” spaced pin strip (male and female)	Connect the LED and spectral sensor PCBs to Raspberry Pi	0.9	1	0.9
Digikey	Thermistor	Temperature feedback	7.22	1	7.22
Digikey	4 channel 16-bit ADC	Convert voltage to digital values	6.35	1	6.35
Digikey	Spectral sensor	Fluorescence detection	7.5	8	60
Digikey	DC barrel jack	Power connector	0.76	1	0.76
Digikey	0.1 and 10 µF SMD capacitors and 10k ohm SMD resistor	Passive components for power regulation of ICs	0.02	40	0.8
Digikey	2 ohm 1% 25W power resistor	Heat generation	7.63	4	30.52
Digikey	Raspberry Pi Microprocessor board	Electronic coordination and data logging	10	1	10
Digikey	SPDT switch	Power delivery	0.07	1	0.07
OSH park	Two PCBs for LED and spectral sensor	Interface LED and spectral sensor with Raspberry Pi	12	1	12
Hubs	Machined aluminum heat block	Aluminum reaction tube incubator	65.5	1	65.5
In-house	Housing fabrication	3D-printed housing of the analyzer	7	1	7
				Total cost ($)	214.08

Supplementary video

Supplementary video V1. The RT-LAMP-based diagnostic test’s overall workflow for identifying three respiratory viruses.