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

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

***** This is ongoing research to develop a testing strategy for the SARS-CoV-2 virus. The Wellcome Sanger Institute is not in a position to develop this into a commercial product, but would be open to discussions with third parties about how they could develop this further to achieve the societal benefits of this work. *****

*** This is a work in progress and will be regularly updated (version date: 31/05/2021). ***

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.

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic is caused by the SARS-CoV-2 virus [1]. Pandemic control has been challenging due to the long incubation period and high percentage of asymptomatic carriers of this disease [2, 3]. Nucleic acid testing is essential to identify and isolate infected individuals at an early stage to stop the spread of the virus. At the moment, the mainstream nucleic acid test relies on a reverse transcription polymerase chain reaction (RT-PCR) assay, performed on nasopharyngeal or oropharyngeal swabs [4]. It requires labour-intensive RNA extraction and expensive equipment such as a thermocycler. The complexity, cost and availability of RNA extraction kits and thermocyclers have limited the throughput of such RT-PCR assays. Hence, even though the current testing regime, in conjunction with the lockdown measures, has successfully brought down the reproduction number R_0 of the disease to below 1 in many countries, ramping up testing capacity sufficiently to maintain R_0 below 1 will be challenging once social activities return to normal. At the same time, a prolonged lockdown is highly detrimental to the economy and the physical and mental health of individuals. Regular, high-throughput testing with rapid results is one way out of the current conundrum. Several point-of-care diagnostic tests have been proposed and some already authorised for use around the world, including SAMBA II, Abbott ID NOW and many others. However, they typically require relatively expensive instruments or reagents, thus limiting their widespread adoption at a population level.

The ideal test would have the following five features: it would be accurate, cheap, scalable, portable and fast. This would allow for decentralised and frequent testing of a large proportion of the population, even in countries with limited medical resources. Here we propose INSIGHT (Isothermal NASBA-Sequencing based hIGH-througput Test): a two-stage testing strategy, using a combination of Nucleic Acid Sequence-Based Amplification (NASBA) and Next Generation Sequencing (NGS) technologies, to achieve the five desired features of an ideal test (Figure 1a). The first stage of INSIGHT is the NASBA reaction, which can potentially be incorporated into low-cost and portable testing devices that can generate rapid test results on the spot in 1-2 hours. The second stage employs next generation sequencing to improve the test accuracy in a highly scalable way, and may also provide some insight into viral evolution, relevant for monitoring of viral strains and their spreading and transmission patterns. Below we describe the two stages of INSIGHT in detail.

The first stage consists of an isothermal NASBA reaction (Figure 1b) with crude saliva as sample input that could be incorporated into a point of care or home-based kit. NASBA employs reverse transcription and T7 RNA polymerase-mediated *in vitro* transcription to rapidly amplify RNA of interest. More than a billion-fold amplification can be typically achieved in under two hours [5]. Compared to RT-PCR, the isothermal nature of NASBA means that special equipment, such as thermocyclers, are not needed. A fluorescent molecular beacon can be used as a readout to monitor the amplification in real time and yield rapid test results [6]. Furthermore, we also explored the possibility of using a lateral flow assay for a relatively quick and cheap dipstick-based readout.

The first stage is highly specific due to the use of a specific pair of primers, and a molecular beacon or capture oligo that is complementary to part of the amplified sequence (amplicon). While molecular beacons are generally very sensitive in detecting the presence of target amplicons, we propose a second NGS stage to further improve the sensitivity and specificity of the test in a scalable way. To achieve this, a sample-specific barcode sequence can be integrated into the primers and incorporated into the amplicon during the reaction. The reaction

end product from different samples can then be sent to a central facility for pooled sequencing, in theory allowing up to hundreds of thousands of samples to be analysed on a single sequencer. The DNA barcodes allow identification of each sample in the pool and the amplicon sequence ensures the specificity of the test. If required, another layer of sequencing library indexing can be performed to further expand the scale and multiplexing possibilities, potentially up to a population level. The highly sensitive nature of NGS may also significantly reduce any possible false negative or false positive results. At the same time, the sequencing stage could be used to monitor the mutations within the short amplified region, which may contribute to vaccine development and our general understanding of viral spread.

Materials and reagents

Reagent	Source	Identifier
QuickExtract DNA Extraction Solution	Lucigen	QE09050
NASBA liquid kit	Life Sciences Advanced Technologies Inc.	SKU: NWK-1
Tris (1 M), pH = 8, RNase free	Invitrogen	AM9855G
Sodium Hydroxide	Sigma-Aldrich	71687
1M $MgCl_2$	Invitrogen	AM9530G
2M KCl	Invitrogen	AM9640G
DTT	Sigma-Aldrich	43816
DMSO	Sigma-Aldrich	276855
dNTP set, 100 mM	Invitrogen	10297018
NTP set, 100 mM	Thermo Scientific	R0481
Bio-11-UTP (75 mM)	Invitrogen	AM8451
RNase H	NEB	M0297L
ProtoScript II reverse transcriptase	NEB	M0368S
T7 RNA polymerase	NEB	M0251L
BSA 20 mg/ml	NEB	B9000S
Direct-zol RNA Miniprep Plus	Zymo Research	R2070
PCRD lateral flow assay	Abingdon Health	FG-FD51673
Qubit RNA HS Assay Kit	Invitrogen	Q32852
PowerUp™ SYBR™ Green qPCR Master Mix	Applied Biosystems	15340939

Primers (P8)	
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 FAM labelled RNA capture oligo for PCRD
FAM-AAAAGTCTACTAATTTGGTTAAAAACAAATGTGTCAATTTCAACTTC

*Molecular beacons were synthesised in the ATDBio laboratory using a K&A H-8 SE DNA Synthesiser, and purified by reversed-phase HPLC.

Experimental protocol

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. 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 including the enzyme mix is prepared and incubated directly at 41 °C for a total of 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.

   Life Sciences reaction mixture (RM)

	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		

   In-house reaction mixture (RM)

	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		

   *see Supplementary Table S1 for detailed composition

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 (see table below). Take 75 µl of mix to the sample well of a PCRD test cassette. Results will be shown within 10 min.

	vol.	**final conc.**
purified RNA from NASBA	4.2 µl	
FAM labelled RNA capture oligo	1.8 µl	20 nM
PCRD extraction buffer	84 µl	

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 (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 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 (Figure 3b). Here, if needed, another layer of indexing barcodes can be added to further increase the multiplexing capacity.

This protocol is also available at:
www.protocols.io/view/insight-a-scalable-isothermal-nasba-based-platform-bghsjt6e

The software code for barcode generation is available at: github.com/suochenqu/INSIGHT

Results

Optimising the NASBA reaction

Primers were designed to target the SARS-CoV-2 S gene, encoding the viral envelope spike glycoprotein. The S gene is one of the most highly expressed viral RNAs [7], and at the same time is a promising target for SARS-CoV-2 vaccine development [8]. In addition, the S gene sequence is an informative sentinel for viral genome evolution with respect to increased or decreased affinity to the human viral entry receptors such as ACE2 [9]. We designed 53 pairs of primers based on the NASBA primer selection criteria reported in the literature [6, 10] and selected the most efficient pair to optimise our in-house NASBA reaction (Supplementary Note, Supplementary Figure S1a, b). All subsequent reactions and figures shown in this paper were carried out with primer pair P8 (sequence shown in Materials and Reagents). Furthermore, we also optimised the primer concentration used in the NASBA reaction by quantifying the NASBA end product RNA concentration with a molecular beacon (Supplementary Figure S1c). We found a primer concentration of 25 nM each, which is 10 times less than typically used in the literature [11-13], increased the reaction efficiency.

Additional optimisation has been carried out for our in-house NASBA reaction mixture (Supplementary Note). This includes the choice of enzymes (Protoscript II reverse transcriptase instead of AMV reverse transcriptase, Supplementary Figure S1d), the concentration of enzymes (higher concentration with better yield and more consistent results, Figure 2a) and the buffer pH (Supplementary Figure S1e).

COVID-19 detection with NASBA

With synthetic SARS-CoV-2 RNA as the input, we have achieved target RNA amplification with both our in-house NASBA mixture and a commercial ready-made mixture. The NASBA reaction product was confirmed by RNA urea gel (Figure 2b), showing a specific product of the expected size in the presence of viral template RNA only. We then established two forms of specific readouts for COVID NASBA reaction: a molecular beacon with fluorescence detection, and a dipstick-based lateral flow assay.

Molecular beacons are hairpin-shaped molecules with a fluorophore and a quencher covalently attached, and brought into close proximity due to the secondary structure formed by the hairpin. Upon recognition of the target, the fluorophore and the quencher will be spatially separated due to hybridisation, which results in fluorescence (Figure 2c). Here, four types of beacons were designed to target the P8 amplicon: a conventional DNA beacon, a toehold DNA beacon, a conventional 2'-O-methyl RNA beacon and a toehold 2'-O-methyl RNA beacon. The toehold provides an initial anchor point for the beacon to latch onto its target and assists in the unwinding of the stem of the beacon, and the 2'-O-methyl modification increases target affinity and provides stability against oligonucleotide degradation. All four types were first tested with *in vitro* transcribed RNA, and the 2'-O-methyl RNA toehold beacon was found to achieve the best sensitivity (Figure 2d). Therefore, we chose the RNA toehold beacon for our COVID-19 NASBA assay. An essential feature of this beacon is a 3'-propyl group which prevents any possibility of polymerase extension of the toehold sequence.

We have also titrated the concentration of the toehold RNA beacon in the reaction mixture to achieve the best real-time results, as a high beacon concentration inhibits the NASBA reaction (Supplementary Figure S1f). We found that 20 nM of molecular beacon resulted in the best

assay sensitivity. With this beacon concentration, the fluorescence plateaus at around 60 min into the reaction. The real-time detection limit is around 100-1000 copies/reaction for our in-house mixture, and around 10 copies/reaction for the commercial mixture (Figure 2e).

Nucleic acid lateral flow assays are a simple, portable and cheap platform for detecting analytes. Here, we use a format of lateral flow assay which detects nucleic acid utilising neutravidin-conjugated carbon nanoparticles (NA-CNPs), a test line comprising an anti-FAM antibody and a biotin control line to capture excess NA-CNPs (Figure 2f). Biotinylated UTP can be incorporated into the NASBA RNA products during the reaction. Subsequently, a FAM-labelled RNA capture oligo is hybridised to the single-stranded RNA NASBA product. The dual tagging of FAM and biotin results in aggregation of NA-CNP at the test line, resulting in a visible signal in the assay (Figure 2f). Here, the concentration of biotinylated UTP was first titrated, since a higher concentration of bio-UTP inhibits the NASBA reaction, but a lower concentration reduces the sensitivity of end-product detection (Figure 2g). We found 12% biotin-UTP resulted in an optimal compromise between NASBA reaction efficiency and lateral flow sensitivity. A positive lateral flow assay showed a clearly visible line at test line 2 in addition to the control line (C-line) (Figure 2h).

For the second stage of the test using an NGS readout, by using combinatorial barcoding, a 5-nt barcode sequence on each primer can generate up to a million unique barcodes, or 30,000 Hamming-distance 3-separated barcodes. The latter allows single nucleotide substitution error in the barcodes to be corrected after sequencing. If needed, a 6-nt barcode on each primer can generate 450,000 Hamming-distance 3-separated unique barcode pairs. (The software code for barcode generation is available at: *github.com/suochenqu/INSIGHT.*)

We tested two pairs of primers with 5-nt barcodes flanked on each side, and an Illumina handle on the forward primer. The detection threshold was around 10-100 copies/reaction for the commercial NASBA mixture (Figure 3c). Finally, we amplified our barcoded NASBA product with PCR using primers flanked with sequencing adapters. A distinctive band at the desired product size was shown on the DNA gel, indicating a clean sequencing library (Figure 3d). During this step, another layer of indexing can be added, enabling further multiplexing, as well as control of cross-contamination between runs. We are currently carrying out studies using barcoded NASBA products and analysing the NGS result in the lab.

Towards a home-based COVID-19 assay: isothermal testing on crude saliva lysate

To work towards a version of the INSIGHT test to be used in the home, and to simplify the assay workflow and improve its scalability, the NASBA reaction would ideally be applied to a crude saliva lysate. This would alleviate the need for complex and expensive processes to purify RNA. Commercially acquired human saliva can be mixed with QuickExtract buffer and heat treated at 95 °C for 5 min to generate saliva lysate. Here, we demonstrate that saliva lysate is compatible with the NASBA reaction. Furthermore, using saliva lysate as input, we have also tested a completely isothermal NASBA reaction without the 65 °C RNA denaturation step.

Following the protocol in Ladha et al. [14], we mixed commercially available human saliva from healthy individuals with QuickExtract DNA Extraction Solution (Figure 4a), which contains detergents and proteinase K that have been shown to expose viral RNA. We then spiked in synthetic viral RNA and carried out the NASBA reaction with a real-time molecular beacon detection. Saliva with lysis buffer was compatible with the downstream NASBA reaction, albeit with a slightly reduced detection threshold of 10-100 copies/reaction (Figure

4b, c). Furthermore, we also tested maintaining the NASBA reaction completely isothermal at 41 °C (i.e. using option (b) in Step 2 of the protocol, Figure 4d) with crude saliva lysate as input. While the amplification appeared to take slightly longer, the detection threshold remained at 10-100 copies/reaction (Figure 4e).

Discussion

INSIGHT was developed to achieve a portable, scalable, accurate, fast and cheap diagnosis for SARS-CoV-2. The use of an isothermal NASBA reaction allows the amplification to be achieved in any portable device that can maintain a constant temperature of 41 °C. In addition, we have simplified the assay procedure, allowing the use of crude saliva as the assay input. In the future, we hope that these two features will allow the assay to be scalable and broadly accessible to the general public in the home. INSIGHT's dual-stage design allows the first stage rapid result to be confirmed by a second stage NGS analysis, thus further improving the accuracy of the test. We have shown that a 5-nt barcode on each of the forward and reverse primers dose not compromise the assay sensitivity, and early barcoding allows pooling before library construction for sequencing.

The NASBA reaction is rapid and gives a detectable fluorescence signal within 10 to 50 min, depending on the input viral RNA amount. With the QuickExtract lysis step, the entire procedure from saliva to result can be carried out within 2 hours, making it substantially faster than conventional RNA extraction and RT-qPCR based assays. Finally, NASBA only requires off-the-shelf reagents. Based on list prices and excluding labour and instrument costs, our in-house and commercial NASBA mix with molecular beacon costs £2.67 and £9.29 per reaction respectively. The lateral flow assay option would cost an extra £8.66 per reaction for the biotin-11-UTP and the PCRD detection strip.

INSIGHT has shown promising results in experimental settings. However, additional work is required to bring it into practical use. Most importantly, the current experiments were carried out using saliva from healthy individuals with spiked-in viral RNA. The accuracy of INSIGHT remains to be tested on actual patient samples, and false positive and negative rates remain to be determined for each stage. In addition, if a molecular beacon readout is chosen in the first stage of INSIGHT, an inexpensive fluorescence detector needs to be incorporated into the test device, adding to the cost and time necessary for developing such a device.

The alternative option of dipstick readout requires less engineering effort, but currently requires additional steps of RNA purification and sample dilution. Work is ongoing to simplify the assay procedure to remove these steps so as to minimise sample handling. Finally, in the sequencing stage, we will also need to examine the extent of index swapping during the universal indexing PCR and bridge PCR on the flow cell. This may require modifications to the reaction conditions or use of two layers of unique dual indexing.

Other isothermal methods have been proposed for SARS-CoV-2 detection. Most of them are based on the RT-LAMP technology with a colourimetric or turbidimetric readout [15-18]. These assays could potentially suffer from false positive results generated from non-specific primer binding or primer dimers. A major advantage of INSIGHT is to enable a rapid readout whilst still giving high specificity for SARS-CoV-2. In the first stage, the addition of a molecular beacon for fluorescence readout or RNA capture oligo for lateral flow readout provides an additional layer of sequence specific detection. In the second stage, NGS further improves the assay sensitivity and specificity by unambiguously identifying the viral sequence.

The most comparable methods in the literature to our approach are the STOPCovid test [19] and LAMP-Seq method [20]. Similar to our first stage, STOPCovid has an added layer of sequence specific detection using a CRISPR-mediated technology. On the other hand, LAMP-Seq uses NGS to detect LAMP-amplified viral genome with high specificity. It is unclear

whether STOPCovid and LAMP-Seq could be combined in a way similar to the two-stage design in INSIGHT. However, we note that unlike the concatemer amplification product in LAMP, the NASBA reaction used in INSIGHT generates a single well-defined amplicon and can easily incorporate barcodes in both forward and reverse primers allowing combinatorial barcoding and thus making it particularly suited for high-throughput pooled NGS analysis.

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Declaration of conflicts of interest

Q.W., C.S., S.A.T. and A.R.B. are inventors on a patent application (GB2007724.4) submitted by Genome Research Limited, with the aim to make this technology freely available for research and deployment. In the past three years, S.A.T. has consulted for Genentech and Roche, and sits on Scientific Advisory Boards for ForeSite Labs, Biogen and GlaxoSmithKline. T.B. is a consultant at ATDBio.

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Figures

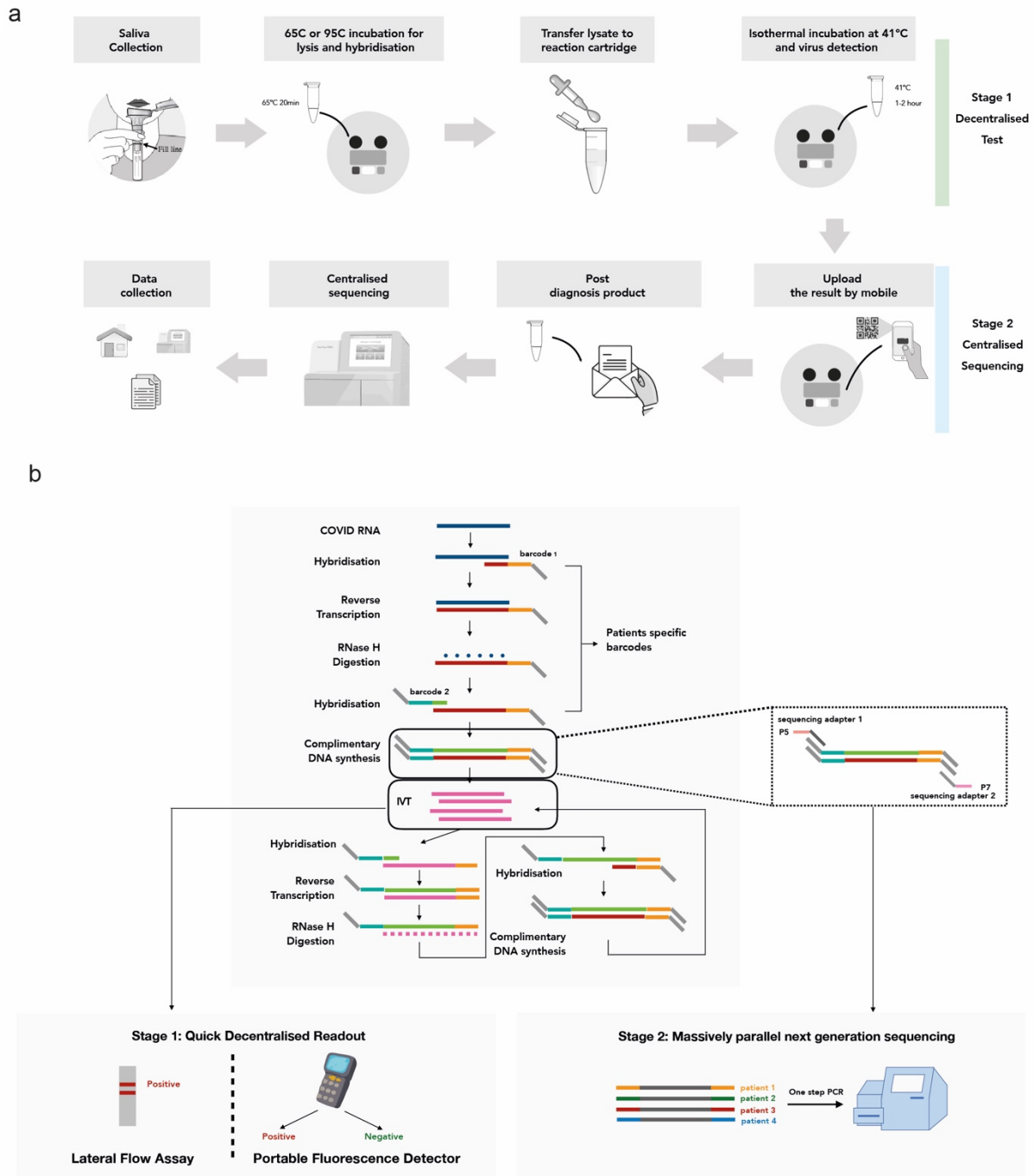

Figure 1. **INSIGHT: an all-in-one platform for two-stage COVID-19 detection**. (a) Schematic outline of our proposed two-stage testing strategy. Stage 1 is a portable decentralized COVID-19 rapid test. Saliva can be collected directly into a tube containing QuickExtract lysis buffer at home or point-of-care. After heating up to 95 °C to lyse the virus and inactivate proteinase K, the lysate can be transferred into the NASBA reaction and incubated at 41 °C. After 1-2 hours, result can be directly visualised with a portable fluorescence detector or lateral flow assay. In Stage 2, individuals can post the used reaction mixture to a local sequencing centre. All samples are pooled and sequenced after a one-step

universal PCR. (b) Barcoded NASBA with dual readout for COVID-19 detection. In the NASBA reaction, a sample-specific barcode 1 is inserted between the sequencing adaptor and the forward primer, and barcode 2 is inserted between the T7 promoter and the reverse primer. The NASBA *in vitro*-transcribed RNA product can be used to carry out either a portable fluorescence detection assay or a dipstick-based lateral flow assay for rapid results. Afterwards, the NASBA DNA products can be pooled and sequenced.

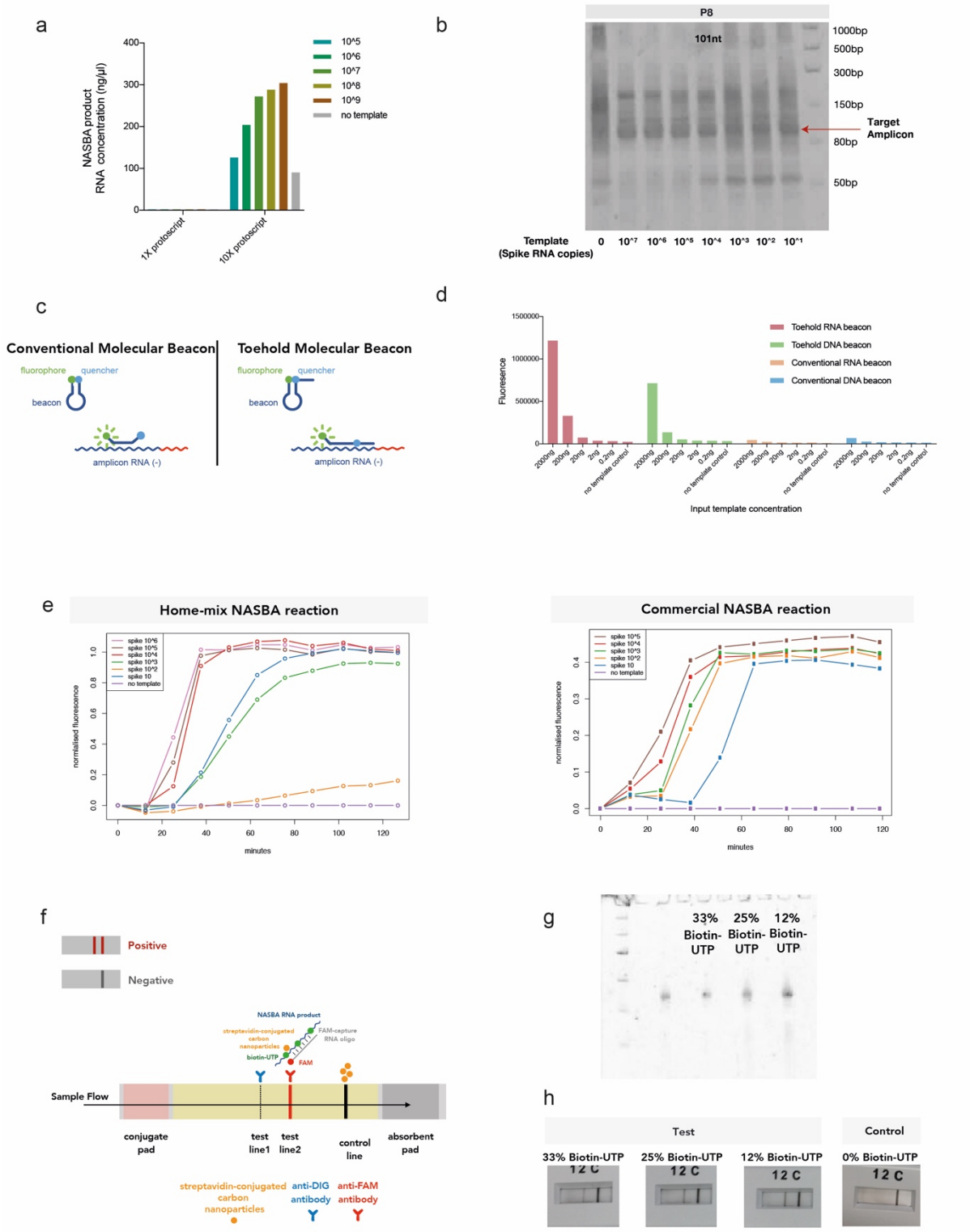

Figure 2. NASBA with instant beacon- or dipstick-based COVID-19 detection. (a) High enzyme concentrations improve NASBA efficiency. By adding ten times the amount of Protoscript II reverse transcriptase, ten times more T7 polymerase and three times increased RNase H, as reported in [11], a significantly higher NASBA end product concentration is detected by Qubit RNA HS kit at all input template amounts. (b) RNA urea gel for NASBA end product with primer pair P8 and different input template amounts ranging from 10^1 to 10^7

copies of viral RNA templates. Amplicon-sized band (101nt) is boxed. (c) Illustration of conventional and toehold molecular beacons. (d) Sensitivity of different types of molecular beacons. Four different types of beacons were mixed with various amount (0.2ng to 2000ng) of *in vitro*-transcribed target amplicon RNA for fluorescence readout. (e) Realtime molecular beacon results using in-house and commercial NASBA mixtures with various amounts (10^1 to 10^6 copies) of input viral RNA templates. With a final concentration of 20 nM of beacon in the reaction, fluorescence was detectable at around 30 mins, and reached saturation at around 60 mins. (f) Illustration of the nucleic acid lateral flow assay. (g) RNA urea gel for NASBA end product with various proportions (12%, 25%, 33%) of biotin-UTP within the UTP in the reaction. Input template quantity was 10^3 copies of viral RNA for all three reactions. (h) Lateral flow assay readout of purified RNA from NASBA reactions. Different proportions (12%, 25%, 33%) of biotin-UTP were used. Input template quantity was 10^3 copies of viral RNA in all three reactions.

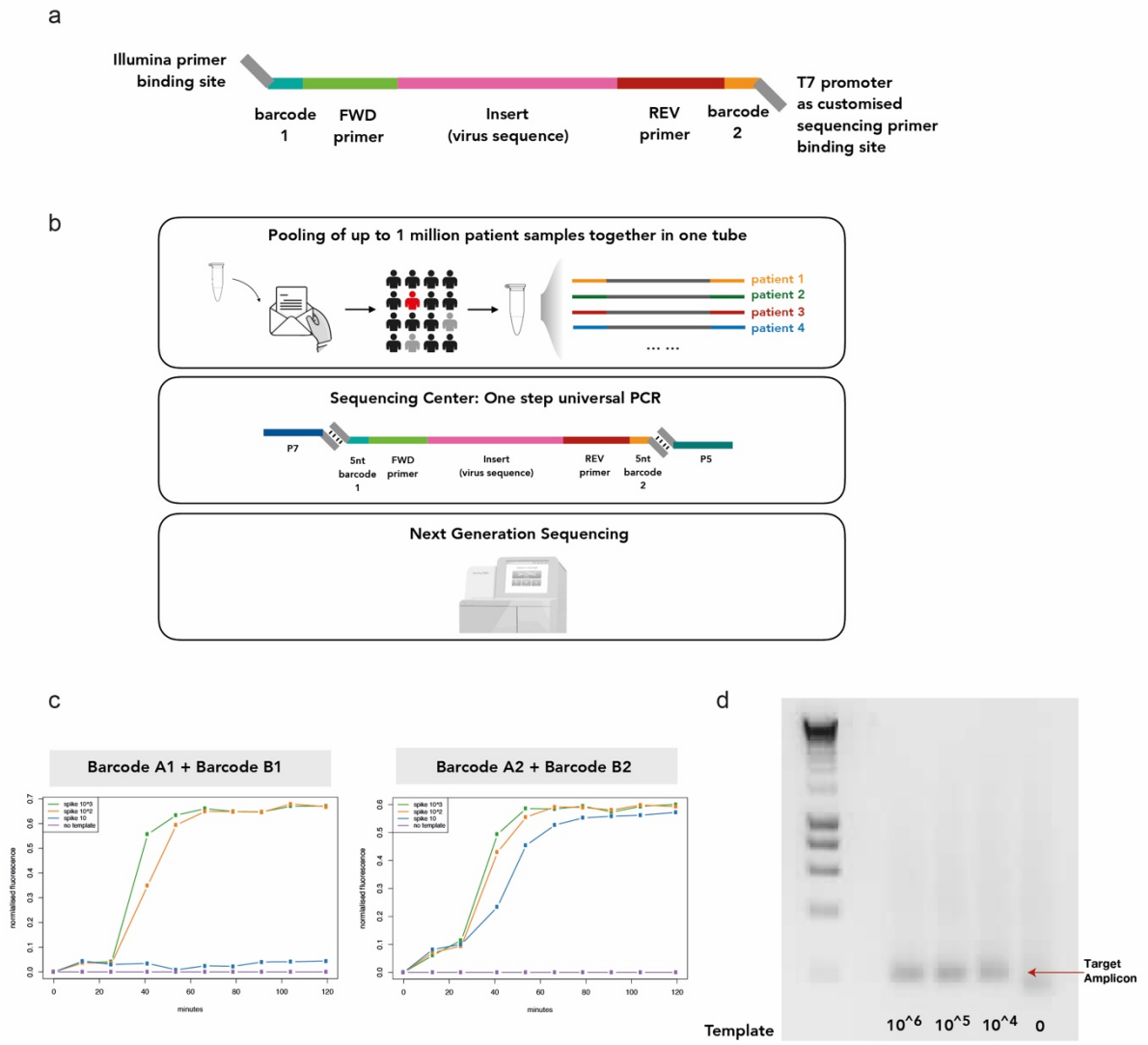

Figure 3. Barcoded NASBA for next generation sequencing. (a) Schematic illustration of the barcoded NASBA DNA product. Using primer pair P8, the forward primer contains a 24-nt sequence complementary to part of the SARS-CoV-2 S gene, a 5-nt barcode 1 and a 33-nt sequence of the Illumina sequencing adaptor. The reverse primer contains a 22-nt sequence complementary to part to SARS-CoV-2 S gene, a 5-nt barcode 2 and a 31-nt T7 promoter. Customised sequencing primers can be used for the reverse read. (b) Strategy for Stage 2 NGS based detection. Up to 1 million samples can be pooled together, and a universal indexing PCR can be carried out at local sequencing centre. (c) Real time molecular beacon readout for two representative barcoded primer pairs with various amounts (10 to 10^3 copies) of input viral RNA templates. (d) DNA gel with amplified NASBA sequencing library. The specific product at 156 nt is marked with an arrowhead.

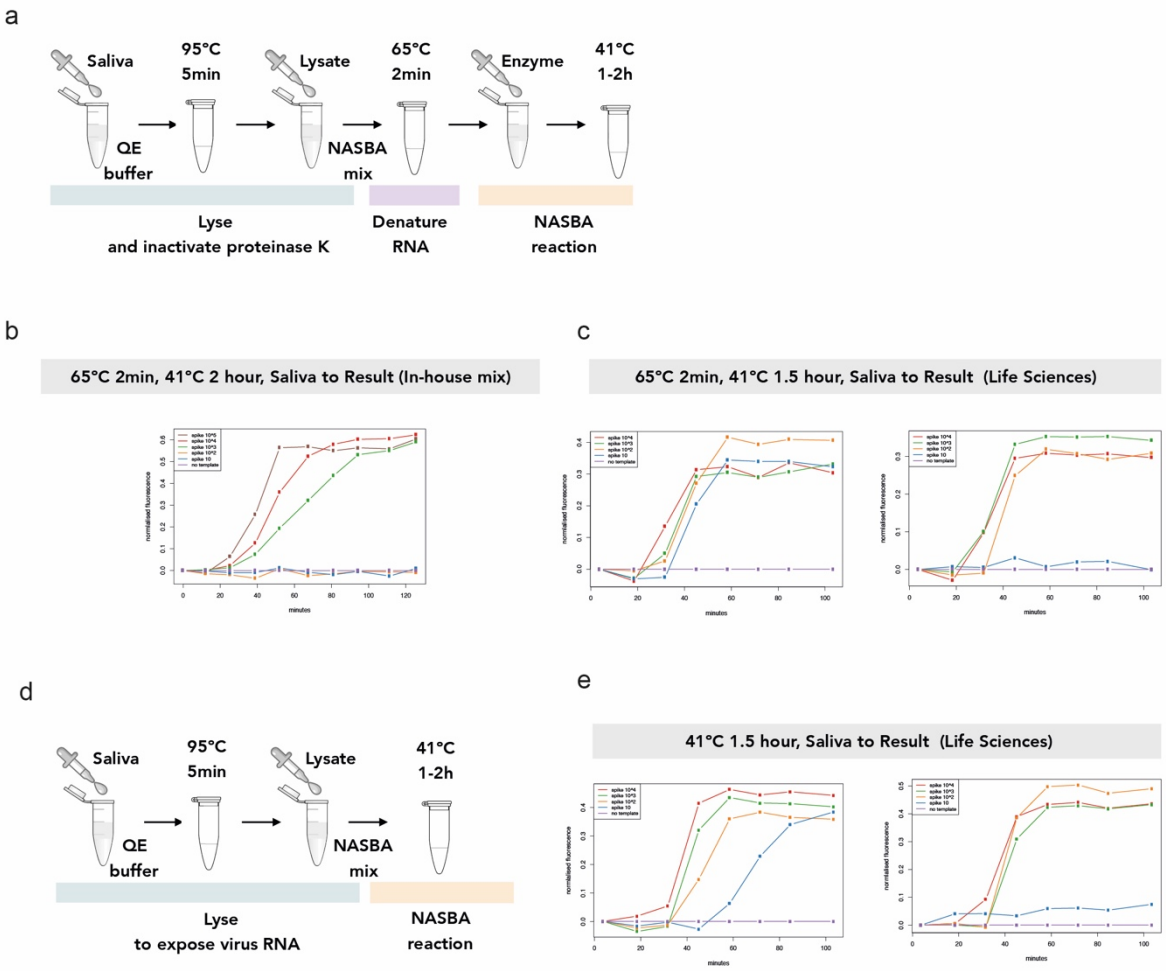

Figure 4. Towards a home-based COVID-19 assay. (a) Protocol of simple viral lysis in saliva followed by conventional NASBA reaction. (b) Realtime molecular beacon detection using saliva lysate as input and an in-house NASBA mix. (c) Realtime molecular beacon fluorescence readings using saliva lysate as input and a commercial NASBA mixture. The commercial NASBA assay can reliably detect 100 copies of input viral RNA templates, while 10 copies of input RNA can only be detected in one of the two technical replicates. (d) Protocol of viral lysis in saliva followed by a completely isothermal NASBA reaction. NASBA mixtures were directly combined with viral lysates and enzymes without the initial 65 ºC RNA denaturation step. (e) Real time molecular beacon fluorescence reading using crude saliva lysate as input, and commercial NASBA mixture under completely isothermal reaction conditions. Reliable detection is achieved for 100 copies of input viral RNA templates.

Supplementary Information

Supplementary Figure

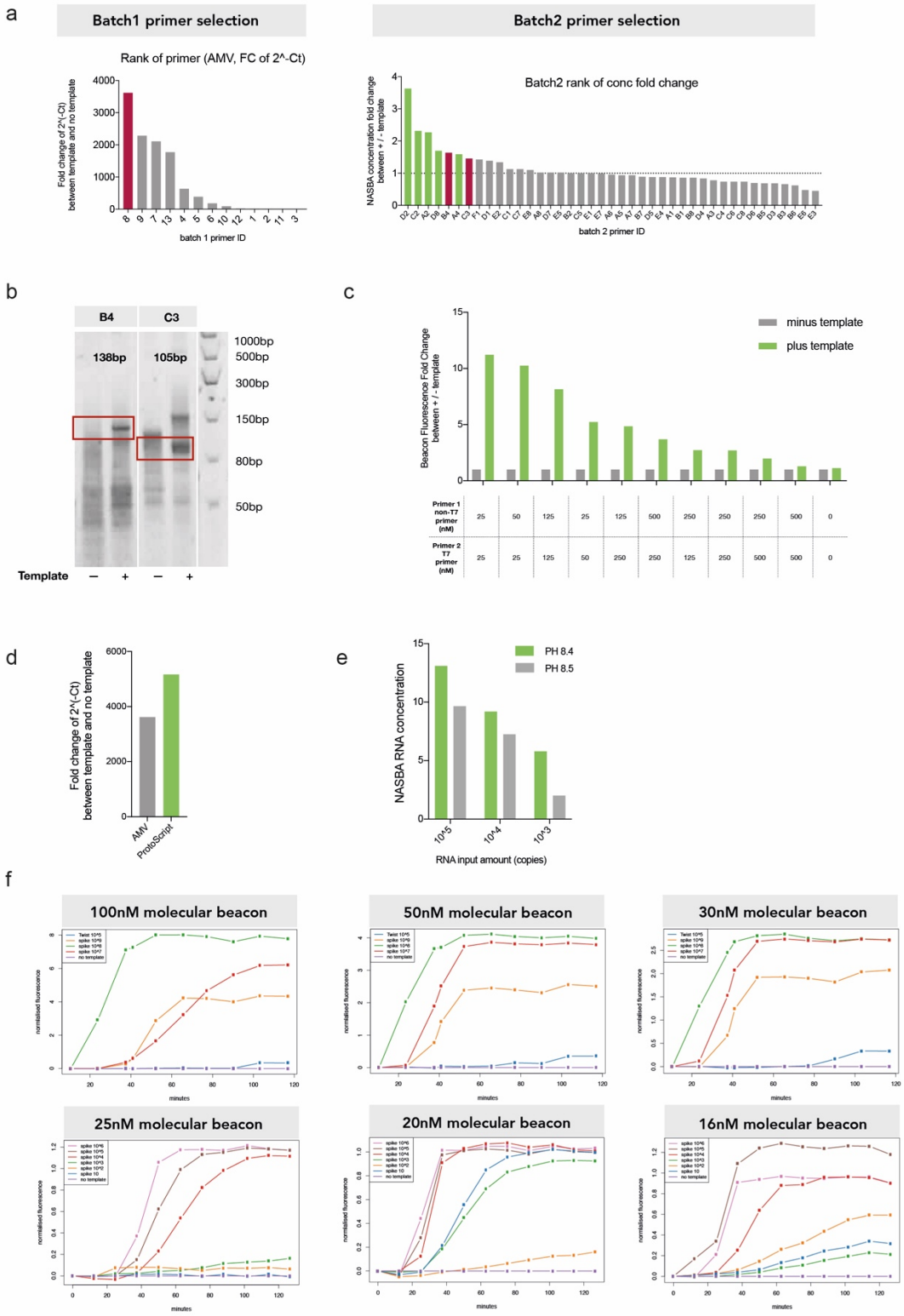

Figure S1. (a) Left: Batch 1 primer efficiency test with qPCR as readout. The NASBA end product was used to carry out qPCR with a pair of inner primers. The fold change between the

template-containing sample and a sample without template was calculated and ranked. The most efficient primer pair (P8) was used in this work. Right: Batch 2 primer efficiency test with RNA concentration as readout. The NASBA end product RNA concentration was measured by a Qubit RNA HS assay. The fold change between the template-containing and no-template samples was calculated and ranked. Among the 40 pairs of Batch 2 primers, the top 7 primer pairs were tested on RNA urea gel. Primer pair A2 and primer pair B4 amplify a similar viral RNA region. Primer pair A4 amplifies a similar region as Batch 1 primer pair P8. (b) RNA urea gel for B4 and C3 batch 2 primers, with target amplicon-sized bands boxed. (c) Primer concentration optimisation. Different amounts of primer concentration were used to test the NASBA reaction efficiency. Previous studies typically used 0.2 - 0.25 μM as a final primer concentration. However, we found ten times lower concentrations resulted in increased NASBA efficiency. The NASBA reaction end-point products were quantified using a molecular beacon. The fold change between template-containing and no-template samples was calculated and used to select the optimal primer concentration. (d) Comparison between two types of reverse transcriptase: AMV and ProtoScript II. The NASBA end product was used to carry out qPCR with a pair of inner primers. The fold change between the template-containing sample and no-template samples was calculated for both enzymes. A higher fold change is observed with Protoscript II. (e) The NASBA end product RNA concentration at different buffer pH, measured with a Qubit RNA HS assay. A more efficient NASBA reaction was observed with a buffer pH of 8.4 for various amounts of input viral RNA templates. (f) Real time molecular beacon fluorescence readings for NASBA reaction with different molecular beacon concentrations (16 - 100 nM). The best detection sensitivity was observed with a beacon concentration of 20 nM.

Supplementary Note

Primer selection

We initially screened a batch of 13 pairs of primers, and selected one pair (P8) based on total RNA quantification with RNA HS Qubit and DNA quantification post-NASBA with qPCR (Supplementary Figure S1a) as the NASBA end product includes both RNA and DNA. We also designed an additional 40 pairs of primers based on NASBA primer selection criteria reported in the literature [6, 10]. The principle of primer selection is listed below. We screened these 40 primer pairs and selected 4 pairs based on total RNA quantification (Supplementary Figure S1a, sequences of all 4 primer pairs shown in Supplementary Table S2) and denaturing RNA gel (Supplementary Figure S1b). We have been mainly using P8 (left primer without Illumina handle and right primer with T7 handle) in further NASBA optimisation and subsequent reactions and figures shown in this paper were done with primer pair P8.

NASBA primer selection rules

1. GC content between 40-60%
2. Template hybridization regions of 20- to 24-nts and with DNA melting temperatures above 41°C
3. No consecutive runs of four or more nucleotides
4. An A base at the final 3' nucleotide
5. Minimal DNA primer internal secondary structure, including the T7 promoter region
6. Minimal DNA primer dimer formation probability
7. Higher GC content in the 6-nts at the 5' end of the primer that hybridized to the template
8. Higher AT content in the 6-nts at the 3' end of the primer
9. Minimise secondary structure of amplicon

Table S1. Composition of in-house NASBA reaction mixture.

Buffer with DMSO

	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		

*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.

Buffer without DMSO

	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		

Nucleotide mix (incl. 10% excess)

	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		

Enzyme 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		

Diluted Rnase H

	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	

Table S2. Primer pairs selected from second batch

A2	
FWD primer	CCATCATTGCCTACACTATGTCA
REV primer with T7 handle	aattctaatacgactcactatagggagaaggTCATAGACACTGGTAGAA
C3	
FWD primer	GTGGGTTATCTTCAACCTAGGA
REV primer with T7 handle	aattctaatacgactcactatagggagaaggTTCTGAGAGAGGGTCAAGTGCA
B4	
FWD primer	GTCAATCCATCATTGCCTACA
REV primer with T7 handle	aattctaatacgactcactatagggagaaggTCATAGACACTGGTAGAA
A4	
FWD primer	TTGAACTTCTACATGCACCA
REV primer with T7 handle	aattctaatacgactcactatagggagaaggCACCTGTGCCTGTTAAACCA