A thermostable Cas12b from *Brevibacillus* leverages one-pot discrimination of SARS-CoV-2 variants of concern

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eBioMedicine 2022;77: 103926
Published online xxx
https://doi.org/10.1016/j.ebiom.2022.103926

Summary

Background Current SARS-CoV-2 detection platforms lack the ability to differentiate among variants of concern (VOCs) in an efficient manner. CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) based detection systems have the potential to transform the landscape of COVID-19 diagnostics due to their programmability; however, most of these methods are reliant on either a multi-step process involving amplification or elaborate guide RNA designs.

Methods Three Cas12b proteins from *Alicyclobacillus acidoterrestris* (AacCas12b), *Alicyclobacillus acidiphilus* (AapCas12b), and *Brevibacillus sp.* SYP-B805 (BrCas12b) were expressed and purified, and their thermostability was characterised by differential scanning fluorimetry, cis-, and trans-cleavage activities over a range of temperatures. The BrCas12b was then incorporated into a reverse transcription loop-mediated isothermal amplification (RT-LAMP)-based one-pot reaction system, coined CRISPR-SPADE (CRISPR Single Pot Assay for Detecting Emerging VOCs).

Findings Here we describe a complete one-pot detection reaction using a thermostable Cas12b effector endonuclease from *Brevibacillus sp.* to overcome these challenges detecting and discriminating SARS-CoV-2 VOCs in clinical samples. CRISPR-SPADE was then applied for discriminating SARS-CoV-2 VOCs, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) and validated in 208 clinical samples. CRISPR-SPADE achieved 92·8% sensitivity, 99·4% specificity, and 96·7% accuracy within 10−30 min for discriminating the SARS-CoV-2 VOCs, in agreement with S gene sequencing, achieving a positive and negative predictive value of 99·1% and 95·1%, respectively. Interestingly, for samples with high viral load (Ct value $\leq$ 30), 100% accuracy and sensitivity were attained. To facilitate dissemination and global implementation of the assay, a lyophilised version of one-pot CRISPR-SPADE reagents was developed and combined with an in-house portable multiplexing device capable of interpreting two orthogonal fluorescence signals.

Interpretation This technology enables real-time monitoring of RT-LAMP-mediated amplification and CRISPR-based reactions at a fraction of the cost of a qPCR system. The thermostable *Brevibacillus sp.* Cas12b offers relaxed primer design for accurately detecting SARS-CoV-2 VOCs in a simple and robust one-pot assay. The lyophilised

Abbreviations: SPADE, Single Pot Assay for Detecting Emerging VOCs; CRISPR-SPADE, CRISPR-Single Pot Assay for Detecting Emerging VOCs

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reagents and simple instrumentation further enable rapid deployable point-of-care diagnostics that can be easily expanded beyond COVID-19.

Keywords: CRISPR; Cas12b; RT-LAMP; Diagnostics; COVID-19; SARS-CoV-2; Variants of concern; One-pot detection; Thermophillic; Dual-detection

Research in context

Evidence before this study

Efforts into developing rapid amplification-free CRISPR tests have been achieved, such as FIND-IT (Fast Integrated Nuclease Detection In Tandem) that supplements Csm6 protein into a Cas13a reaction to trigger a cascade of amplified fluorescence signal. However, these tests rely on guide RNA targeting multiple regions of the SARS-CoV-2 RNA genome, which can be difficult to design to discriminate the variants of concerns (VOCs) due to the low number of mutations. Having the ability to differentiate the VOCs is still dependent on the enrichment of target viral genomes. STOPCovid (SHERLOCK Testing in One Pot) technology can potentially overcome these challenges by employing a thermostable Cas12b derived from *Alicyclobacillus acidiphilus* (AapCas12b) that can be combined with RT-LAMP into a one-pot detection assay. One major disadvantage of STOPCovid is that the wild-type AapCas12b collateral activity ceases to work above 60 °C and requires additional additives, such as taurine, and a longer incubation time to perform robustly due to suboptimal temperature conditions, leading to slower diagnostics. Additionally, the protein's performance at this temperature range restricts LAMP primer designs since RT-LAMP reactions are typically optimised at 60 °C−65 °C.

Added value of this study

This study identifies the unique thermostability and trans-cleavage activity of BrCas12b and its application in diagnostics. Here we present a CRISPR-based one-pot test that can clinically discriminate between Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) VOCs of SARS-CoV-2 in as low as 10-30 min. A multiplexed point-of-care device was developed for tracking RT-LAMP amplification and Cas12b-mediated cleavage using dual orthogonal fluorescence channels.

Implications of all the available evidence

The ultra-thermostable *Brevibacillus sp.* Cas12b offers relaxed primer design for accurately detecting SARS-CoV-2 VOCs in a simple and robust one-pot assay. The lyophilized reagents and simple instrumentation further enable rapid deployable point-of-care diagnostics that can be easily expanded beyond COVID-19.

Introduction

Since the beginning of the COVID-19 pandemic, many strategies have been explored to develop rapid and sensitive detection kits to drive the diagnostics towards home-based testing.[1–5] Current gold-standard Reverse Transcription - quantitative Polymerase Chain Reaction (RT-qPCR) tests require sophisticated instrumentation as well as intensive labour training, and therefore become a major hurdle for deployment in remote areas. CRISPR-based detection technologies hold promises for future rapid point-of-care diagnosis of infectious diseases and cancer.[6,7] This is in part due to their versatility in implementation and design that lie in the programmability of the guide RNA sequence. Taking advantage of the collateral cleavage property, many Cas effectors have been repurposed for nucleic acid detection such as Cas12a in DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter)[5,8] and HOLMESv1 (a one-HOur Low-cost Multipurpose highly Efficient System),[9] Cas12b in HOLMESv2,[10] and Cas13a in SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing).[11,12] Many of these tests employ a two-pot assay in which a pre-amplification step such as RPA (reverse transcription recombinase polymerase amplification) or RT-LAMP (reverse transcription loop-mediated isothermal amplification) is required prior to CRISPR detection. This strategy increases reaction time and chances of carryover contamination.

Methods

Expression plasmid construction

BrCas12b gene sequence derived from *Brevibacillus* sp. SYP-B805 was obtained from the National Center for Biotechnology Information (GenBank ID: WP_165214399·1). The gene sequence was then codon-optimised using the GenSmart™ Codon Optimisation tool (Genscript) for bacterial protein expression, synthesised by Twist Bioscience, and cloned into PET28a^{+} vector.

Protein expression and purification

The BrCas12b expression plasmid was transformed into BL21(DE3) competent *E. coli* cells. Individual colonies were picked and inoculated in 8 mL Luria Broth (Fisher

Scientific) for 12–15 h. The culture was added to a 2 L homemade Terrific Broth for scale-up and shaken until the OD600 reached 0·5–0·8. The culture was then placed on ice for 15–30 min prior to the addition of 0·2 mM IPTG (Isopropyl β- d-1-thiogalactopyranoside) followed by overnight expression at 16 °C for 14–18 h.

The next day, cells were harvested by centrifugation at 10,000 xg for 5 min. Cell pellets were then resuspended in lysis buffer (0·5 M NaCl, 50 mM Tris-HCl, pH = 7·5, 0·5 mM TCEP, 1 mM PMSF, 0·25 mg/ml lysozyme, and 10 ug/mL deoxyribose nuclease I). The suspended cell mixture was disrupted by sonication and centrifuged at 39,800 xg for 30 min at 4 °C. The supernatant was collected and filtered through a 0·22 µm filter (Milipore Sigma) prior to injecting into a Histrap 5 mL FF column (Cytiva) pre-equilibrated with Wash Buffer (0·5 M NaCl, 50 mM Tris-HCl, pH = 7·5, 0·5 mM TCEP, 20 mM Imidazole). The purification process was performed via an FPLC Biologic Duoflow system (Biorad). After lysate injection and washing with Wash Buffer A, the column was eluted with 40 mL of Elution Buffer B (0·5 M NaCl, 50 mM Tris-HCl pH = 7·5, 0·5 mM TCEP, 300 mM imidazole). The eluted fractions were pooled together, transferred to a 10–14 KDa dialysis bag, and dialysed in Dialysis Buffer (0·25 M NaCl, 40 mM HEPES, pH = 7, 1 mM DTT) overnight at 4 °C.

Following overnight dialysis, the BrCas12b mixture was concentrated down to 15 mL in a 30 kDa MWCO Vivaspin® 20 concentrator via centrifugation with the speed of 2000 xg at 4 °C. The concentrate was mixed at 1:1 ratio with Buffer C (150 mM NaCl, 50 mM HEPES, pH = 7, 0·5 mM TCEP) before injecting into a Hitrap Heparin 1 mL HP column (Cytiva). The protein was eluted over a gradient buffer exchange from Buffer C to Buffer D (2 M NaCl, 50 mM HEPES, pH = 7, 0·5 mM TCEP). Purest elution fractions of BrCas12b were collected, concentrated down, snap frozen, and stored at -80 °C until use. Experiment-ready BrCas12b was diluted in storage buffer (500 mM NaCl, 20 mM sodium acetate, 0·1 mM EDTA, 0·1 mM TCEP, 50% glycerol, pH 7 @ 25 °C) which can be stored at -20 °C.

AacCas12b and AapCas12b expression plasmids were obtained as a gift from Jennifer Doudna (Addgene plasmid # 113433) and Wei Li (Addgene plasmid # 121949), respectively. These two proteins were expressed and purified following Chen et al.[8] and Teng et al.[13]

Nucleic acid preparation

For *in vitro* cleavage assay and differential scanning fluorimetry experiments, single-guide RNA (sgRNA) was synthesised using PCR (Takara) and *in vitro* transcription (IVT) using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). The IVT reaction was purified using the RNA Clean and Concentrator Kit (Zymo Research).

For patient sample detection, the sgRNA was either chemically synthesised by Integrated DNA Technologies (IDT) or enzymatically synthesised by an *in vitro* transcription (IVT) reaction followed by HPLC purification via the HPLC 1100 system (Agilent). The control genomic RNA for Beta, Delta, and Omicron variants were obtained from Twist Bioscience. The control genomic RNA for wild-type SARS-CoV-2 and Alpha were obtained from BEI Resources. The control genomic RNA for Gamma was obtained from Salemi Lab at the University of Florida.

In vitro dsDNA cleavage assay

Cas12b, sgRNA and dsDNA were combined in 1x NEBuffer 2·1 on ice to a final concentration of 100 nM, 125 nM, and 7 nM, respectively. The reaction mixture was immediately transferred to a pre-set thermocycler and incubated for 1 h at different temperatures (37 °C, 47 °C, 52 °C, 57 °C, 59 °C, 61 °C, 63 °C, 65 °C, 70 °C, and 75 °C). After incubation, the reaction mixture was treated with 6x quenching buffer (30% glycerol, 1·2% SDS, 250 mM EDTA). The reaction products were analysed on 1% agarose gel pre-stained with GelRed (Biotium).

Differential scanning fluorimetry

Cas12b and sgRNA were combined in a 1:2·5 ratio (1 µM: 2·5 µM final concentration) in a mixture of Protein Thermal Shift™ buffer (Thermofisher) and 1x reaction buffer (100 mM NaCl, 50 nM Tris-HCl, pH = 7·5, 1 mM DTT, and 10 mM $MgCl_2$). The reaction was incubated at 37 °C for 30 min to ensure complexation of sgRNA and Cas12b prior to adding the Protein Thermal Shift™ dye (Thermofisher). The reaction mixture was then transferred to qPCR StepOne Plus system (Thermofisher), and the binary complex temperature melting profile was recorded over a temperature range of 25–99 °C with a ramp rate of 1%/s. The experiment was carried out in duplicates and repeated twice.

Cas12b trans-cleavage kinetic assay

The trans-cleavage kinetic experiment was carried out following Cofsky et al.[14] with modifications. In short, BrCas12b, sgRNA, and dsDNA activator were combined to a final concentration of 100 nM : 125 nM : 1 nM, respectively in 1x NEBuffer 2·1 (New England Biolabs) and incubated at 62 °C for 30 min. HEX-polyT-Quencher reporter (FQ) at various concentrations (10 nM, 100 nM, 200 nM, 500 nM, 1 µM, and 2 µM) was added to the reaction mixture containing Cas12b trans-activated complex; and the entire reaction was then immediately transferred to a plate reader. Fluorescence measurements (λ_{ex}: 483/30 nm, λ_{em}: 530/30 nm) were read every 30 s using the Biotek Synergy Neo (Agilent) that was pre-heated to 62 °C. Initial velocity for

each FQ concentration was determined by establishing the slope for all components and subtracting the slope for the no-activator control. The cleaved HEX-polyT reporter was titrated to different concentrations (10 nM, 100 nM, 200 nM, 500 nM, 1 µM, and 2 µM) and measured in the same experiment for the conversion of FQ fluorescence to concentration, eliminating non-linearity at high reporter concentrations.

Patient sample collection

Saliva samples were collected and processed by following the guidelines approved by the University of Florida Institutional Review Board through Evaluating the Molecular Epidemiology of Coronavirus (COVID-19) in Florida (IRB202000633), Saliva Collection to Support Disease Diagnostics (IRB202002224), and Detecting SARS-CoV-2 RNA in human samples using an engineered CRISPR-based paper-based test (IRB202000781). Samples were extracted following CDC-recommended procedures and sequenced (described below) to identify SARS-CoV-2 lineages.

RNA extraction, library preparation and sequencing

Viral RNA was extracted from 180 µl of each saliva sample using the QIAamp 96 Viral RNA Kit with the QIAcube HT (Qiagen) using the following settings with a filter plate: the lysed sample was pre-mixed 8 times before subjecting to vacuum for 5 min at 25 kPa and vacuum for 3 min at 70 kPa. Following 3 washes using the same vacuum conditions above, the samples were eluted in 75 µl AVE buffer followed by a final vacuum for 6 min at 60 kPa. Next, nine microliters of RNA were used for cDNA synthesis and library preparation using the Illumina COVIDSeq Test kit (Illumina) and Mosquito HV Genomics Liquid Handler (SPT Labtech Inc.). The size and purity of the library were determined using the 4200 TapeStation System (Agilent) and the Qubit dsDNA HS Assay Kit (Life Technologies), according to the manufacturer's instructions. Constructed libraries were pooled and sequenced using the NovaSeq 6000 Sequencing System SP Reagent Kit and the NovaSeq Xp 2-Lane Kit. Illumina's DRAGEN pipeline was used to derive sample consensus sequences, which were filtered based on a minimum of 70% coverage of the genome.

SARS-CoV-2 viral load quantification

Levels of SARS-CoV-2 were determined using the 2019-nCoV_N1 assay (primer and probe set) with 2019-nCoV_N_positive control (IDT). Viral RNA was extracted as previously described then subjected to first-strand synthesis using ProtoScript II Reverse Transcriptase according to the manufacturer's instructions (New England Biolabs). Quantitative PCR was performed using TaqMan Fast Advanced Master Mix (ThermoFisher Scientific) according to the manufacturer's instructions. A standard curve was generated using N1 quantitative standards 10-fold diluted to determine viral copies. The assay was run in triplicate including one non-template control.

One-pot BrCas12b detection assay

LAMP primers targeting wild-type SARS-CoV-2 and variants of concern were designed using NEB® LAMP Primer Design Tool (New England Biolabs) and PrimerExplorer v5 at https://primerexplorer.jp/e/(15).

For the one-pot detection assay that tracks both target amplification and BrCas12b trans-cleavage, the reaction was assembled by combining the following reagents:

Item #	Reagents	Volume (µL)	Final Concentration
1	WarmStart® Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG)	12·5	1X
2	10X LAMP Primer Mix	2·5	1X
3	100 µM HEX-polyT-Quencher Reporter	0·5	2 µM
4	25 µM SYTO™ 9 Dye	1	1 µM
5	10 µM BrCas12b	0·25	0·1 µM
6	10 µM sgRNA	0·5	0·2 µM
7	RNase-free Water	2·75	————
	Total	**20**	

For the rapid one-pot detection assay monitoring BrCas12b trans-cleavage only, the reaction was assembled by combining the following reagents:

Item #	Reagents	Volume (µL)	Final Concentration
1	WarmStart® Multi-Purpose LAMP/RT-LAMP 2X Master Mix (with UDG)	12·5	1X
2	10X LAMP Primer Mix	2·5	1X
3	100 µM HEX-polyT-Quencher Reporter	0·5	2 µM
4	10 µM BrCas12b	0·25	0·1 µM
5	10 µM sgRNA	0·5	0·2 µM
6	RNase-free Water	3·75	————
	Total	**20**	

For SARS-CoV-2 variant specificity testing, the one-pot reaction was added with LAMP primer and sgRNA

corresponding to a SARS-CoV-2 variant and tested against other variants. For Limit of Detection (LoD) testing, RNA controls were subjected to serial dilution in triplicates and added to the one-pot reaction. For patient sample detection testing, 5 µL of extracted nucleic acid from clinical samples were added to the one-pot reaction.

The reaction mixture was transferred to a Bio-rad CFX96 Real-Time system with C1000 Thermal Cycler module. The reaction was isothermally incubated at 62 °C for all variant detection except Omicron, which was carried out at 60 °C. Fluorescence measurements were read every 30 s per cycle over 120 cycles. The FAM channel (λ_{ex}: 470/20 nm, λ_{em}: 520/10 nm) was used to monitor SYTO9^{TM}, and the HEX channel (λ_{ex}: 525/10 nm, λ_{em}: 570/10 nm) was used to detect BrCas12b trans-cleavage via FRET-based HEX reporter.

Lyophilisation of BrCas12a one-pot detection assay

Developmental lyo-ready Warmstart® Master Mix with Uracil-DNA glycosylases (UDG) (New England Biolabs) was combined with 100 nM BrCas12b, 200 nM sgRNA, and 1·25 µmol Trehalose in ice. The mixture was placed in an aluminium cooling block and kept in dry ice for 30 min. The cooling block containing samples was then transferred to a freeze-drying system (Labconco) and lyophilised for 4 h or overnight. To initiate the reaction, the lyophilised master mix was reconstituted in 20 µL RNase-free water.

Imaging using a mobile phone and a lens

One-pot BrCas12b reagents were combined in an optically clear PCR tube (Applied Biosystems) followed by the addition of the extracted RNA sample and 62 °C incubation for 20 min. The reaction tube was then imaged using a battery-operated 410 nm–415 nm UVA-blue flashlight on a mobile phone in a dark setting. To visualise a sample containing FRET-based HEX reporter, the imaging system was assembled by attaching a combination of yellow and orange lenses onto the flashlight camera (NestEcho).

Portable multiplexing detection prototype (FISSH)

To build a portable detection device that is capable of multiplexing (in this study the device enables monitoring both LAMP amplification and BrCas12b collateral cleavage), the optical system was assembled with two wavelengths. The first one was a MCPCB-mounted LED on 490 nm with a bandwidth of 26 nm, 205 mW driven by 199 mA with impulse length 150 ms for exposure of FITC (Thorlabs). The second LED worked on 625 nm with a bandwidth of 17 nm, 700 mW driven by 215 mA with the same length of impulse for exposure of Cy5 (Thorlabs). The beam of light from the LED was collimated (straightened) by the lens before it travelled to the dichroic mirror and reflected the light into an excitation filter, which cut off the parasitic light. The light beam went to the second dichroic mirror which reflected the beam of light to the collimating lens, concentrating light into the vial with the sample. When the sample started emitting the light from the fluorophore, it then travelled through the collimating lens into the second dichroic mirror, allowing the beam light to go through directly into the emission filter, cutting off the parasitic light. Next, the beam went through another collimating lens prior to being focused and detected by a photodiode sensor/detector (Thorlabs). A schematic diagram of the device is presented in Figure 2f, and the specifications of device parts are indicated in supplementary Table S2.

Ethics

This study was conducted by strictly following the ethical guidelines approved by the University of Florida Institutional Review Board under the IRB202000633: "Evaluating the Molecular Epidemiology of Coronavirus (COVID-19) in Florida", IRB202002224: "Saliva Collection to Support Disease Diagnostics", and IRB202000781: "Detecting SARS-CoV-2 RNA in human samples using an engineered CRISPR-based paper-based test". IRB202000633 and IRB202000781 protocols were approved as an exempt study with de-identified/coded samples under confidentiality agreements signed by investigator (recipient investigator) and the code owner (collecting investigator). The IRB202002224 was approved as a banking study with the informed consent form by following the Institutional Review Board guidelines.

Statistics

Patient samples containing different VOCs and wild-type strains were randomised and blinded. Data were visualised using GraphPad Prism 8 (GraphPad Software, San Diego, CA) and results were expressed as means ± SD.

Role of funders

This project was funded in part by the United States-India Science & Technology Endowment Fund-USISTEF/COVID-I/247/2020 (P.K.J.), Florida Breast Cancer Foundation- AGR00018466 (P.K.J.), National Institutes of Health- NIAID 1R21AI156321-01 (P.K.J.), United States Centers for Disease Control and Prevention- U01GH002338 (R.R.D., J.A.L., & P.K.J.), University of Florida, Herbert Wertheim College of Engineering (P.K.J.), University of Florida Vice President Office of Research and CTSI seed funds (M.S.), and University of Florida College of Veterinary Medicine and Emerging Pathogens Institute (R.R.D.). The funding sources did not have a role in the design of the

study, the collection, analysis, or interpretation of data, nor in writing of the manuscript.

Results

Here, we report the development of a complete one-pot RT-LAMP-coupled CRISPR detection reaction using a novel Cas12b derived from unclassified *Brevibacillus* sp. SYP-B805 (GenBank ID: WP_165214399·1) named BrCas12b. BrCas12b exhibits phenomenal stability at high temperatures (up to 70 °C in optimal buffers) which is suitable for coupling with an RT-LAMP reaction. Notably, BrCas12b is shown to have high collateral cleavage (referred to as trans-cleavage) up to the temperature of 64 °C without the need for supplemental additives. In addition, BrCas12b works robustly in isothermal amplification buffer, which is an ideal scenario for its incorporation into a complete one-pot reaction. The one-pot RT-LAMP-coupled BrCas12b reaction provides two detection checkpoints: (1) amplification by RT-LAMP that can be tracked by SYTO^{TM} dye and (2) BrCas12b:sgRNA complex detecting amplified targets that can emit a different signal by a Fluorescence Resonance Energy Transfer (FRET)-based reporter. This dual-checkpoint one-pot assay provides a highly accurate level of nucleic acid detection. The broad versatility along with the high specificity of BrCas12b enables us to detect SARS-CoV-2 and distinguish its variants of concerns Alpha (B.1.1.7), Beta (B.1.352), Gamma (P1), Delta (B.1.617.2), and Omicron (B.1.1.529). Finally, we develop two low-cost detection methods for fluorescence visualisation. The first one requires a colour-filtered lens attached to a mobile phone camera and a handheld flashlight. By engaging the flashlight, samples can be detected in the dark by the naked eye or enhanced and magnified through the use of filters and the camera. The second method utilises an in-house, portable and inexpensive prototype that allows for quantitative dual checkpoints of amplification and BrCas12b trans-cleavage activity using FITC and Cy5 channels.

Tian et al. first reported and characterised the BrCas12b from a hot spring bacterium *Brevibacillus* sp. SYP-B805 that exerts high enzymatic target cleavage (cis-cleavage) activity at elevated temperature (up to 65·5 °C); however, the trans-cleavage activity remained to be investigated.[16] BrCas12b forms a complex with crRNA and tracrRNA (~130 nt) to recognise and cleave dsDNA target containing a TTN PAM sequence upstream of the complementary binding site (Figure 1a). We sought to verify the reported BrCas12b cis-cleavage activity by carrying out a temperature-dependent *in vitro* cleavage assay compared to Cas12b from *Alicyclobacillus acidoterrestris* (AacCas12b) and *Alicyclobacillus acidiphilus* (AapCas12b), two thermostable effector endonucleases with considerable cleavage activity as reported in STOPCovid (Figure S1). A more restrictive PAM sequence TTTN was selected to investigate these three effectors' performance. Corroborating the study by Tian et al., BrCas12b showed cleavage up to 70 °C in Bovine Serum Albumin-containing buffer compared to 52 °C and 59 °C for AacCas12b and AapCas12b, respectively (Figure 1b). Thermal stability for AacCas12b, AapCas12b, and BrCas12b were confirmed by differential scanning fluorimetry where the melting temperatures were found to be 55·3 °C, 58·3 °C, and 63·4 °C, respectively (Figure 1c, Table S2). Fascinated by the robust performance of BrCas12b, we proceeded to test the effector's trans-cleavage activity to determine if it is suitable for a one-pot RT-LAMP-coupled detection reaction. As shown by trans-cleavage kinetic analysis, we observed a turnover number of 14·2 s^{-1} and a catalytic efficiency of 1·74 × 10^7 ($M^{-1}s^{-1}$) at 62 °C, indicating fast enzymatic activity (Figure 1d, Table S3).

The above observations provide advantages when coupling with an RT-LAMP reaction for detection purposes. First, the high optimum temperature of BrCas12b lies within the range of ideal RT-LAMP operating conditions. Additionally, we noticed that BrCas12b performed well in isothermal amplification buffer used in the RT-LAMP reaction. After iterative optimisation using many common additives such as proline, taurine, BSA, and betaine, we did not observe enhancement in the trans-cleavage activity (Figure S4). Moreover, FAM- and HEX-based reporters exhibited significantly higher fluorescence signal compared to a Cy5-based reporter (Figure S5). Therefore, we formulated an RT-LAMP-coupled BrCas12b reaction in the absence of additives and used HEX-based reporter for our assay. By multiplexing the assay with two different reporters, we were able to track both target amplification and BrCas12b trans-cleavage activity in a one-pot reaction. This allowed us to confirm the presence of nucleic acid targets via the two checkpoints. Since the LAMP reaction is prone to non-specific primer-dimer amplification, the BrCas12b trans-cleavage activity serves as a final verdict for detection (Figure 1e). We observed that the trans-cleavage activity of BrCas12b has a 1–3 min delay after amplification, indicating minimal inhibition by the LAMP reaction on activity. Multiple studies have shown that when combining RT-LAMP/RT-RPA with the CRISPR reaction, the sensitivity of detection is reduced significantly, possibly due to operating temperature differences, an inhibitory effect of excessive amount of amplified product, differences in optimal buffer and salt conditions, and unwanted nonspecific trans-cleavage of Cas effector on primers.[1,17–20] However, having observed such a robust trans-cleavage with a small delay after amplification, we consider that BrCas12b has circumvented many of these issues.

We then systematically tested the one-pot reaction at various temperatures ranging from 60 °C to 64 °C to evaluate detection performance among Cas12b. To compare with STOPCovid, we tested its LAMP primers at

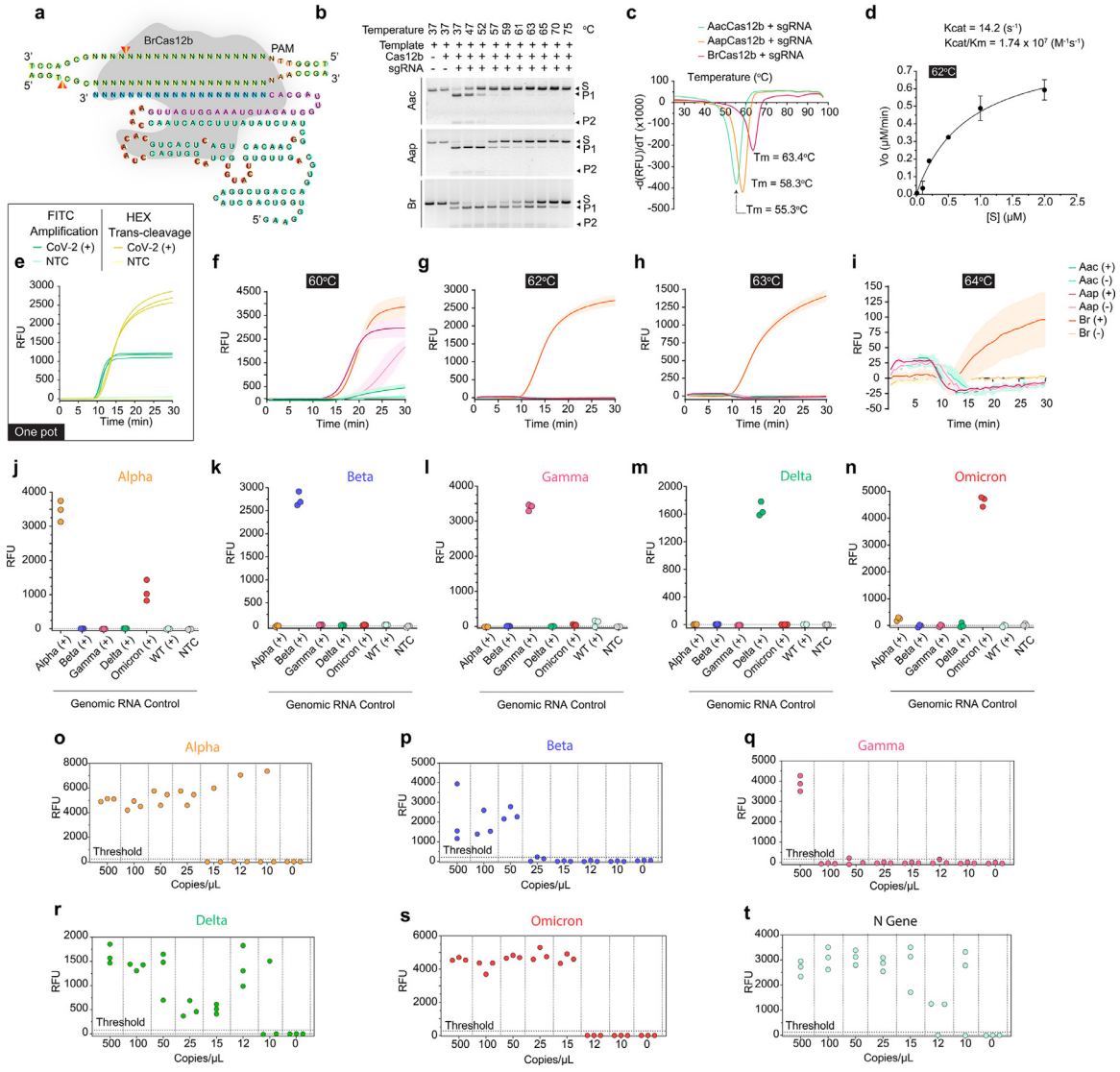

Figure 1. BrCas12b characterisation and one-pot specificity & sensitivity testing. (a) Schematic of binary complex illustrating cleavage pattern of dsDNA target. (b) Temperature-dependent *in-vitro* cleavage assay of AacCas12b, AapCas12b, and BrCas12b targeting more restrictive PAM TTTG. 125 nM sgRNA:100 nM Cas12b:7 nM dsDNA was combined in 1X NEBuffer 2·1. The experiment was repeated (n = 2 biological replicates) with similar results. (c) Michaelis-Menten Kinetics of BrCas12b trans-cleavage at 62 °C. (d) Differential scanning fluorimetry of apo AacCas12b, apo AapCas12b, and apo BrCas12b and their corresponding Cas12b:sgRNA complexes. (e) Multiplexing using FITC to detect LAMP amplification and HEX to monitor trans-cleavage of BrCas12b. (f)−(i) Detection capability of BrCas12b via trans-cleavage against AacCas12b and AapCas12b at various temperatures. Fluorescence kinetics within 30 min was monitored using HEX-based reporter. Shaded regions represent standard deviation (n = 3 biological replicates). STOPCovid LAMP primers were used in (f) to compare performance with AapCas12b. A different set of LAMP primers (DETECTR) with optimal performance at temperature higher than 60 °C were used in (g), (h), and (i). (+) denotes the presence of the SARS-CoV-2 genomic RNA control, and (-) signifies the non-template control (NTC). (j)−(n) Specificity testing using a pair of sgRNA and LAMP primers targeting SARS-CoV-2 variants of concern (n = 3 biological replicates). (o) − (t) Limit of detection of non-N gene targeting Alpha (B.1.1.7), Beta (B.1.352), Gamma (P1), Delta (B.1.617.2), Omicron (B.1.1.529) and N gene, respectively (n = 3 biological replicates).

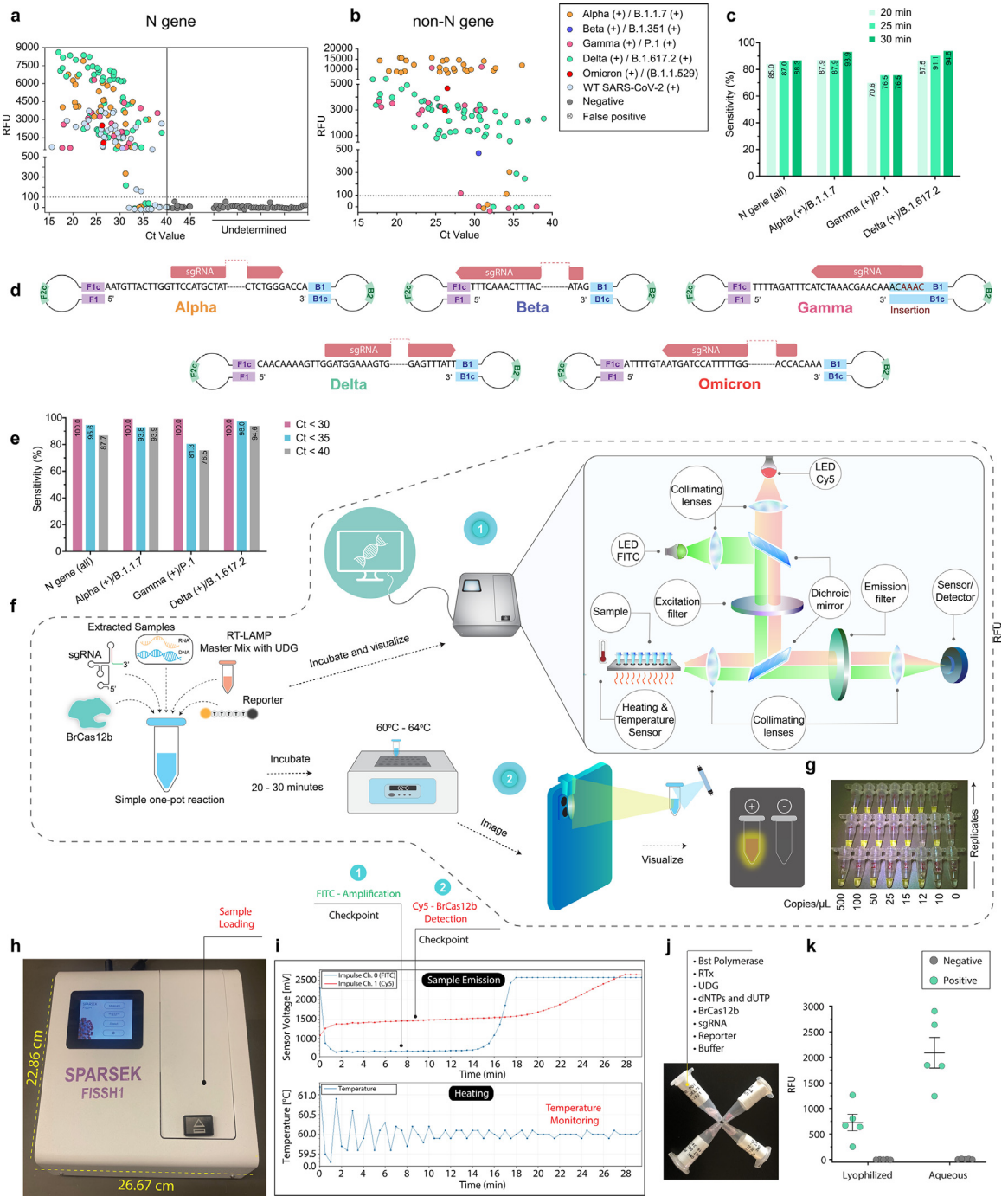

Figure 2. Clinical validation of one-pot BrCas12b detection assay and portable diagnostic systems. (a) and (b) One-pot patient sample detection. The N gene target indicates the presence of SARS-CoV-2 while non-N gene targets indicate detection of variants. Fluorescence measurements were taken at t = 30 min. (c) Percent accuracy of the one-pot detection at 20-, 25-, and 30 min targeting variants and WT SARS-CoV-2. (d) Self-hybridising loop structure acts as a seed for exponential LAMP amplification which subsequently serves as a basis for BrCas12b one-pot detection of VOCs. Guide RNA designed to target the dumbbell region of the LAMP products are shown. (e) Percent accuracy with respect to C_t value for SARS-CoV-2 variant detection. (f) One-pot assembly and comparison of a portable in-house detection instrument (FISSH) and an at-home detection method using a mobile phone with a simple lens. (g) Depiction of image taken by the mobile phone in (e). (h) and (i) FISSH footprint and dual-channel graph generated from a positive sample. (j) Representation of lyophilised one-pot detection reaction. (k) Lyophilised samples compared to the standard one-pot reaction. Fluorescence measurements were taken at t = 30 min and the Mean±SD (n = 5 technical replicates) are indicated.

60 °C but with higher concentration of Cas protein and sgRNA. We selected the LAMP primers used in DETECTR for the remaining temperatures because STOPCovid primers are not optimal above 60 °C (Figure 1f–i).[5] BrCas12b was observed to work robustly up to 63 °C and with reduced activity at 64 °C, whereas, as expected, AacCas12b and AapCas12b failed at temperatures above 60 °C (Figure 1f–i). Additionally, false positives were occasionally observed when STOPCovid LAMP primers and high concentrations of AapCas12b and sgRNA were used (Figure 1f).

Thermal stability of BrCas12b enabled us to design LAMP primers with less restrictive parameters compared to STOPCovid to comprehensively distinguish SARS-CoV-2 VOCs from its original strain. We systematically tested each variant including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P1), Delta (B.1.6127.2), and Omicron (B.1.1.529) against one another and the original strain (Figure S6). The pair of LAMP primers and sgRNA targeting each variant exhibited high specificity with no relative cross-target detection (Figure 1j–n) apart from the Alpha variant showing fluorescence signal for the Omicron variant. Prior to the emergence of the Omicron variant in late 2021, we had designed the sgRNA and LAMP primers targeting the Alpha variant based on a 6-base deletion associated with H69del of the spike protein (Figure S6). Coincidentally, multiple sequence alignment revealed that the Omicron variant possesses the same deleted region in its spike protein, resulting in the positive signal when detected with Alpha-targeted LAMP primers and sgRNA. To overcome this nonspecificity, we designed a new pair of LAMP primers and sgRNA targeting the Omicron variant at another unique base-deleted region in the spike protein with high specificity, allowing for the discrimination between the Alpha-detected samples and Omicron-detected samples. For sensitivity testing, we sought to determine the limit of detection (LoD) of nucleocapsid gene (N) for the presence of SARS-CoV-2 virions and non-N mutated genes for the prevalent VOCs. The assay confirmed the estimated LoD of 25 copies/µL, 50 copies/µL, 500 copies/µL, 12 copies/µL, 15 copies/µL, and 15 copies/µL for Alpha, Beta, Gamma, Delta, Omicron, and the universal N gene, respectively (Figure 1o–t).

We hereafter refer the RT-LAMP-coupled BrCas12b test as CRISPR-SPADE (Single Pot Assay for Detecting Emerging VOCs). We proceeded to validate CRISPR-SPADE in clinical samples including 57 Delta positive (B.1.617.2), 33 Alpha positive (B.1.1.7), 17 Gamma positive (P.1), 1 Beta positive (B.1.351), 45 wild-type SARS-CoV-2, and 53 negative samples. At the time of the study (October 2021), the Omicron variant was not clinically detected. N gene and non-N genes were tested in parallel. The detection of N gene served as a basis for the presence of SARS-CoV-2, whereas the non-N genes were used to differentiate the variants. An 88·3 % sensitivity in N gene for all samples was reached (Figure 2a–c); likewise, a 93·9%, 100%, 76·5%, 94·6%, and 100% sensitivity was achieved within 30 min in non-N gene for Alpha, Beta, Gamma, Delta, and Omicron samples, respectively (Figure 2a–c, Table 1). LAMP primers play a crucial role in the sensitivity and specificity of the BrCas12b one-pot detection (Figure 2d). Quality in LAMP primer design can aid in the reduction of primer-dimer amplification.[15] During the development of the one-pot detection reaction, we occasionally observed non-specific amplification, which was the main cause for false negatives in low copy samples due to the consumption of resources. The high optimum temperature of BrCas12b alleviates some of the LAMP primer design restrictions and thus allows for more flexibility in primer selection. Specifically, in samples with a C_t value less than 30, 100% sensitivity was accomplished, and in samples above a C_t value of 30, we observed a reduced performance in sensitivity (Figure 2e, Table S2). With negative samples considered, we achieved an overall 96·7% accuracy and 99·4 % specificity with one false positive detected out of a total of 208 samples (Figs. S7, S8, Tables 1,3). Patient samples detected with variants of concern were confirmed through next-generation sequencing (Figure S9).

We further developed two methods of fluorescence-based detection: an inexpensive lens attached to a mobile phone camera, and a multiplexing detection prototype engineered to track RT-LAMP amplification and BrCas12b trans-cleavage simultaneously (Figure 2f–i). The phone-based detection activates via UVA-blue flashlight (410 nm–415 nm) and a combination of low-cost yellow and orange filtered lenses with a clip (cost < $5). This combined lens system allowed for the detection of FRET-based HEX reporters in a dark setting (Figure 2g). We envision that this approach, with proper safety precautions, could be used for home-based testing, as demonstrated by others.[3,4,19,21] The portable multiplexing detection prototype was built with two wavelengths in the optical system: a LED working at 490/26 nm excitation for FITC, and a second LED working at 625/17 nm with the same length of impulse for Cy5 excitation. A combination of reporter pairs could be used for this portable device such as SYTO™ 62 using Cy5 channel for tracking amplification and FRET-based FAM reporter using the FITC channel for detection of BrCas12b trans-cleavage activity, or the SYTO™ 9 and Cy5 reporter pair for the opposite channels. This prototype came with a built-in touch screen for ease of use (Figure 2f) and could potentially be programmed to connect to a mobile phone or a tablet via an app. The device was built with a significant reduction in cost compared to qPCR systems. We anticipate that it will be most suitable in a professional setting such as a clinic.

Additionally, we took one step further by lyophilising the one-pot reagent to facilitate in transportation,

Clinical characteristics of SARS-CoV-2 VOC with non-N gene					
VOC	**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**	**PPV (%)**	**NPV (%)**
α	31/33 (93.9%)	29/29 (100%)	60/62 (96.8%)	31/31 (100%)	29/31 (93.5%)
β	1/1 (100%)	44/44 (100%)	45/45 (100%)	1/1 (100%)	44/44 (100%)
γ	13/17 (76.5%)	42/42 (100%)	55/59 (93.2%)	13/13 (100%)	42/46 (91.3%)
δ	53/56 (94.6%)	33/34 (97.1%)	86/90 (95.6%)	53/54 (98.1%)	33/36 (91.7%)
o	2/2 (100%)	..	2/2 (100%)	2/2 (100%)	..
Other	16/16 (100%)	28/28 (100%)	44/44 (100%)	16/16 (100%)	28/28 (100%)
All combined	116/125 (92.8%)	176/177 (99.4%)	292/302 (96.7%)	116/117 (99.1%)	176/185 (95.1%)

***Table 1*: Clinical characteristics of SPADE compared to genomic sequencing for discriminating SARS-CoV-2 VOC with non-N gene.**
TP = true positive; TN = true negative; FP = false positive; FN = false negative; PPV= positive predictive value; NPV= negative predictive value; Sensitivity = TP/(TP+FN); Specificity = TN/(TN+FP); Accuracy = (TP+TN)/(TP+TN+FP+FN); PPV = TP/(TP+FP); NPV = TN/(TN+FN)

N gene for detecting SARS-CoV-2						
SAR-CoV-2		C_t Value (RT-qPCR)				Total
		Positive			Negative	
		< 30	< 35	< 40	>40 or ND	
SPADE	Positive	96 (TP)	130 (TP)	136 (TP)	0 (FP)	136
	Negative	0 (FN)	6 (FN)	19 (FN)	53 (TN)	72
Total		96	136	155	53	208

***Table 2*: Clinical validation of CRISPR-SPADE with N gene for detecting SARS-CoV-2.**
TP = true positive; TN = true negative; FP = false positive; FN = false negative

distribution, and deployment without the need for cold-chain requirements in remote or austere settings. We obtained the lyo-ready Warmstart® reagent currently in development from New England Biolabs that is comprised of glycerol-free LAMP reaction mixture. Prior to lyophilisation, BrCas12b, sgRNA, and cryoprotectant were added to the lyo-ready mixture. Under our testing conditions, we observed a slightly reduced detection signal from BrCas12b to that of the glycerol version, and the LAMP exhibited a five-minute delay in reaction time (Figure 2j, k), possibly due to incorporation of several enzymes in the one-pot master mix. Additionally, the freeze-dried reagents were shown to maintain their activity at 4 °C for up to two months (Figure S10).

Discussion

As the COVID-19 pandemic progresses with a growing number of VOCs circulating, the need for a rapid, one-pot detection system to differentiate among the strains is paramount.[22–24] Currently, the primary method to reliably detect a VOCs is via next-generation sequencing (NGS).[25–27] Although NGS-based methods are vital in identification and confirmation of new variants, they are labour-intensive and require several hours of processing time, which limit their cost-effectiveness for real time molecular epidemiology surveillance. Due to several advantages of BrCas12b such as high specificity, and robust trans-cleavage at RT-LAMP reaction temperatures, we applied this effector towards a one-pot detection reaction. BrCas12b was observed to have minimal inhibitory effects on RT-LAMP reaction and perform well in isothermal amplification buffer. In ≤ 30 min, samples with high viral load (C_t value ≤ 30) exhibited 100% accuracy, and we achieved ≥ 95% sensitivity detecting VOCs for samples with C_t value ≤ 32. When combined with Uracil-DNA Glycosylase (UDG), the one-pot detection reaction minimises carryover contamination as one false positive were observed in our clinical validation (Table 3). Additionally, the freeze-drying process reduces the need for low-temperature logistics. Although lyophilised reactions offer convenience in transport and distribution, there is a slight reduction in fluorescent signal after extended periods of storage. Further research is needed to optimise the lyophilisation conditions. We envision that CRISPR-SPADE with the portable detection instrument will move us closer to providing a cost-effective, rapid point-of-care test (Table 2).

Contributors

P.K.J and L.T.N designed the experiments. L.T.N, N.C.M, B.L.M.P carried out experiments and performed data analyses. L.T.N, B.L.M.P, and N.C.M wrote the primary manuscript. M.S. and R.D. provided the patient samples. M.R.M. and J.A.L. processed the patient samples

			Non-N gene for detecting SARS-CoV-2 VOC						
			Genomic sequencing results						Total
			α	β	γ	δ	o	Other	
SPADE	α	Positive	31 (TP)	0 (FP)	0 (FP)	0 (FP)	..	0 (FP)	31
		Negative	2 (FN)	0 (TN)	2 (TN)	11 (TN)	..	16 (TN)	31
	β	Positive	0 (FP)	1 (TP)	0 (FP)	0 (FP)	..	0 (FP)	1
		Negative	15 (TN)	0 (FN)	2 (TN)	11 (TN)	..	16 (TN)	44
	γ	Positive	0 (FP)	0 (FP)	13 (TP)	0 (FP)	..	0 (FP)	13
		Negative	15 (TN)	0 (TN)	4 (FN)	11 (TN)	..	16 (TN)	46
	δ	Positive	1 (FP)	0 (FP)	0 (FP)	53 (TP)	..	0 (FP)	54
		Negative	15 (TN)	0 (TN)	2 (TN)	3 (FN)	..	16 (TN)	36
	o	Positive	..	..	..	..		..	2
		Negative	..	..	..	..	..	..	..
	Other	Positive	0 (FP)	0 (FP)	0 (FP)	0 (FP)	..	16 (TP)	16
		Negative	15 (TN)	0 (TN)	2 (TN)	11 (TN)	..	0 (FN)	28
Total			94	1	25	100	2	80	302

***Table 3*: Clinical validation of CRISPR-SPADE with non-N gene for detecting SARS-CoV-2.**
TP = true positive; TN = true negative; FP = false positive; FN = false negative

in a BSL3 facility. M.N.C. and M.S. performed the sequencing analysis and viral load quantification of patient samples. J.K. and J.S. co-developed the portable multiplexing device (FISSH). The manuscript was edited, refined, and approved by all authors. L.T.N, N.C. M, B.L.M.P, R. D, M.S, and P.K.J have verified all presented data, and all authors have access to all research materials associated with this study.

Data sharing

Data related to this study are in the main text and the supplementary materials. The BrCas12b expression plasmid has been deposited and will be made available on Addgene (plasmid #170819). Sequencing Data of tested patient samples have been deposited into GISAID (Table S4). The BrCas12b protein sequence derived from unclassified *Brevibacillus* sp. SYP-B805 can be accessed at NCBI database (GenBank ID: WP_165214399·1). A complete list of detailed resources and reagents can be found in Table S5.

Declaration of interests

L.T.N. and P.K.J. are listed as inventors on the multiple patent applications related to the content of this work. J. S. and J.K. are both co-founders of Sparsek s.r.o. J.K. is also the founder of SCIERING s.r.o. R.R.D. and P.K.J are co-founders of Genable Biosciences, LLC. The remaining authors declare no competing interests.

Acknowledgments

We are thankful to Mr. Christopher Dervinis in the Forest Genomics group and Dr. Whitney Stoppel in the department of Chemical Engineering for the use of the lyophilisers and providing us with a written consent. Additionally, we appreciate the support from New England Biolabs for the use of lyo-ready Warmstart® reagents. Finally, we are grateful to the Health Care Centre at the University of Florida and members of Jain Lab for their insightful feedback. The following reagents were obtained through BEI Resources, NIAID, NIH: Genomic RNA from SARS-Related Coronavirus 2, Isolate USA-WA1/ 2020, NR-52285, (lineage B.1.1.7), NR-55244, contributed by Centres for Disease Control and Prevention. This work was supported in part by funds of the University of Florida Vice President Office of Research and CTSI seed funds, the University of Florida Office of Research and Health Science Centre with resources from the Interdisciplinary Centre for Biotechnology Research Gene Expression Core (RRID: SCR_019145), NextGen Sequencing Core (RRID: SCR_019152) and Bioinformatics Core (RRID: SCR_019120).

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103926.

References

1 Joung J, Ladha A, Saito M, et al. Detection of SARS-CoV-2 with SHERLOCK one-pot testing. *N Engl J Med*. 2020;383(15):1492–1494.
2 Nguyen PQ, Soenksen LR, Donghia NM, et al. Wearable materials with embedded synthetic biology sensors for biomolecule detection. *Nat Biotechnol*. 2021.

3 de Puig H, Lee RA, Najjar D, et al. Minimally instrumented SHERLOCK (miSHERLOCK) for CRISPR-based point-of-care diagnosis of SARS-CoV-2 and emerging variants. *Sci Adv*. 2021;7(32).
4 Fozouni P, Son S, Diaz de Leon Derby M, et al. Amplification-free detection of SARS-CoV-2 with CRISPR-Cas13a and mobile phone microscopy. *Cell*. 2021;184(2):323–333. e9.
5 Broughton JP, Deng X, Yu G, et al. CRISPR-Cas12-based detection of SARS-CoV-2. *Nat Biotechnol*. 2020;38(7):870–874.
6 Kaminski MM, Abudayyeh OO, Gootenberg JS, Zhang F, Collins JJ. CRISPR-based diagnostics. *Nat Biomed Eng*. 2021;5(7):643–656.
7 Abudayyeh OO, Gootenberg JS. CRISPR diagnostics. *Science*. 2021;372(6545):914–915.
8 Chen JS, Ma E, Harrington LB, Da Costa M, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science*. 2018;360(6387):436–439.
9 Li SY, Cheng QX, Wang JM, et al. CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discov*. 2018;4:20.
10 Li L, Li S, Wu N, et al. HOLMESv2: a CRISPR-Cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synth Biol*. 2019;8(10):2228–2237.
11 Gootenberg JS, Abudayyeh OO, Lee JW, et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science*. 2017;356(6336):438–442.
12 Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO, Zhang F. SHERLOCK: nucleic acid detection with CRISPR nucleases. *Nat Protoc*. 2019;14(10):2986–3012.
13 Teng F, Cui TT, Feng GH, et al. Repurposing CRISPR-Cas12b for mammalian genome engineering. *Cell Discov*. 2018;4.
14 Cofsky JC, Karandur D, Huang CJ, Witte IP, Kuriyan J, Doudna JA. CRISPR-Cas12a exploits R-loop asymmetry to form double-strand breaks. *Elife*. 2020;9.
15 Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 2000;28(12):E63.
16 Tian Y, Liu RR, Xian WD, Xiong M, Xiao M, Li WJ. A novel thermal Cas12b from a hot spring bacterium with high target mismatch tolerance and robust DNA cleavage efficiency. *Int J Biol Macromol*. 2020;147:376–384.
17 Li Z, Zhao W, Ma S, Li Z, Yao Y, Fei T. A chemical-enhanced system for CRISPR-Based nucleic acid detection. *Biosens Bioelectron*. 2021;192: 113493.
18 Hardinge P, Murray JAH. Full dynamic range quantification using loop-mediated amplification (LAMP) by combining analysis of amplification timing and variance between replicates at low copy number. *Sci Rep*. 2020;10(1). Uk.
19 Liu TY, Knott GJ, Smock DCJ, et al. Accelerated RNA detection using tandem CRISPR nucleases. *Nat Chem Biol*. 2021;17(9):982.
20 Ali Z, Aman R, Mahas A, et al. iSCAN: an RT-LAMP-coupled CRISPR-Cas12 module for rapid, sensitive detection of SARS-CoV-2. *Virus Res*. 2020;288: 198129.
21 Young RM, Solis CJ, Barriga-Fehrman A, et al. Smartphone screen testing, a novel pre-diagnostic method to identify SARS-CoV-2 infectious individuals. *Elife*. 2021;10.
22 Fontanet A, Autran B, Lina B, Kieny MP, Karim SSA, Sridhar D. SARS-CoV-2 variants and ending the COVID-19 pandemic. *Lancet*. 2021;397(10278):952–954.
23 Tregoning JS, Flight KE, Higham SL, Wang Z, Pierce BF. Progress of the COVID-19 vaccine effort: viruses, vaccines and variants versus efficacy, effectiveness and escape. *Nat Rev Immunol*. 2021;21(10):626–636.
24 Chookajorn T, Kochakarn T, Wilasang C, Kotanan N, Modchang C. Southeast Asia is an emerging hotspot for COVID-19. *Nat Med*. 2021;27(9):1495–1496.
25 Bhoyar RC, Jain A, Sehgal P, et al. High throughput detection and genetic epidemiology of SARS-CoV-2 using COVIDSeq next-generation sequencing. *PLOS One*. 2021;16(2).
26 Otu A, Agogo E, Ebenso B. Africa needs more genome sequencing to tackle new variants of SARS-CoV-2. *Nat Med*. 2021;27(5):744–745.
27 Chiara M, D'Erchia AM, Gissi C, et al. Next generation sequencing of SARS-CoV-2 genomes: challenges, applications and opportunities. *Brief Bioinform*. 2021;22(2):616–630.