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INSIGHT: a scalable isothermal NASBA-based platform for COVID-19 diagnosis V.1

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Coronavirus Method De...

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We use this protocol and it's working

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Abstract

We present here INSIGHT (Isothermal NASBA-Sequencing based hIGH-througput Test): a two-stage COVID-19 testing strategy, using a combination of an isothermal NASBA reaction and next generation sequencing. From commercially acquired human saliva with spiked-in viral RNA as input, the first stage employs isothermal amplification of viral RNA to give a rapid result in one to two hours, using either fluorescence detection or a dipstick readout, whilst simultaneously incorporating sample-specific barcodes into the amplification product. In the first stage, fluorescent viral RNA detection can be consistently achieved at 10-100 copies per 20 µl reaction. The second stage pools post-amplification barcoded products from multiple samples for scalable sequencing that could be centralised, to further improve the accuracy of the test in a massively parallel way. Our two-stage testing strategy is suitable for further development into a home-based or point-of-care assay, and is potentially scalable to population level.

Materials

MATERIALS

- QuickExtract DNA Extraction Solution **Lucigen Catalog #**QE09050
- NASBA liquid kit **Life Sciences Advanced Technologies Inc. Catalog #**SKU: NWK-1
- Tris (1 M) pH = 8 RNase free **Invitrogen - Thermo Fisher Catalog #**AM9855G
- Sodium Hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**71687
- 1M MgCl2 **Invitrogen - Thermo Fisher Catalog #**AM9530G
- 2M KCl **Invitrogen - Thermo Fisher Catalog #**AM9640G
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43816
- DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**276855
- dNTP set 100 mM **Invitrogen - Thermo Fisher Catalog #**10297018
- NTP set 100 mM **Thermo Scientific Catalog #**R0481
- Bio-11-UTP (75 mM) **Invitrogen - Thermo Fisher Catalog #**AM8451
- RNase H **NEB Catalog #**M0297L
- ProtoScript II reverse transcriptase **NEB Catalog #**M0368S
- T7 RNA polymerase **NEB Catalog #**M0251L
- BSA 20 mg/ml **NEB Catalog #**B9000S
- Direct-zol RNA Miniprep Plus **Zymo Research Catalog #**R2070
- PCRD lateral flow assay **Abingdon Health Catalog #**FG-FD51673
- Qubit RNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32852
- PowerUp™ SYBR™ Green qPCR Master Mix **Applied Biosystems (ThermoFisher Scientific) Catalog #**15340939

Primers pair sequence:

	FWD primer	CCAGCAACTGTTTGTGGACCTA
	REV primer with T7 handle	aattctaatacgactcactata

		ggg agaa ggA CAC CTG TGC CTG TTA AAC CAT
	FWD primer with 5-nt barcode and Illumina handle	tgac tgga gttc agac gtgt gctc ttcc gatc tnnn nnC CAG CAA CTG TTT GTG GAC CTA
	REV primer with 5-nt barcode and T7 handle	aattc taata cgac tcac tata ggg agaa ggn nnn nAC ACC TGT GCC TGT TAA ACC AT

Molecular beacon:

FAM-AUUGACAGUCUACUAAUUUGGUUAAAAACAAAUGUGUCAA-BHQ1dT-UUCAACUUCAAUG-propyl

FAM labelled RNA capture oligo for PCRD

FAM-AAAAGTCTACTAATTTGGTTAAAAACAAATGTGTCAATTTCAACTTC

Safety warnings

***** IMPORTANT: This protocol has not been validated on patient samples and should not be used for clinical diagnosis without further validation and certification. *****

1 **Lysis of saliva samples**

Mix crude saliva at 1:1 ratio with QuickExtract DNA Extraction Solution. Incubate at 95 °C for 5 min to ensure complete lysis of virus and inactivation of proteinase K.

2 **NASBA reaction**

Take 1 µl from the product of Step 1 and add into the NASBA reaction mixture to make a total volume of 20 µl. Reaction mixture can either be prepared in-house or from the Life Sciences NASBA liquid kit (see tables below).

A fluorescence plate reader can be used to monitor the reaction in real-time, or as an endpoint assay.

		vol.	**stock conc.**	**conc. in RM**
	sample	1 µl		
	primers*	1 µl	0.5 µM each	25 nM each
	water +/- beacon	3 µl		20 nM for beacon
	buffer (NECB-24)	6.7 µl		
	nucleotide (NECN-24)	3.3 µl		
	enzyme mix (NEC-1-24)	5 µl		
	total volume	20 µl		

Life Sciences reaction mixture (RM)

* Primer sequence available in Materials.

		vol.	**stock conc.**	**conc. in RM**
	sample	1 µl		
	primers*	1 µl	0.5 µM each	25 nM each

	water +/- beacon	4 µl		20 nM for beacon
	buffer with DMSO*	5 µl		
	nucleotide mix*	4 µl		
	enzyme mix*	5 µl		
	total volume	20 µl		

In-house reaction mixture (RM)
* For detailed mixture composition, see tables below. Primer sequence available in Materials.

		vol.	**stock conc.**	**conc. in RM**
	Tris-HCl pH 8.4*	120 µl	1 M	40 mM
	MgCl2	39.6 µl	1 M	13.2 mM
	KCl	112.5 µl	2 M	75 mM
	DTT	30 µl	1 M	10 mM
	DMSO	450 µl	100%	11%
	water	247.9 µl		
	total volume	1000 µl		

Buffer with DMSO
*Tris-HCl pH 8.4 is made in-house by titrating Tris-HCl pH 8.0 with NaOH pellet and pH determined by pH meter.

		vol.	**stock conc.**	**conc. in RM**

	Tris-HCl pH 8.4	120 µl	1 M	40 mM
	MgCl2	39.6 µl	1 M	13.2 mM
	KCl	112.5 µl	2 M	75 mM
	DTT	30 µl	1 M	10 mM
	water	697.9 µl		
	total volume	1000 µl		

Buffer without DMSO

		vol.	**stock conc.**	**conc. in RM**
	dNTP	0.22 µl each	100 mM	1 mM each
	NTP	0.88 µl each	100 mM	4 mM each
	total volume	4.4 µl		

Nucleotide mix (incl. 10% excess)

		vol.	**stock conc.**	**conc. in RM**
	diluted Rnase H	0.17 µl	500 U/ml	3.75 U/ml
	Photoscript RT	0.28 µl	200000 U/ml	2500 U/ml
	T7 polymerase	2.75 µl	50000 U/ml	6250 U/ml
	BSA	0.13 µl	20 mg/ml	0.12 mg/ml

	buffer without DMSO	1.78 µl		
	water	0.40 µl		
	total volume	5.5 µl		

Enzyme mix (incl. 10% excess)

		vol.	**stock conc.**
	Rnase H	5 µl	5000 U/ml
	BSA (0.48mg/ml)	1.2 µl	20 mg/ml
	buffer without DMSO	16.67 µl	
	water	27.13 µl	
	total volume	50 µl	

Diluted Rnase H

STEP CASE

(a) With denaturation step 2 steps

Reaction mixture without the enzyme mix is incubated at 65 °C for 2 min followed by a 10-min incubation at 41 °C. Following that, 5 µl enzyme mix is added into the reaction and incubated at 41 °C for a further of 90-120 min.

3 **Detection with lateral flow dipstick (if desired)**

For detection with lateral flow assay, Bio-11-UTP can be added into the NASBA nucleotide mixture in Step 2 at a final concentration of 0.5 mM. At the end of the NASBA reaction, RNA is purified from the end-product using Direct-zol RNA Miniprep kit, and eluted with 30 µl of RNase free water. After purification, 4.2 µl of purified RNA

is mixed with 1.8 µl of FAM labelled RNA capture oligo and 84 µl of PCRD extraction buffer. Take 75 µl of mix to the sample well of a PCRD test cassette. Results will be shown within 10 min.

4 **Sequencing stage**

To allow for pooled sequencing of NASBA reaction end products, barcode sequences are added upstream of each of the forward and reverse primers. In addition, an Illumina sequencing adaptor is added upstream of the forward primer barcode sequence as a universal PCR handle (see Materials and Reagents for the exact sequence).

Uniquely barcoded NASBA end products from different samples are pooled and purified. A one step PCR can be carried out at local sequencing centres using universal P5 and P7 primers. Here, if needed, another layer of indexing barcodes can be added to further increase the multiplexing capacity.