INSIGHT: a population scale COVID-19 testing strategy combining point-of-care diagnosis with centralised high-throughput sequencing V.2

Version 2
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1 *Works for me* This protocol is published without a DOI.

Coronavirus Method Development Community

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ABSTRACT

We present INSIGHT (Isothermal NASBA-Sequencing-based hIGH-througput Test): a two-stage COVID-19 testing strategy, using a barcoded isothermal NASBA reaction that combines point-of- care diagnosis with next generation sequencing, aiming to achieve population-scale COVID-19 testing. INSIGHT combines the advantages of near-patient with centralised testing. Stage 1 allows a quick decentralised readout for early isolation of pre-symptomatic or asymptomatic patients. The same reaction products can then be used in a highly multiplexed sequencing-based assay in Stage 2, confirming the near-patient testing results and facilitating centralised data collection. Based on experiments using commercially acquired human saliva with spiked-in viral RNA as input, the INSIGHT platform gives Stage 1 results within one to two hours, using either fluorescence detection or a lateral flow (dipstick) readout, whilst simultaneously incorporating sample-specific barcodes into the amplification product. INSIGHT Stage 2 can be performed by directly pooling and sequencing all post-amplification barcoded Stage 1 products from hundreds of thousands of samples with minimal sample preparation steps. The 95% limit of detection (LoD-95) for INSIGHT is estimated to be below 50 copies of viral RNA per 20 µl of reaction. Our two-stage testing strategy is suitable for further development into a rapid home-based and point-of-care assay, and is potentially scalable to the population level.

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MATERIALS

NAME	CATALOG #	VENDOR
QuickExtract DNA Extraction Solution	QE09050	Lucigen
NASBA liquid kit	SKU: NWK-1	Life Sciences Advanced Technologies Inc.
Tris (1 M) pH = 8 RNase free	AM9855G	Invitrogen - Thermo Fisher
Sodium Hydroxide	71687	Sigma-aldrich
1M MgCl2	AM9530G	Invitrogen - Thermo Fisher
2M KCl	AM9640G	Invitrogen - Thermo Fisher
DTT	43816	Sigma-aldrich
DMSO	276855	Sigma-aldrich
dNTP set 100 mM	10297018	Invitrogen - Thermo Fisher
NTP set 100 mM	R0481	Thermo Scientific
RNase H	M0297L	NEB
ProtoScript II reverse transcriptase	M0368S	NEB
T7 RNA polymerase	M0251L	NEB
BSA 20 mg/ml	B9000S	NEB
PCRD lateral flow assay	FG-FD51673	Abingdon Health
Qubit RNA HS Assay Kit	Q32852	Invitrogen - Thermo Fisher
PowerUp™ SYBR™ Green qPCR Master Mix	15340939	Applied Biosystems
Twist synthetic SARS-CoV-2 RNA control	Mt007544.1	Twist Bioscience
NASBA lyophilised kit	SKU: NLK	Life Sciences Advanced Technologies Inc.
Qubit dsDNA HS Assay Kit	Q32851	Invitrogen
Normal human saliva	MBS170210	MyBioSource
QIAquick PCR Purification Kit	28104	Qiagen
KAPA HiFi HotStart ReadyMix PCR Kit	KK2600	Kapa Biosystems
AMPure XP	A63882	Bechman Coulter
MiSeq Reagent Kits v2 (300-cycles)	MS-102-2002	illumina

MATERIALS TEXT

NASBA primers (P8) sequence:

FWD primer	CCAGCAACTGTTTGTGGACCTA
REV primer with T7 handle	aattctaatacgactcactatagggagaaggACACCTGTGCCTGTTAAACCAT
FWD primer with 5-nt barcode and Illumina handle	tgactggagttcagacgtgtgctcttccgatctnnnnnCCAGCAACTGTTTGTGGACCTA
REV primer with 5-nt barcode and T7 handle	aattctaatacgactcactatagggagaaggnnnnnACACCTGTGCCTGTTAAACCAT

Toehold molecular beacon (2'-O-methyl RNA):

FAM-AUUGACAGUCUACUAAUUUGGUUAAAAACAAAUGUGUCAA-BHQ1dT-UUCAACUUCAAUG-propyl

P8 RNA capture oligos for PCRD:

Probe A	FAM-AAAAGTCTACTAATTTGGTTAAAA
Probe B	ACAAATGTGTCAATTTCAACTTCA-Biotin

Library construction PCR primers:

P5 end primer	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNAGCCAGCTCTGGAGAATTCTAATACGACTCACTATAGGGAGAAGG
P7 end primer	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Customised NGS primer (T7containing)	AGCCAGCTCTGGAGAATTCTAATACGACTCACTATAGGGAGAAGG

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 (commercial pooled human saliva from healthy individuals) 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 (Option A) NASBA Saliva lysatereaction with fluorescence detection

Take 1 µl from the product of Step 1 (saliva lysate) and add into the NASBA reaction mixture (without the enzyme mix) to make a total volume of 15 µl. Reaction mixture can either be prepared in-house or from the Life Sciences NASBA liquid kit (see tables below) using one of the two temperature settings below.

a. 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.

b. Alternatively, reaction mixture without the enzyme mix is incubated at 95 °C for 5 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 90-120 min.

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

	vol.	stock conc.	conc. in RM
Saliva lysate	1 µl		
primers*/beacon# mix	1 µl	500 nM each primer, 400 nM beacon	25 nM each primer, 20 nM beacon
spiked-in viral RNA/water	3 µl		
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.
# Molecular beacon is reconstituted with annealing buffer (10 mM Tris pH 8 with 10 µM $MgCl_2$) to the final concentration of 10 µM. Beacon is then annealed by incubation at 85 °C for 5 min, then gradual cooling to 4 °C by 0.1 °C/s before the NASBA reaction.

	vol.	stock conc.	conc. in RM
Saliva lysate	1 µl		
primers*/beacon# mix	1 µl	25 nM each primer 20 nM beacon	25 nM each primer 20 nM beacon
spiked-in viral RNA/water	4 µl		
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.
# Molecular beacon is reconstituted with annealing buffer (10 mM Tris pH 8 with 10 µM $MgCl_2$) to the final concentration of 10 µM. Beacon is then annealed by incubation at 85 °C for 5 min, then gradual cooling to 4 °C by 0.1 °C/s before the NASBA reaction.

	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

(Option B) NASBA reaction with lateral flow dipstick detection

For detection with a lateral flow assay, a NASBA lyophilised kit is used with the constitution of the reaction mixture shown below.

Take 4 µl from the product of Step 1 (saliva lysate) and add into the NASBA reaction mixture (without the enzyme mix) to make a total volume of 60 µl. Incubate at 95 °C for 5 min followed by a 10-min incubation at 41 °C.

Following that, 20 µl enzyme mix is added into the reaction and incubated at 41 °C for a further of 90-120 min. Take the reaction product to the sample well of a PCRD test cassette. Results will be shown within 10 min.

3 Library construction for NGS

To allow for pooled sequencing of NASBA reaction end products, barcode sequences are added upstream of each of the forward and reverse primers (Figure 3a). In addition, an Illumina sequencing adaptor is added upstream of the forward primer barcode sequence as a universal PCR handle (see Materials and reagents section for the exact sequence).

Here, 2 µl NASBA end products from each sample are first pooled into a single tube. Pooled products are then column purified to remove residual NASBA primers (QIAquick PCR Purification Kit). PCR is performed on the column purified pooled sample using two NGS indexing primers. Here, we have designed a customised NGS primer containing the T7

polymerase promoter sequence (see Materials and reagents section for the exact sequence) at the P5 end and used a standard TruSeq sequencing primer at the P7 side. A PCR mix is made based on the table below. A standard PCR program is used with longer elongation time and minimal cycle number to reduce barcode hopping.

	vol.
2x PCR mix (KAPA HIFI HotStart ReadyMix)	20 µl
P5 end primer (10 µM)	1 µl
P7 end primer (10 µM)	1 µl
Column purified NASBA product (~3.5 ng dsDNA)	4 µl
Nuclease free water	14 µl

Library construction PCR

temperature	**time**	**cycle number**
95 °C	3 min	1
98 °C	20 s	15
60 °C	15 s	
72 °C	30 s	
72 °C	4 min	1

After the PCR, an AMPure bead-based double size selection is carried out (0.55x and 0.75x) to enrich for products of interest. In this study, a MiSeq Reagent Kit v2 (300- cycles) was used for NGS.

4 Analysis of NGS results

To analyse the INSIGHT NGS data, sequences in FASTQ files are first trimmed to leave the first 80 nucleotides for both read 1 and read 2 using FASTX_trimmer. The trimmed read 1 and paired read 2 are then merged by FLASH. The merged sequence is compared with the reference viral genome sequence (NNNNNACACCTGTGCCTGTTAAACCATTGAAGTTGAAATTGACACATTTGTT TTTAACCAAATTAGTAGACTTTTTAGGTCCACAAACAGTTGCTGGNNNNN, N stands for the barcode position), and only those with a hamming distance less than or equal to 2 are extracted. Here, only substitutions were allowed while insertion- and deletion- containing reads were filtered out. The first 5 nt and the final 5 nt regions of all extracted sequences correspond respectively to the right barcode and the reverse complement of the left barcode. Diagnostic results for sequenced NASBA samples are determined according to the read counts of their corresponding sample-specific barcode pairs. More details can be found in *www.github.com/suochenqu/INSIGHT*.