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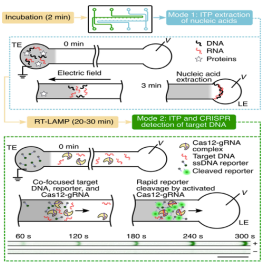
ITP-CRISPR detection of SARS-CoV-2 RNA



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Disclaimer

Abstract

The rapid spread of COVID-19 across the world has revealed major gaps in our ability to respond to new virulent pathogens. Rapid, accurate, and easily configurable molecular diagnostic tests are imperative to prevent global spread of new diseases. CRISPR-based diagnostic approaches are proving to be useful as field-deployable solutions. In one basic form of this assay, the CRISPR-Cas12 enzyme complexes with a synthetic guide RNA (gRNA).This complex becomes activated only when it specifically binds to target DNA and cleaves it. The activated complex thereafter non-specifically cleaves single-stranded DNA reporter molecules labeled with a fluorophore-quencher pair. We discovered that electric field gradients can be used to control and accelerate this CRISPR assay by co-focusing Cas12-gRNA, reporters, and target within a microfluidic chip. We achieve an appropriate electric field gradient using a selective ionic focusing technique known as isotachophoresis (ITP) implemented on a microfluidic chip. We also use ITP for automated purification of target RNA from raw nasopharyngeal swab samples. We here combine this ITP purification with loop-mediated isothermal amplification (LAMP) and the ITP-enhanced CRISPR assay to achieve detection of SARS-CoV-2 RNA (from raw sample to result) in about 30 min for both contrived and clinical nasopharyngeal swab samples. Our goal is to use validated LAMP primers and gRNAs and implement them on our ITP-CRISPR microfluidic platform. The on-chip electric field control enables a new modality for a suite of microfluidic CRISPR-based diagnostic assays and makes the technology amenable to automation and point-of-care applications.

Materials

Materials list:

	Reagent or Consumable	Supplier	Catalog #	Amount
	WarmStart® LAMP Kit (DNA & RNA)	New England Biolabs	E1700S	1.25 mL
	Wash Solution 1	Custom	N/A	10 mL
	Wash Solution 2	Custom	N/A	10 mL
	Wash Solution 3	Custom	N/A	10 mL
	Wash Solution 4	Custom	N/A	10 mL
	EnGen® Lba Cas12a (Cpf1)	New England Biolabs	M0653T	2000 pmol
	LAMP primer N gene (10x mix)	Elim Biosciences	N/A	10 mL
	LAMP primer E gene (10x mix)	Elim Biosciences	N/A	10 mL
	LAMP primer RNase P gene (10x mix)	Elim Biosciences	N/A	10 mL
	E gene gRNA (10 x)	IDT	N/A	5 mL
	N gene gRNA (10 x)	IDT	N/A	5 mL
	RNase P gene gRNA (10 x)	IDT	N/A	5 mL
	Reporter ssDNA (10 x)	IDT	N/A	10 mL
	Nuclease free water	Thermo Fisher	10977015	500 mL
	Lysing buffer (10 x)	Custom	N/A	10 mL
	LE1 extraction	Custom	N/A	50 mL
	LE1 elution	Custom	N/A	50 mL

	TE1 (10 x)	Custom	N/A	5 mL
	LE2	Custom	N/A	50 mL
	TE2	Custom	N/A	10 mL

Equipment list:

	Equipment	**Supplier**	**Model**
	Microscope	Nikon	TE200
	sCMOS camera + software	Hamamatsu	ORCA-Flash4.0
	Sourcemeter	Keithley	2410
	Blue LED light souce	Thorlabs	M470L3
	Microfluidic chip	Caliper life sciences	NS12AZ
	Waterbath	Custom	N/A
	Vacuum pump	Custom	N/A

Additional equipment and consumables:

- Pipette (P10, P20 and P200)
- Pipette tips (10 µL, 20 µL and 200 µL)

	LAMP primer	**Sequence (5'-3')**
	N-gene F3	AAC ACA AGC TTT CGG CAG
	N-gene B3	GAA ATT TGG ATC TTT GTC ATC C
	N-gene FIP	TGC GGC CAA TGT TTG TAA TCA GCC AAG GAA ATT TTG GGG AC
	N-gene BIP	CGC ATT GGC ATG GAA GTC ACT TTG ATG GCA CCT GTG TAG
	N-gene LF	TTC CTT GTC TGA TTA GTT C

	N-gene LB	ACC TTC GGG AAC GTG GTT
	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
	RNaseP POP7 F3	TTG ATG AGC TGG AGC CA
	RNaseP POP7 B3	CAC CCT CAA TGC AGA GTC
	RNaseP POP7 FIP	GTG TGA CCC TGA AGA CTC GGT TTT AGC CAC TGA CTC GGA TC
	RNaseP POP7 BIP	CCT CCG TGA TAT GGC TCT TCG TTT TTT TCT TAC ATG GCT CTG GTC
	RNaseP POP7 LF	ATG TGG ATG GCT GAG TTG TT
	RNaseP POP7 LB	CAT GCT GAG TAC TGG ACC TC
	qPCR primer	Sequence (5'-3')
	E_Sarbeco_F1	ACA GGT ACG TTA ATA GTT AAT AGC GT
	E_Sarbeco_R2	ATA TTG CAG CAG TAC GCA CAC A

	E_Sarbeco_P1	5 -FAM/ACA CTA GCC ATC CTT ACT GCG CTT CG/3 -BHQ-1
	RP-F	AGA TTT GGA CCT GCG AGC G
	RP-R	GAG CGG CTG TCT CCA CAA GT
	RP-P	5 -FAM/TTC TGA CCT GAA GGC TCT GCG CG/3 -BHQ-1
	gRNA	**Sequence (5'-3')**
	E gene	UAA UUU CUA CUA AGU GUA GAU GUG GUA UUC UUG CUA GUU AC
	N gene	UAA UUU CUA CUA AGU GUA GAU CCC CCA GCG CUU CAG CGU UC
	RNase P	UAA UUU CUA CUA AGU GUA GAU AAU UAC UUG GGU GUG ACC CU
	Template and reporter	Sequence (5'-3')
	ssDNA reporter	/56-FAM/TTATT/3IABkFQ/

Primers and guide RNA sequences used in this assay

Troubleshooting

Before start

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

Chip preparation before testing

1 Rinse the NS12AZ channel in the following order: Wash solution 1 for 2 min, Wash solution 2 for 2 min, Wash solution 3 for 2 min, Wash solution 2 for 2 min, Wash solution 4 for 2 min, and Wash solution 3 for 2 min.
Between each rinse step, dry the channel using vacuum.

> Note
>
> In the current protocol, the NS12AZ caliper chips can be reused for multiple samples. Wash proceduce described here ensures no cross contamination. Future iterations of the protocol will invovle the use of pre-prepared disposable chips for which this wash step can be skipped.

ITP extraction of total nucleic acids

2 Mix 25 µL of raw NP swab sample in VTM with 3 µL of 10 x Lysing buffer, pipette mix, and incubate at 62 °C for 00:02:00 in a water bath.

3 Add 3 µL of 10x TE1 to the lysate, pipette mix, and load 20 µL of this mix into the Reservoir 1 of the chip

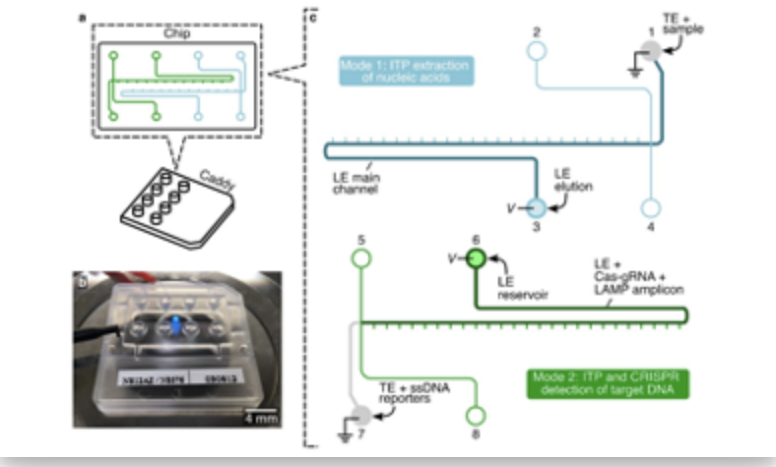

Figure 1. Chip layout and loading procedure.

4 Load 20 µL of LE1 in reservoirs 2 and 4 each, as shown in Figure 1, and apply vacuum using a vacuum pump for 00:00:15 at reservoir 3 till the main channel is completely filled as depicted in Figure 1.

5 Empty Reservoir 3 of any residual liquid, and load 20 µL of LE1 elution buffer in it.

6 Apply 1000 V voltage between reservoirs 1 and 3 as shown in Figure 1 for approximately 00:03:00 . Visualize ITP peak containing total nucleic acids using the microscope and a camera. Turn off voltage when the ITP peak reaches the elution reservoir 3.

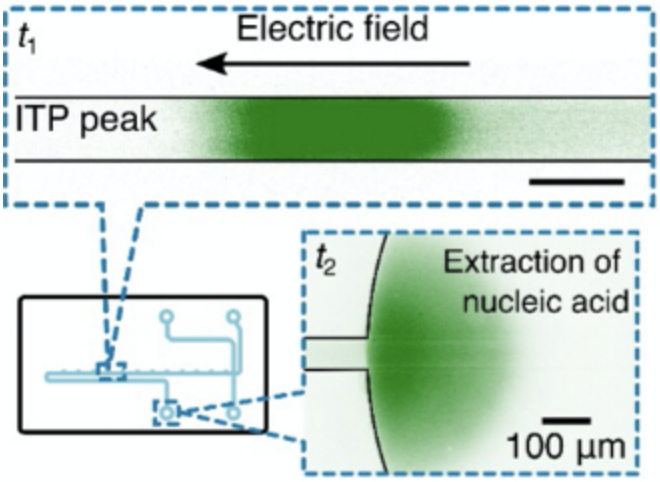

Figure 2. Visualization of ITP peak during extraction and elution of total nucleic acids from raw NP swab samples

7 Pipette out 20 µL of elution volume containing extracted nucliec acids into an Eppendorf tube and place on ice until further use.

RT-LAMP for N, E, and RNase P genes

8 Prepare the following RT-LAMP mixtures each for N, E, and RNase P genes. Template is obtained from the eluate in the previous step.

	Reagent	Volume (per E gene reaction)	Volume (per N gene reaction)	Volume (per RNa

				se P gen e reac tion)
	WarmStart LAMP 2X Master Mix	10 µL	10 µL	10 µL
	LAMP Primer Mix (10X)	2 µL (E)	2 µL (N)	2 µL (RNa se P)
	Template from Eluate	6 µL	6 µL	6 µL
	Nuclease free water	2 µL	2 µL	2 µL
	Total Volume	20 µL	20 µL	20 µL

8.1

	LAMP primer component	**10x concentration**	**1x con cetr atio n**
	FIP	16 µM	1.6 µM
	BIP	16 µM	1.6 µM
	F3	2 µM	0.2 µM
	B3	2 µM	0.2 µM
	LOOP F	8 µM	0.8 µM
	LOOP B	8 µM	0.8 µM

LAMP 10x primer mix for N, E, and RNase P genes

9 Incubate the above mixtures at 62 °C for 00:20:00 to 00:30:00 and perform LAMP for N, E and RNase P genes in independent tubes. Place tubes on ice after

LAMP.

ITP-CRISPR detection of cDNA of LAMP amplicons

10 Prepare 10x RNP mix as follows for each N, E, and RNase P genes

	Reagent	Volume (per E gene reaction)	Volume (per N gene reaction)	Volume (per RNase P gene reaction)
	NEBuffer 2.1 (10x)	2 uL	2 uL	2 uL
	NEB LbCas12a (100 uM)	0.2 uL	0.2 uL	0.2 uL
	gRNA (10 x)	17.8 uL (E)	17.8 uL (N)	17.8 uL (RNase P)
	total volume	20 uL	20 uL	20 uL

Incubate the mixtures in 37 °C for 00:30:00 and then place on ice.

11 Add 3 µL of the 10x RNP mixes prepared above to 27 µL of LE2, independently for N, E and RNase P genes, and then place the three mixtures on ice.

12 Add 2 µL of 10x reporter ssDNA to 18 µL of TE2.

13 Load 20 µL of LE2 in reservoirs 5 and 8 (as per Figure 1).

14 Mix 2 µL of LAMP amplicon for N gene with 18 µL of the RNP+LE2 mix in Step 11 corresponding to the N gene, and load this 20 µL in reservoir 6.

Note

Note that a lower volume of 0.5 µL for RNP+LE2 containing CRISPR regaents can alternately be used in reservoir 6 to minimize reagent consumption. In this case, apply vacuum at reservoir 7 (step 15) for only 00:00:01 after loading RNP+LE2 , and then load the remainder of reservoir 6 with 19.5 µL of LE2.

15 Apply vaccum for 00:00:10 at reservoir 7 using the vacuum pump. Clear reservoir 7 of any residual liquid.

16 Load 20 µL of the mix prepared in Step 12 to reservoir 7.

17 Apply 4 µA of current between reservoirs 6 and 7 (as per figure 1) and monitor the fluorescence of the ITP peak using a custom microscope-LED-camera system.

Expected result

A positive sample shows a rapid increase in fluorescence signal of the ITP peak and a value above the threshold value. The threshold is determined using prior calibration experiments. A negative sample has low or minimal increase in fluorescence signal of the ITP peak.

18 Perform wash step 1 and repeat steps 14 to 18 for the E gene and Rnase P gene.

Note

Future iterations of this protocol will involve multiplexed detection of the N, E and RNase P genes on a single chip.

19 Expected result for positive and negative detection of SARS-CoV-2 samples.

Expected result

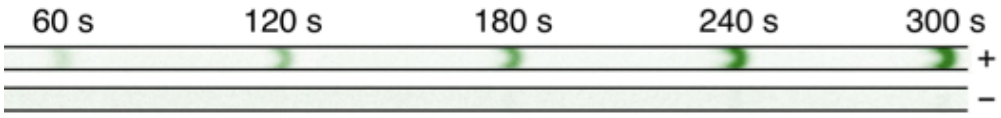

Figure 3. Raw experimental visualization of the ITP peak fluorescence intensity

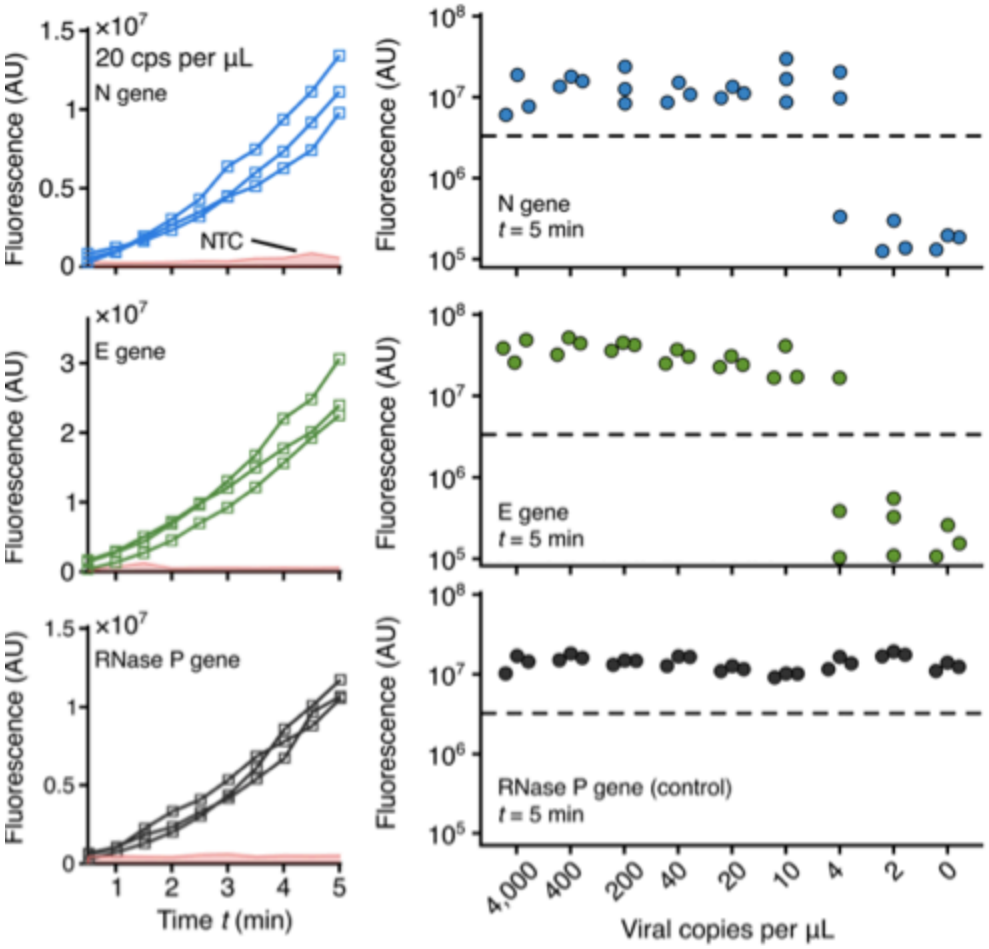

Figure 4. Measured kinetic profiles of fluorescence and end-point fluorescence readout for the N, E and RNase P genes. LOD is 10 copies per uL

Note

Current protocol is optimized for testing one sample per run. Future version of our protocol will multiplex 96 target reactions and samples in a single run.