Rapid and accurate nucleobase detection using FnCas9 and its application in COVID-19 diagnosis

Mohd. Azhar [a,b,1], Rhythm Phutela [a,b,1], Manoj Kumar [a,b,1], Asgar Hussain Ansari [a,b,1], Riya Rauthan [a,b], Sneha Gulati [a], Namrata Sharma [a], Dipanjali Sinha [a,b], Saumya Sharma [a,b], Sunaina Singh [a], Sundaram Acharya [a,b], Sajal Sarkar [a], Deepanjan Paul [a], Poorti Kathpalia [a], Meghali Aich [a,b], Paras Sehgal [a,b], Gyan Ranjan [a,b], Rahul C. Bhoyar [a], on behalf of Indian CoV2 Genomics & Genetic Epidemiology (IndiCovGEN) Consortium[2], Khushboo Singhal [a,b], Harsha Lad [c], Pradeep Kumar Patra [c], Govind Makharia [d], Giriraj Ratan Chandak [e], Bala Pesala [g], Debojyoti Chakraborty [a,b,*,1], Souvik Maiti [a,b,f,**,1]

[a] CSIR-Institute of Genomics & Integrative Biology, Mathura Road, New Delhi, 110025, India
[b] Academy of Scientific & Innovative Research (AcSIR), Ghaziabad, 201002, India
[c] CSIR-Sickle Cell Anemia Mission Laboratory, Chhattisgarh Institute of Medical Sciences, Bilaspur, 495001, Chhattisgarh, India
[d] All India Institute of Medical Sciences, Ansari Nagar East, New Delhi, 110029, India
[e] CSIR-Center for Cellular and Molecular Biology, Uppal Road, Hyderabad, Telengana, 500007, India
[f] CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune, 411008, India
[g] CSIR-Central Electronics Engineering Research Institute, Chennai, India

ARTICLE INFO

ABSTRACT

Rapid detection of DNA/RNA pathogenic sequences or variants through point-of-care diagnostics is valuable for accelerated clinical prognosis, as witnessed during the recent COVID-19 outbreak. Traditional methods relying on qPCR or sequencing are tough to implement with limited resources, necessitating the development of accurate and robust alternative strategies. Here, we report FnCas9 Editor Linked Uniform Detection Assay (FELUDA) that utilizes a direct Cas9 based enzymatic readout for detecting nucleobase and nucleotide sequences without trans-cleavage of reporter molecules. We also demonstrate that FELUDA is 100% accurate in detecting single nucleotide variants (SNVs), including heterozygous carriers, and present a simple web-tool JATAYU to aid end-users. FELUDA is semi-quantitative, can adapt to multiple signal detection platforms, and deploy for versatile applications such as molecular diagnosis during infectious disease outbreaks like COVID-19. Employing a lateral flow readout, FELUDA shows 100% sensitivity and 97% specificity across all ranges of viral loads in clinical samples within 1hr. In combination with RT-RPA and a smartphone application True Outcome Predicted via Strip Evaluation (TOPSE), we present a prototype for FELUDA for CoV-2 detection closer to home.

1. Introduction

The rise of CRISPR Cas9 based approaches for biosensing nucleic acids has opened up a broad diagnostic portfolio for CRISPR products beyond their standard genome editing abilities (Chertow, 2018; Y. Li et al., 2019). In recent times, CRISPR components have been successfully used to detect a wide variety of nucleic acid targets obtained from pathogenic microorganisms or disease-causing mutations in various biological specimens (Pardee et al., 2016; Gootenberg et al., 2018; Sashital, 2018; Qiu et al., 2018; Teng et al., 2019; Quan et al., 2019; Kellner et al., 2019; L. Li et al., 2019). Almost exclusively, CRISPR based detection procedures that have been successfully validated in the lab

and the field employ the property of certain classes of CRISPR proteins to bind to target DNA or RNA and cleave reporter molecules to generate a signal outcome (Abudayyeh et al., 2016; Csx et al., 2017; Li et al., 2018; Chen et al., 2018; Yan et al., 2018). In order to enable the detection methodology to be precise, sensitive, and reproducible across a large variety of targets, the accuracy of nucleic acid interrogation and subsequent enzyme activity is extremely critical, especially when clinical decisions are to be made based on these results (Chertow, 2018; Y. Li et al., 2019).

The COVID-19 pandemic has sparked the development of alternative testing strategies to augment traditional gold standard tests. Among the Cas proteins that have been explored so far, Cas12 and Cas13 along with their variants have obtained Emergency Usage Authorization (EUA) for diagnostics during the COVID-19 pandemic (Broughton et al., 2020; Joung et al., 2020). However, each of these approaches has its own strengths and limitations that are either related to sensitivity to mismatches or the ease of design for a wide variety of targets beyond SARS CoV-2 (L. Li et al., 2019). Importantly both these enzymes unleash an uncontrolled secondary reporter activity upon activation, leading to possible loss of information about starting copy numbers of the target (Broughton et al., 2020; Joung et al., 2020). Taken together, the development of a detection pipeline utilizing a highly specific Cas protein with direct binding or cleavage-based readout can significantly increase the sensitivity of detection along with reduced time and cost of CRISPR based diagnostics (CRISPRDx). That is especially crucial for point-of-care (POC) applications where complex experimentation or reaction setup are not feasible.

We and others have recently reported a Cas9 ortholog from *Francisella novicida* (FnCas9) showing very high mismatch sensitivity under both in vitro and *in vivo* conditions (Hirano et al., 2016; F. Chen et al., 2017; Acharya et al., 2019) This is based on its negligible binding affinity to substrates that harbor mismatches, a property to distinguish it from other engineered Cas proteins showing similar specificity (Chen et al., 2017). We reasoned that FnCas9 mediated DNA interrogation and subsequent cleavage can be both adapted for accurately identifying nucleotide sequences or any single nucleotide variants (SNVs). We name this approach FnCas9 Editor Linked Uniform Detection Assay (FELUDA) and demonstrate its utility in various pathological conditions alongside genetic disorders. Notably, due to its ease of design, implementation, and reproducibility for new targets, we were successful in its rapid deployment for a ready-to-use diagnostic kit during the COVID-19 outbreak that has successfully completed regulatory validation (Petherick, 2019; Babiker et al., 2020; Guo et al., 2020).

2. Materials and methods

2.1. Study design and molecular analysis

The study was designed to evaluate the efficacy of FELUDA on clinical samples. A detailed description of the study parameters and molecular assays are presented in Supplementary Note 1.

2.2. Oligos

A list of all oligos used in the study can be found in Supplementary Table 1.

2.3. SCA FELUDA detection assays

Sequences containing WT, SCT, and SCA were amplified using primers with/without 5' biotinylation from genomic DNA extracted from saliva or blood samples. Details about cleavage and fluorescence based detection is presented in Supplementary Note 1.

2.4. JATAYU

JATAYU is a web tool to design sgRNAs and target amplicon primers for the SNV detection. When provided with a valid genomic DNA sequence along with position and type of variation, JATAYU returns the primers and sgRNA sequences for FELUDA based detection. JATAYU user interface has been created using Bootstrap 4 and jQuery. The tool runs with a customized python-based Flask framework, together with genome analysis tools like BWA (Burrows-Wheeler aligner) and bedtools (Li and Durbin, 2010; Quinlan and Hall, 2010). The source code is available at https://github.com/asgarhussain/JATAYU.

2.5. RT-PCR-FELUDA primer and crRNA design

To design crRNAs specific to SARS-CoV 2, genome sequences were downloaded from GISAID (Shu and McCauley, 2017) and crRNAs were designed by scanning for a stretch of 20 nucleotides followed by NGG PAM. To remove non-specific crRNAs the off-target analysis was done by mapping them to viruses from Influenza Virus Database (Brister et al., 2015) and human transcriptome (GENCODE GRCh38). Finally, 21 crRNAs with higher conservation frequency across SARS-CoV2, genome sequences in the GISAID dataset were selected (Shu and McCauley, 2017). Target PCR Primer sequences flanking these crRNAs are also investigated for off-targets on the human transcriptome (GENCODE GRCh38).

Mutational frequencies of the crRNA were routinely checked across GISAID datasets (1,65,799 genomes as of Nov. 29, 2020). Custom python script and SeqMap (Jiang and Wong, 2008) were used to design crRNAs and calling mutations (Supplementary Table 2).

2.6. SARS-CoV2 detection via lateral flow assay (RT-PCR)

N and S gene SARS-CoV2 RNA regions were reverse transcribed and amplified using the 5′ biotin-labeled primers. Chimeric gRNA (crRNA: TracrRNA) was prepared by mixing crRNA (N and S genes) and synthetic 3′-FAM-labeled TracrRNA in an equimolar ratio within annealing buffer (100 mM NaCl, 50 mM Tris-HCl pH 8 and 1 mM $MgCl_2$), heated at $95°C$ for 2–5 min and then allowed to cool at room temperature for 15–20 min. Chimeric gRNA-dead FnCas9 RNP complex was prepared by equally (Protein:sgRNA molar ratio, 1:1) mixing them in buffer (20 mM HEPES, pH7.5, 150 mM KCl, 1 mM DTT, 10% glycerol, 10 mM $MgCl_2$) and incubated for 10 min at RT. Target biotinylated amplicons were then incubated with the RNP complexes for 10 min at 37 °C. An Dipstick buffer (80 μl) was added to the reaction mix along with Milenia HybriDetect 1 lateral flow strip. The solution is allowed to migrate into the strip for 2-5 min at room temperature followed by visual or app-based detection. Details about RT-RPA FELUDA and LOD studies are presented in Supplementary Note 1.

2.7. TOPSE based image quantification

Details of app development are mentioned in Supplementary Note 2. For single-gene FELUDA before machine learning, a cut-off of 5 (Test-NTC) was used for calling samples positive or negative based on LOD vs intensity values. After training the app on 473 single gene FELUDA readouts and correlating with qPCR values, the threshold was increased to 6.7 for increased sensitivity (refer to Supplementary Note 1).

3. Results

3.1. FELUDA distinguishes between nucleotide sequences differing by one mismatch

To identify an SNV with high accuracy, we first sought to investigate whether FnCas9 can be directed to cleave the wild-type (WT) allele at an SNV by placing an additional synthetic mismatch (MM) in the sgRNA

sequence with reference to the SNV (Fig. 1A). To test this, we selected sickle cell anemia (SCA), a global autosomal recessive genetic disorder caused by a point mutation (GAG > GTG) (Piel et al., 2017; Andrieu-Soler and Soler, 2020). By fixing the position of the SNV and walking along the entire length of the sgRNA, we discovered that two mismatches at the PAM proximal 2nd and 6th positions completely abrogated the cleavage of the SNV target (SCA) while leaving the WT target intact (Fig. 1B). We tested FELUDA on DNA cloned from 6 SCA patients and a healthy control and obtained a clearly identifiable signature for SNV in every case (Supplementary Fig. 1A). Significantly, the same design principle can be availed universally for searching and discriminating other Mendelian SNVs without the need for optimization (Supplementary Fig. 1B). To aid users with quick design and implement FELUDA for a target SNV. We have also developed a web tool JATAYU (Junction for Analysis and Target Design for Your FELUDA assay), that incorporates the above features and generates primer sequences for amplicon and sgRNA ready for synthesis (http://jatayu.igib.res.in, Supplementary Fig. 1C).

3.2. FELUDA diagnosis is adaptable to direct binding-based outcomes

We next sought to adapt FELUDA for fluorescent detection. Unlike Cas12/Cas13 based CRISPRDx platforms, FnCas9 is reported not to produce collateral activity on substrates, so FELUDA is not suitable for trans-cleavage signal output. To circumvent this, we envisioned FELUDA as a direct, non-cleavage, affinity-based method of detection, working with single nucleotide mismatch sensitivity. We first investigated if a catalytically inactive dead FnCas9 (dFnCas9) tagged with a fluorophore (GFP) is adept in sensing a point mutation on DNA using Microscale Thermophoresis (MST) (Fig. 1C). We observed that using FELUDA specific sgRNAs (having 2 MM with WT and 1 MM to SCA substrate), the WT substrate exhibited negligible dFnCas9-GFP binding ($K_d = 1037.4$ nM $\pm$ 93.3 nM) while the SCD substrate (having 1 MM) showed moderately strong binding ($K_d = 187.2$ nM $\pm$ 3.4 nM) (Fig. 1C and Supplementary Fig. 2A). That is consistent with the in vitro cleavage (IVC) outcomes on the two substrates (Fig. 1B). Importantly, both *Streptococcus pyogenes* Cas9 (SpCas9) and its engineered High-Fidelity variant (dSpCas9-HF1-GFP) showed similar strong binding towards WT substrate with sgRNAs containing both 0 as well as 2 mismatches. (dSpCas9-GFP: 51.5 ± 2.9 nM and 144.9 ± 11.8 nM; dSpCas9-HF1-GFP: 122.8 ± 24.4 nM and 153.6 ± 19.8 nM respectively) Corroborating that the inherent DNA interrogation properties of FnCas9 are responsible for discriminating single mismatched targets with very high specificity (Fig. 1C). We then developed a pipeline to adapt FELUDA for an affinity-based fluorescent readout system, where an amplification step generates biotinylated products that can then be immobilized on magnetic streptavidin beads (Fig. 1D). Upon incubation with dFnCas9-GFP with specific sgRNA, enzymatic binding to the substrate leads to loss of fluorescence signal in the supernatant, which is measured. In this assay too, FELUDA was able to successfully distinguish the SCA and WT substrates with greater than 2-fold difference in signal counts (Fig. 1D). It is possible to perform this assay on immobilized streptavidin molecules on a microplate and collect readout as a gain of fluorescence upon Cas9: Substrate interaction.

Although sickle cell trait (SCT) individuals are generally non-symptomatic, carrier screening is vital to prevent the spread of SCA in successive generations and is widely employed in SCA control programs in various parts of the world (Piel et al., 2017). Since FELUDA outcomes are reflected by binding to substrate molecules, it resulted in clearly distinguishable signatures between the SCA, SCT, and WT DNA obtained from the patient's saliva samples (Supplementary Fig. 2B). To address the robustness of detecting the 3 genotype categories, we performed a blinded experiment using DNA obtained from 49 subjects with all three SCA genotypes from a CSIR-Sickle Cell Anaemia Mission Laboratory in Chhattisgarh state of India. Remarkably, FELUDA identified all three genotypes with 100% accuracy, matching the results with Sanger

sequencing data generated on the same samples in a different laboratory (CSIR-Center for Cellular and Molecular Biology, Genome Research on Complex Diseases Lab) (Fig. 1E).

3.3. PAMmer based amplification abrogates PAM dependency on FELUDA design

We made several improvements to FELUDA for simplifying and expanding its detection spectrum. To detect non-PAM proximal SNVs, we installed an in-built PAM site in the primers during the amplification step of FELUDA (PAMmer) and successfully validated this approach using 2 SNVs (A2142G and A2143G) present in *Helicobacter pylori* 23s *rRNA* gene, which confers variable clarithromycin resistance in patients with gastric ulcers (Ribeiro et al., 2003; Landrum et al., 2014; Bińkowska et al., 2018) (Supplementary Fig. 2C and D). Next, we tried to reduce PAM dependency on sgRNA design further by exploring if FELUDA could be performed with a single mismatch in the sgRNA. In line with our previous reports, we found that FnCas9 shows negligible cleavage with sgRNAs containing mismatches at the PAM distal end, and in particular, a mismatch at PAM distal 16th base showed complete absence of cleavage or binding (Supplementary Fig. 3A and B). We confirmed this strategy by targeting the SNV rs713598 (G > C) in different individuals and successfully identified their genotypes (Perna et al., 2018) (Supplementary Fig. 3C). We also show that FELUDA based detection can work robustly across a wide temperature range and up to 3 days post thawing of reaction components (at room temperature). Thus, field studies using FELUDA can be conducted in diverse climatic conditions, and reaction components can be successfully used following cold chain transportation (Supplementary Fig. 4A and B).

### 3.4. Combination of FELUDA with paper strip chemistry for lateral flow readout

The recent outbreak of Coronavirus disease 19 (COVID-19) due to the SARS-CoV-2 virus provided an opportunity to expand the scope of the above-mentioned approach of FELUDA and make a difference in ongoing public health emergencies throughout the world.

In addition to the general social distancing, detecting infected individuals and screening their contacts for possible quarantine measures is one of the foremost steps in reducing community transmission of this virus (Lewnard and Lo, 2020; Chan et al., 2020; Wu et al., 2020). Although quantitative Real-Time (qRT) PCR is considered a gold-standard test for detecting active COVID-19 cases, such tests are expensive and have long turn-around times requiring a dedicated qRT-PCR machine, hence is of limited utility in handling an emergency of this scale. We sought to repurpose FELUDA as a lateral flow assay (LFA) for low-cost SARS-CoV-2 detection, which does not need complex instrumentation and is highly accurate in diagnosis. To enable such a diagnosis on commercially available paper strips we enabled the chemistry of capturing RNP-bound biotinylated substrate molecules on a distinct test line of the paper strip using FAM-labeled chimeric gRNA (Fig. 2A and Supplementary Fig. 5A). In order to make the strip readout reproducible in point of care settings, we chemically modified the chimeric gRNA using synthetic backbone modifications (phosphorothioate) to increase the stability and robustness of the readout (Supplementary Fig. 5B). Finally, using an optimized single step Reverse Transcription-PCR protocol followed by FELUDA, we developed an assay that can detect SARS-CoV-2 sequences from RNA samples within an hour (Fig. 2B and Supplementary Fig. 5C, Methods).

We tested up to 21 targets across the SARS-CoV-2 RNA genome and identified two regions (in the viral N and S genes) which are present in high copy numbers (Snijder et al., 2003; Kim et al., 2020) and are reported with a negligible number of mutations in publicly available datasets (Brister et al., 2015; Shu and McCauley, 2017) (1,65,799 GISAID submissions till November, 2020) (Figure 2C, Supplementary Figure 6A. Supplementary Table 2). Through an extensive optimization
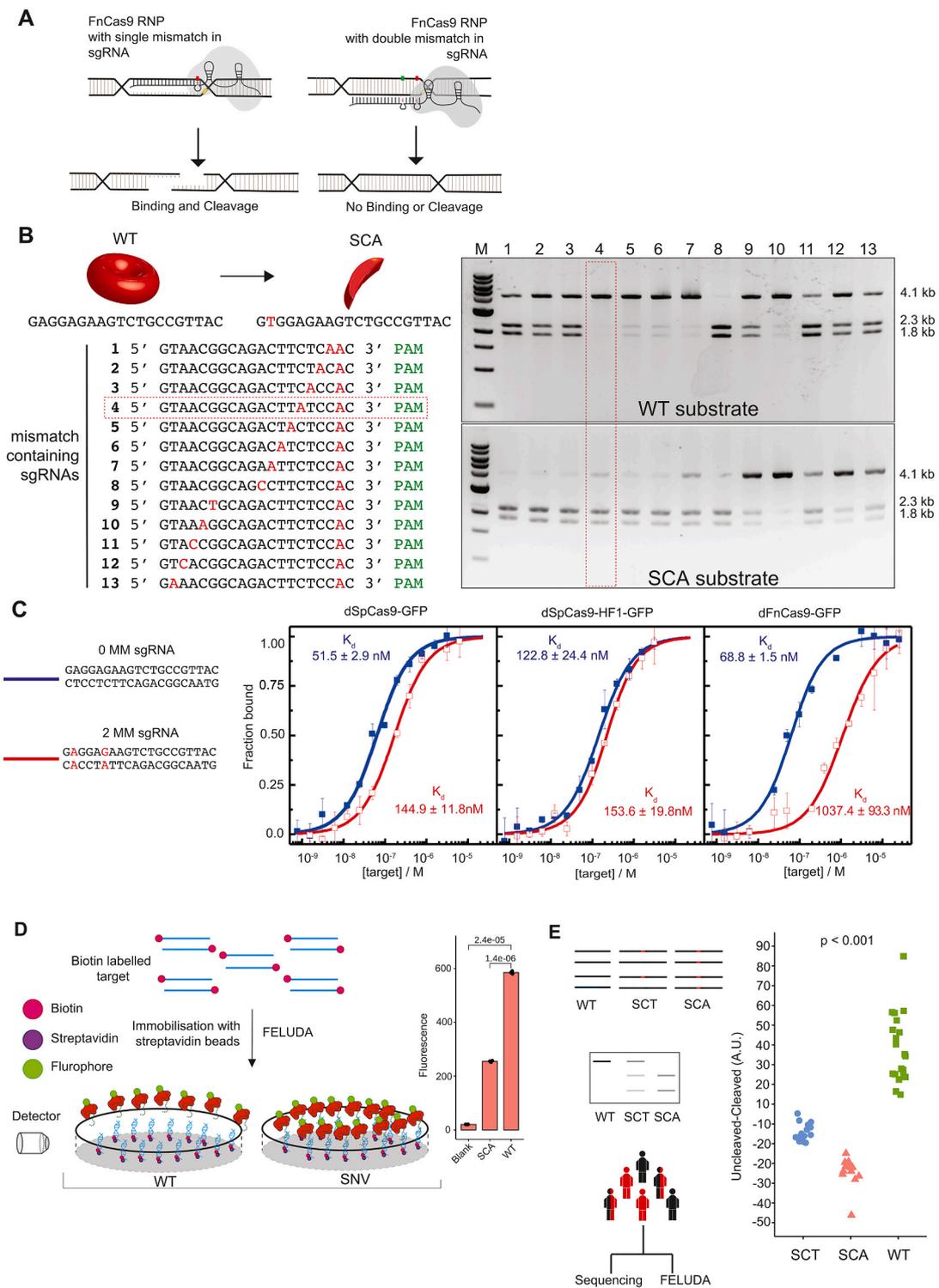
Fig. 1. Schematic for FELUDA detection. Schematic for FELUDA based discrimination of SNVs. **A,** Strategy for discrimination of substrates differing by single mismatch using FnCas9. The presence of 2 mismatches (marked in red and green) at defined positions on the sgRNA prevents the enzyme from binding to the target leading to differential binding and cleavage outcomes. **B,** Left panel shows positions of mismatches sgRNAs containing two mismatches at different positions along their lengths. Representative in-vitro cleavage outcomes on wild type (WT) or sickle cell anemia (SCA) substrates (4.1 kb) are shown on the right. Cleavage with FnCas9 produces 2 products (2.3 kb and 1.8 kb). The red dotted box denotes the sgRNA showing negligible cleavage for WT substrate and maximum cleavage for SCA substrate. **C,** Binding affinity experiments using Microscale Thermophoresis showing the interaction of dSpCas9-GFP, dSpCas9-HF1-GFP, and dFnCas9-GFP with substrates with 0 (blue) or 2 (red) mismatch (MM). Values are expressed as fraction bound protein (y-axis) with respect to varying concentrations of purified DNA substrate (Molar units, M, x-axis). Error bars represent SEM (2 independent experiments). **D,** Schematic for fluorescence-based detection of sickle cell anemia mutation (SCA) using FELUDA. Error bars represent SD. Student's t-test p values are shown (n = 3 independent measurements). **E,** Left panel shows schematic of FELUDA for identifying carriers of SCA mutation. The right panel shows blinded FELUDA results in a mixed cohort of individuals (n = 49, one way ANOVA p-value is shown). SCA, sickle cell anemia individuals; SCT, sickle cell trait individuals; WT, normal subjects; SNV, single nucleotide variation; SEM, standard error of the mean. FELUDA accurately genotypes carriers of Mendelian variants. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
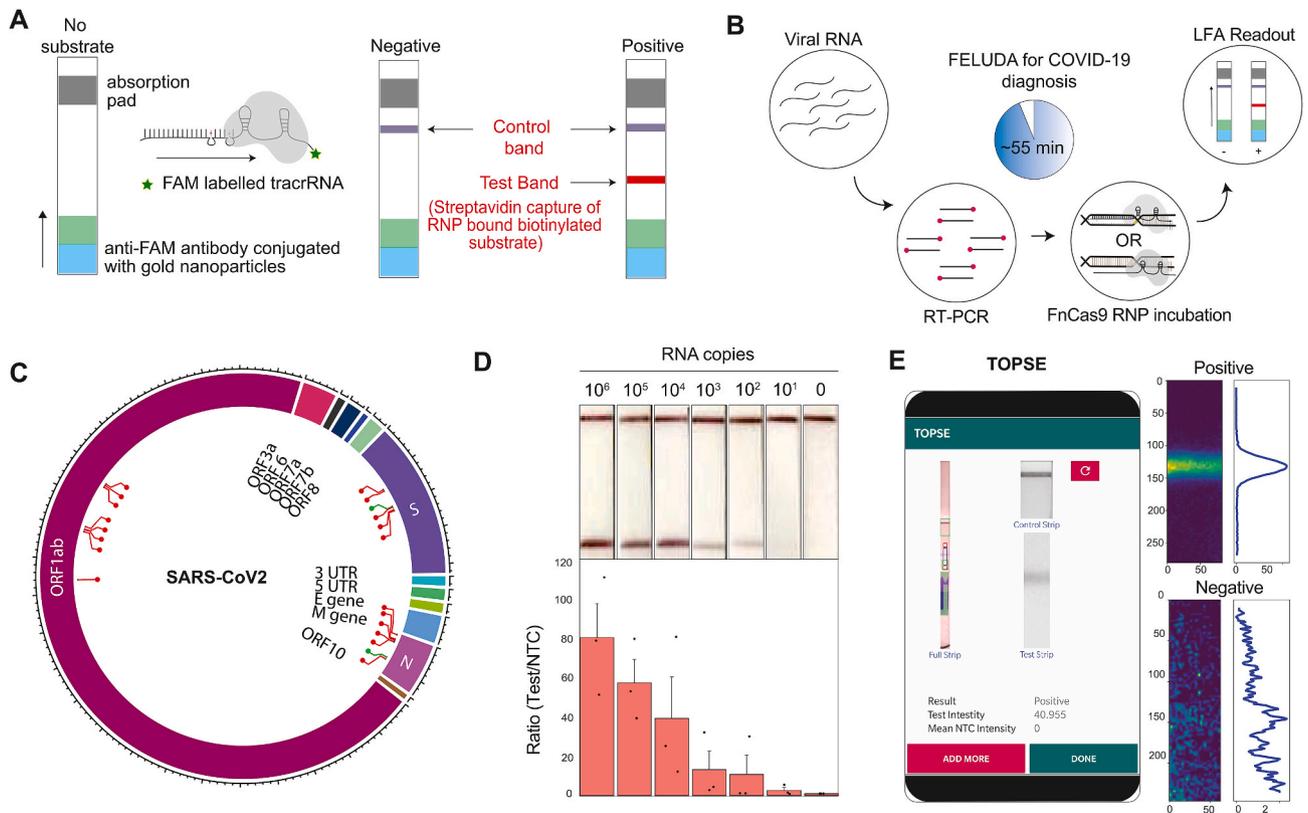
Fig. 2. FELUDA for point-of-care nCOV-2 detection. A, Outline of lateral flow assay using FELUDA showing positions of control and test bands. **B,** Pipeline of FELUDA based detection for SARS-CoV-2 infection from patient samples showing individual steps involved. **C,** Plot showing the regions of SARS-CoV-2 RNA genome tested for FELUDA, the number of tested regions are represented in red while successful regions are highlighted in green. **D,** LOD of FELUDA in purified N gene target RNA. The top panel shows representative LFA readout on strips, and the bottom panel shows Fluorescent intensity ratios. Error bars SEM. (n = 3 independent experiments). **E,** Smartphone GUI for TOPSE showing representative output from a strip image (left). Positive and NTC preprocessed images are shown on the right. FELUDA shows high concordance with gold standard qRT-PCR in detecting nCOV-2 infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
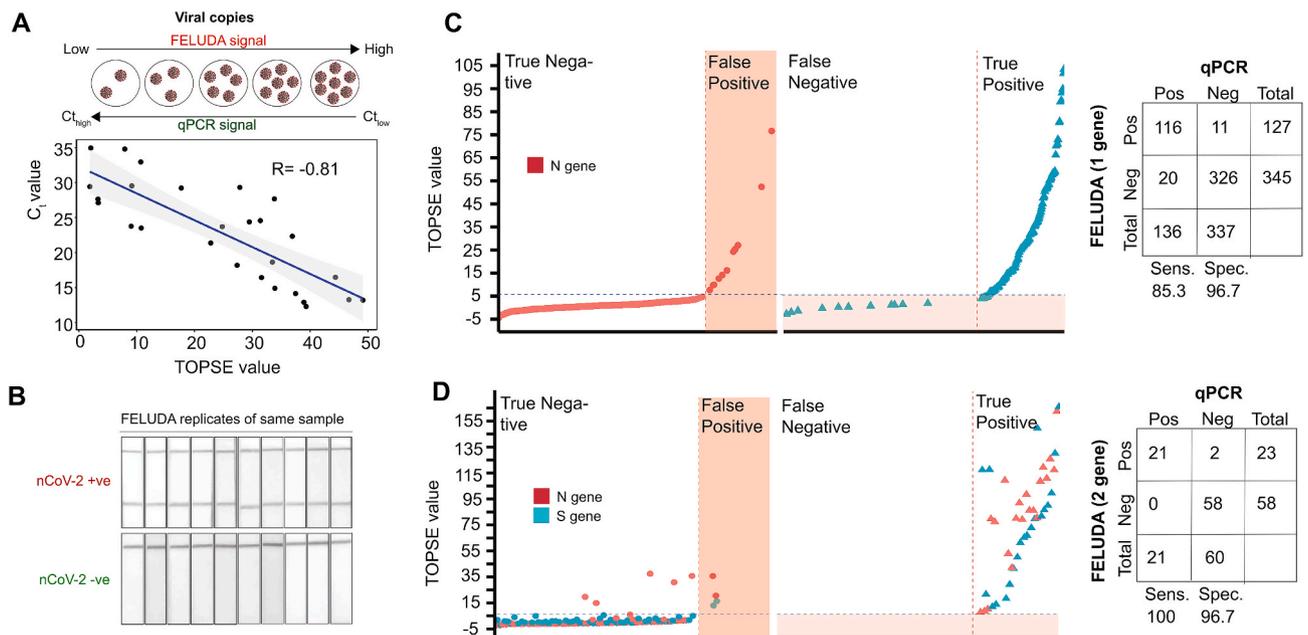
Fig. 3. FELUDA shows high concordance with qRT-PCR for SARS-CoV-2 detection A, FELUDA readouts are semiquantitative. Correlation between Ct values (E gene) and TOPSE values are shown (n = 27). **B,** Strong reproducibility between repeated FELUDA runs on the same positive or negative sample is shown. **C,** One gene (N gene) FELUDA on clinical samples (x-axis) showing the distribution of TOPSE values (y-axis). Analyzed results represented on the right. **D,** Two genes (N and S genes) FELUDA on clinical samples (x-axis) showing the distribution of TOPSE values (y-axis). Analyzed results represented on the right.

of PCR conditions and reaction components, FELUDA reached a limit of detection (LOD) of ~10 copies of purified viral sequence (Fig. 2D, Supplementary Fig. 6B). Upon gradual dilution of patient RNA, both FELUDA and qRT-PCR were able to detect samples till the same dilution range (Supplementary Fig. 6C). Given that, visual detection can occasionally have an operator-bias, mainly when the signal is low. We developed a smartphone app TOPSE, (True Outcome Predicted via Strip Evaluation) to assist detection by returning a predictive score based on background correction (Fig. 2E, Supplementary Note 2, and Supplementary Movie 1).

FELUDA detection is semi-quantitative (due to stoichiometric binding of FnCas9 RNP: target) and therefore shows a strong negative correlation between Ct values and signal intensities (Fig. 3A). That makes it uniquely placed among CRISPRDx platforms to accurately predict the viral load in patient samples with high reproducibility between assays (Fig. 3B) (Broughton et al., 2020) (Joung et al., 2020).

We first performed FELUDA using one gene (N), single-pass assay on qRT-PCR confirmed 473 samples and obtained a sensitivity of 85.3% (116/136) and specificity of 96.7% (326/337) with qRT-PCR (Fig. 3C, Supplementary Dataset 1, Methods). Among the samples that showed discordance between FELUDA with qRT-PCR, 8 false-negative FELUDA samples, and 10 false-positive FELUDA samples were picked for a repeat evaluation using FELUDA and qRT-PCR. Surprisingly a repetition of FELUDA with double the amount of RNA yielded positive signals in 6/8 (75%) of samples and a repeat qPCR yielded positive signals in 5/10 (50%) of initially classified negative samples (Supplementary Fig. 7A and B). That underscores the error rates seen in a single run assay and has been reported elsewhere (Bhoyar et al., 2020). To improve FELUDA accuracy, we combined assays for both N and S genes, doubled the starting RNA amount, and performed FELUDA with 81 qRT-PCR confirmed samples. Remarkably, we obtained a sensitivity of 100% and a specificity of 97% and were able to accurately detect samples up to a high Ct value of 37, showing the robustness of the assay (Fig. 3D, Supplementary Fig. 7C, Supplementary Dataset 2). Based on the above results, licensing of the paper-strip based FELUDA diagnosis technology for SARS CoV2 was done. It completed third party evaluation and regulatory validation in India and is currently available for commercial use.

3.5. FELUDA can adapt to a point-of-care or potential home testing assay

Understanding the need for more testing, especially in the wake of rising numbers and predictions of the second wave of infection (Xu and Li, 2020), we next implemented a thermal cycler free version of FELUDA using Recombinase Polymerase Amplification (RPA) (Zhao et al., 2015; Lobato and O'Sullivan, 2018) (Fig. 4A, Methods). By systematic optimization of reaction components, RPA-FELUDA was able to reach a LOD of ~400 copies of starting RNA substrate (Supplementary Fig. 8A). We then performed RPA-FELUDA using a single gene (N gene) on patient samples (Ct value within 30) and obtained 100% sensitivity (14/14) and 97% specificity (32/33) (Supplementary Dataset 2, Methods). Importantly, contrary to some other reports, we found that the initial RPA amplification using off-the-shelf enzymes did not work consistently for Ct values above 30 (Patchsung et al., 2020), even though such samples were successfully captured by a standard FELUDA assay (Supplementary Fig. 8B). Further, we reasoned that the RPA working temperatures (35 °C–42 °C) overlap with human body temperature and are suitable for on-body amplification. Towards adapting FELUDA for possible home testing in the future, we successfully developed a 15 min benchtop method for RNA extraction from saliva and an on-body 30 min RPA-FELUDA (tested using synthetic RNA fragments). Therefore, generating an end-to-end instrumentation-free testing protocol that can potentially be performed at home (Fig. 4B–C, Supplementary Fig. 8C, Supplementary Movie 2, Supplementary Note 1).

4. Conclusion

In this study, we report a FnCas9 based diagnostic for detecting a wide range of pathogenic SNVs and nucleotide sequences. Our results suggest that FELUDA is suitable for SARS-CoV2 diagnosis with a similar degree of accuracy as the currently accepted gold standard qRT-PCR test and can replace it in resource-limited settings where a qRT-PCR machine is not available or for mass-scale community screening. The ability of FELUDA to genotype single nucleotide variants with high accuracy leads to multiple applications in disease prognosis and pathogen detection which otherwise require extensive optimization of assay conditions (Mamotte, 2006). In combination with microfluidics-based fragment analyzer and portable hand-held PCR machines, the method can be extended for true POC applications (Myers et al., 2013).

FELUDA can be improved further with design of more robust primer and sgRNA pairs, in light of mutations accumulating in the CoV2 genome which might impact traditional qRT PCR readouts (Pachetti et al., 2020). Importantly, we uncovered inconsistencies in qRT PCR Ct values between samples that were measured before and after freezing (Supplementary Fig. 7A and B). This is relevant when comparing the validation of new tests with results of qPCR-validated samples from the freezer, particularly those with high Ct values (Supplementary Fig. 9).
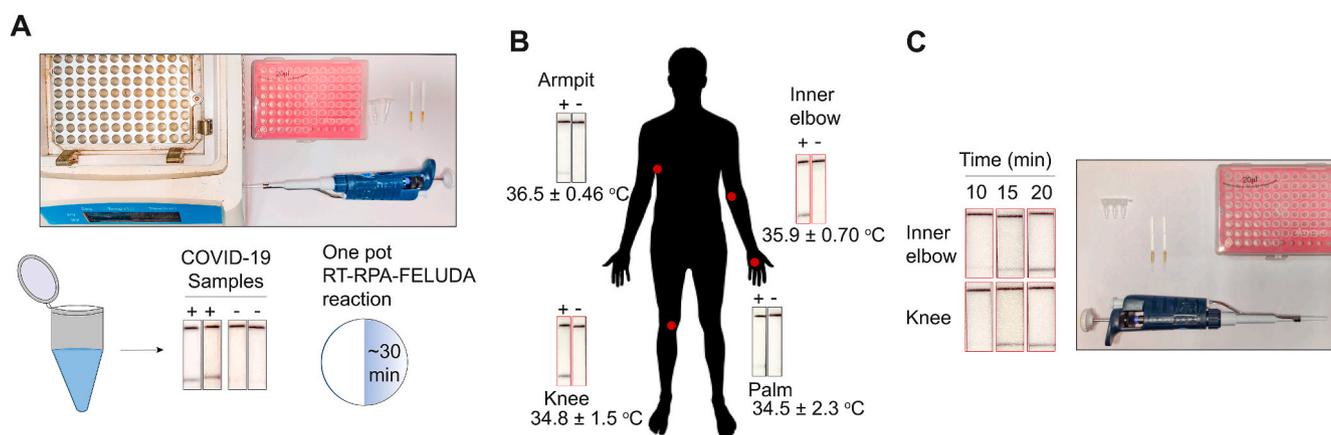
Fig. 4. Prototype for FELUDA for possible home-testing A, One-pot RT-RPA FELUDA. The top panel shows minimum requirements, bottom panel shows the outcome for 2 representative samples. **B,** On-body RT-RPA-FELUDA, left figure shows the variation of temperature in different zones of the body marked in red dots and corresponding RPA-FELUDA for a synthetic RNA fragment as starting material. **C,** Left panel shows representative FELUDA using on body RT-RPA from samples incubated in two different parts of the body. The right panel represents minimum requirements for FELUDA using on-body RT-RPA. All experiments for on body RT-RPA were done by at least 3 different individuals (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Taken together, FELUDA is an accurate and low-cost CRISPR based diagnostic assay to detect nucleic acids and their variations and is an attractive alternative to current technologies that primarily employ Cas12 and Cas13 based systems (Supplementary Table 3). A two-gene FELUDA assay for SARS-CoV2 costs ~7 USD (Supplementary Table 4). Its single-mismatch sensitivity towards nucleic acids expands its application portfolio to other sectors including detection of CoV2 variants and not just limited to healthcare. Its ease of design and implementation, as exemplified by its urgent deployment during the COVID-19 health crisis, offers immense possibilities for rapid and wide-spread testing so far proven to be successful in spreading the progression of the disease in multiple countries.

CRediT author contribution statement

Mohd. Azhar: Conceptualization, Methodology, Investigation, Validation, Visualization. **Rhythm Phutela:** Conceptualization, Methodology, Investigation, Validation, Visualization. **Manoj Kumar:** Conceptualization, Methodology, Investigation, Validation, Visualization. **Asgar Hussain Ansari:** Methodology, Data curation, Visualization, Software. **Riya Rauthan:** Investigation, Validation. **Sneha Gulati:** Investigation, Validation. **Namrata Sharma:** Investigation, Validation. **Dipanjali Sinha:** Investigation. **Saumya Sharma:** Investigation. **Sunaina Singh:** Investigation. **Sundaram Acharya:** Investigation. **Sajal Sarkar:** Investigation. **Deepanjan Paul:** Investigation. **Poorti Kathpalia:** Investigation. **Meghali Aich:** Investigation. **Paras Sehgal:** Resources. **Gyan Ranjan:** Resources. **Rahul C. Bhoyar:** Resources. **Indian CoV2 Genomics & Genetic Epidemiology (IndiCovGEN) Consortium:** Resources. **Khushboo Singhal:** Resources. **Harsha Lad:** Resources, Validation. **Pradeep Kumar Patra:** Resources, Validation. **Govind Makharia:** Investigation, Resources. **Giriraj Ratan Chandak:** Investigation, Resources. **Bala Pesala:** Software, Resources. **Debojyoti Chakraborty:** Conceptualization, Methodology, Visualization, Supervision, Writing – original draft, Funding acquisition. **Souvik Maiti:** Conceptualization, Methodology, Supervision, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be consideredas potential competing interests: A patent application has been filed in relation to this work. The FELUDA technology for CoV2 detection has been licensed non exclusively to TATA Medical and Diagnostics. Mohd. Azhar is currently an employee of TATA Medical and Diagnostics, India.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113207.

References

Abudayyeh, O.O., Gootenberg, J.S., Konermann, S., Joung, J., Slaymaker, I.M., Cox, D.B.T., Shmakov, S., Makarova, K.S., Semenova, E., Minakhin, L., Severinov, K., Regev, A., Lander, E.S., Koonin, E.V., Zhang, F., 2016. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science 353, aaf5573.

Acharya, S., Mishra, A., Paul, D., Ansari, A.H., Azhar, M., Kumar, M., Rauthan, R., Sharma, N., Aich, M., Sinha, D., Sharma, S., Jain, Shivani, Ray, A., Jain, Suman, Ramalingam, S., Maiti, S., Chakraborty, D., 2019. Francisella novicida Cas9 interrogates genomic DNA with very high specificity and can be used for mammalian genome editing. Proc. Natl. Acad. Sci. Unit. States Am. 116, 20959–20968.

Andrieu-Soler, C., Soler, E., 2020. When basic science reaches into rational therapeutic design: from historical to novel leads for the treatment of β-globinopathies. Curr. Opin. Hematol. 27.

Babiker, A., Myers, C.W., Hill, C.E., Guarner, J., 2020. SARS-CoV-2 testing. Am. J. Clin. Pathol. 153, 706–708.

Bhoyar, R.C., Jain, A., Sehgal, P., Divakar, M.K., Sharma, D., Imran, M., Jolly, B., Ranjan, G., Rophina, M., Sharma, S., Siwach, S., Pandhare, K., Sahoo, S., Sahoo, M., Nayak, A., Mohanty, J.N., Das, J., Bhandari, S., Mathur, S.K., Kumar, A., Sahlot, R., Rojarani, P., Lakshmi, J.V., Surekha, A., Sekhar, P.C., Mahajan, S., Masih, S., Singh, P., Kumar, V., Jose, B., Mahajan, V., Gupta, V., Gupta, R., Arumugam, P., Singh, A., Nandy, A., Raghavendran, P.V., Jha, R.M., Kumari, A., Gandotra, S., Rao, V., Faruq, M., Kumar, S., Betsy Reshma, G., Narendra Varma, G., Roy, S.S., Sengupta, A., Chattopadhyay, S., Singhal, K., Pradhan, S., Jha, D., Naushin, S., Wadhwa, S., Tyagi, N., Poojary, M., Scaria, V., Sivasubbu, S., 2020. High throughput detection and genetic epidemiology of SARS-CoV-2 using COVIDSeq next generation sequencing. bioRxiv.

Bińkowska, A., Biernat, M.M., Łaczmański, Ł., Gościniak, G., 2018. Molecular patterns of resistance among Helicobacter pylori strains in south-western Poland. Front. Microbiol. 9, 3154.

Brister, J.R., Ako-Adjei, D., Bao, Y., Blinkova, O., 2015. NCBI viral genomes resource. Nucleic Acids Res. 43, D571–D577.

Broughton, J.P., Deng, X., Yu, G., Fasching, C.L., Servellita, V., Singh, J., Miao, X., Streithorst, J.A., Granados, A., Sotomayor-Gonzalez, A., Zorn, K., Gopez, A., Hsu, E., Gu, W., Miller, S., Pan, C.-Y., Guevara, H., Wadford, D.A., Chen, J.S., Chiu, C.Y., 2020. CRISPR–Cas12-based detection of SARS-CoV-2. Nat. Biotechnol. 38, 870–874.

Chan, J.F.-W., Yip, C.C.-Y., To, K.K.-W., Tang, T.H.-C., Wong, S.C.-Y., Leung, K.-H., Fung, A.Y.-F., Ng, A.C.-K., Zou, Z., Tsoi, H.-W., Choi, G.K.-Y., Tam, A.R., Cheng, V.C.-C., Chan, K.-H., Tsang, O.T.-Y., Yuen, K.-Y., 2020. J. Clin. Microbiol. JCM. 310–320.

Chen, J.S., Dagdas, Y.S., Kleinstiver, B.P., Welch, M.M., Sousa, A.A., Harrington, L.B., Sternberg, S.H., Joung, J.K., Yildiz, A., Doudna, J.A., 2017. Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. Nature 550, 407–410.

Chen, J.S., Ma, E., Harrington, L.B., Da Costa, M., Tian, X., Palefsky, J.M., Doudna, J.A., 2018. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science 360, 436–439.

Chertow, D.S., 2018. Next-generation diagnostics with CRISPR. Science 360, 381. LP – 382.

Csx, P., Smargon, A.A., Cox, D.B.T., Pyzocha, N.K., Makarova, K.S., Koonin, E.V., 2017. Cas13b is a type VI-B CRISPR-associated RNA- differentially regulated by accessory article Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. Mol. Cell 65, 618–630 e7.

Gootenberg, J.S., Abudayyeh, O.O., Kellner, M.J., Joung, J., Collins, J.J., Zhang, F., 2018. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science 360, 439. LP – 444.

Guo, Y.-R., Cao, Q.-D., Hong, Z.-S., Tan, Y.-Y., Chen, S.-D., Jin, H.-J., Tan, K.-S., Wang, D.-Y., Yan, Y., 2020. The origin, transmission and clinical therapies on coronavirus disease 2019 (COVID-19) outbreak – an update on the status. Mil. Med. Res. 7, 1–10.

Hirano, H., Gootenberg, J.S., Horii, T., Abudayyeh, O.O., Kimura, M., Hsu, P.D., Nakane, T., Ishitani, R., Hatada, I., Zhang, F., Nishimasu, H., Nureki, O., 2016. Cell 164, 950–961.

Jiang, H., Wong, W.H., 2008. SeqMap: mapping massive amount of oligonucleotides to the genome. Bioinformatics 24, 2395–2396.

Joung, J., Ladha, A., Saito, M., Segel, M., Bruneau, R., Huang, M.-L.W., Kim, N.-G., Yu, X., Li, J., Walker, B.D., Greninger, A.L., Jerome, K.R., Gootenberg, J.S., Abudayyeh, O.O., Zhang, F., 2020. Point-of-care testing for COVID-19 using SHERLOCK diagnostics. medRxiv Prepr. Serv. Heal. Sci.

Kellner, M.J., Koob, J.G., Gootenberg, J.S., Abudayyeh, O.O., Zhang, F., 2019. SHERLOCK: nucleic acid detection with CRISPR nucleases. Nat. Protoc. 14, 2986–3012.

Kim, D., Lee, J.-Y., Yang, J.-S., Kim, J.W., Kim, V.N., Chang, H., 2020. The architecture of SARS-CoV-2 transcriptome. Cell 181, 914–921 e10.

Landrum, M.J., Lee, J.M., Riley, G.R., Jang, W., Rubinstein, W.S., Church, D.M., Maglott, D.R., 2014. ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Res. 42, D980–D985.

Lewnard, J.A., Lo, N.C., 2020. Scientific and ethical basis for social-distancing interventions against COVID-19. Lancet Infect. Dis. 20, 631–633.

Li, H., Durbin, R., 2010. Fast and accurate long-read alignment with Burrows–Wheeler transform. Bioinformatics 26, 589–595.

Li, L., Li, S., Wu, N., Wu, J., Wang, G., Zhao, G., Wang, J., 2019. HOLMESv2: a CRISPR-cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. ACS Synth. Biol. 8, 2228–2237.

Li, S.Y., Cheng, Q.X., Liu, J.K., Nie, X.Q., Zhao, G.P., Wang, J., 2018. CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA. Cell Res. 28, 491–493.

Li, Y., Li, S., Wang, J., Liu, G., 2019. CRISPR/Cas systems towards next-generation biosensing. Trends Biotechnol. 37, 730–743.

Lobato, I.M., O'Sullivan, C.K., 2018. Recombinase polymerase amplification: basics, applications and recent advances. Trends Anal. Chem. 98, 19–35.

Mamotte, C.D.S., 2006. Genotyping of single nucleotide substitutions. Clin. Biochem. Rev. 27, 63–75.

Myers, F.B., Henrikson, R.H., Bone, J.M., Lee, L.P., 2013. A handheld point-of-care genomic diagnostic system. PloS One 8, e70266.

Pachetti, M., Marini, B., Benedetti, F., Giudici, F., Mauro, E., Storici, P., Masciovecchio, C., Angeletti, S., Ciccozzi, M., Gallo, R.C., Zella, D., Ippodrino, R., 2020. Emerging SARS-CoV-2 mutation hot spots include a novel RNA-dependent-RNA polymerase variant. J. Transl. Med. 18, 179.

Pardee, K., Green, A.A., Takahashi, M.K., Braff, D., Lambert, G., Lee, J.W., Ferrante, T., Ma, D., Donghia, N., Fan, M., Daringer, N.M., Bosch, I., Dudley, D.M., O'Connor, D. H., Gehrke, L., Collins, J.J., 2016. Rapid, low-cost detection of zika virus using programmable biomolecular components. Cell 165, 1255–1266.

Patchsung, M., Jantarug, K., Pattama, A., Aphicho, K., Suraritdechachai, S., Meesawat, P., Sappakhaw, K., Leelahakorn, N., Ruenkam, T., Wongsatit, T., Athipanyasilp, N., Eiamthong, B., Lakkanasirorat, B., Phoodokmai, T., Niljianskul, N., Pakotiprapha, D., Chanarat, S., Homchan, A., Tinikul, R., Kamutira, P., Phiwkaow, K., Soithongcharoen, S., Kantiwiriyawanitch, C., Pongsupasa, V., Trisrivirat, D., Jaroensuk, J., Wongnate, T., Maenpuen, S., Chaiyen, P., Kamnerdnakta, S., Swangsri, J., Chuthapisith, S., Sirivatanauksorn, Y., Chaimayo, C., Sutthent, R., Kantakamalakul, W., Joung, J., Ladha, A., Jin, X., Gootenberg, J.S., Abudayyeh, O.O., Zhang, F., Horthongkham, N., Uttamapinant, C., 2020. Clinical validation of a Cas13-based assay for the detection of SARS-CoV-2 RNA. Nat. Biomed. Eng.

Perna, S., Riva, A., Nicosanti, G., Carrai, M., Barale, R., Vigo, B., Allegrini, P., Rondanelli, M., 2018. Association of the bitter taste receptor gene TAS2R38 (polymorphism RS713598) with sensory responsiveness, food preferences, biochemical parameters and body-composition markers. A cross-sectional study in Italy. Int. J. Food Sci. Nutr. 69, 245–252.

Petherick, A., 2019. World report developing antibody tests for SARS-CoV-2. Lancet 395, 1101–1102.

Piel, F.B., Steinberg, M.H., Rees, D.C., 2017. Sickle cell disease. N. Engl. J. Med. 376, 1561–1573.

Qiu, X.Y., Zhu, L.Y., Zhu, C.S., Ma, J.X., Hou, T., Wu, X.M., Xie, S.S., Min, L., Tan, D.A., Zhang, D.Y., Zhu, L., 2018. Highly effective and low-cost MicroRNA detection with CRISPR-cas9. ACS Synth. Biol. 7, 807–813.

Quan, J., Langelier, C., Kuchta, A., Batson, J., Teyssier, N., Lyden, A., Caldera, S., McGeever, A., Dimitrov, B., King, R., Wilheim, J., Murphy, M., Ares, L.P., Travisano, K.A., Sit, R., Amato, R., Mumbengegwi, D.R., Smith, J.L., Bennett, A., Gosling, R., Mourani, P.M., Calfee, C.S., Neff, N.F., Chow, E.D., Kim, P.S., Greenhouse, B., DeRisi, J.L., Crawford, E.D., 2019. FLASH: a next-generation CRISPR diagnostic for multiplexed detection of antimicrobial resistance sequences. Nucleic Acids Res. 47, e83.

Quinlan, A.R., Hall, I.M., 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26, 841–842.

Ribeiro, M.L., Vitiello, L., Miranda, M.C.B., Benvengo, Y.H.B., Godoy, A.P.O., Mendonca, S., Jr, J.P., 2003. Mutations in the 23S rRNA gene are associated with clarithromycin resistance in Helicobacter pylori isolates in. Brazil 4, 1–4.

Sashital, D.G., 2018. Pathogen detection in the CRISPR-Cas era. Genome Med. 10, 1–4.

Shu, Y., McCauley, J., 2017. GISAID: global initiative on sharing all influenza data@_ from vision to reality. Euro Surveill. 22.

Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., Thiel, V., Ziebuhr, J., Poon, L.L.M., Guan, Y., Rozanov, M., Spaan, W.J.M., Gorbalenya, A.E., 2003. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J. Mol. Biol.

Teng, F., Guo, L., Cui, T., Wang, X.G., Xu, K., Gao, Q., Zhou, Q., Li, W., 2019. CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity. Genome Biol. 20, 1–7.

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H., Pei, Y.-Y., Yuan, M.-L., Zhang, Y.-L., Dai, F.-H., Liu, Y., Wang, Q.-M., Zheng, J.-J., Xu, L., Holmes, E.C., Zhang, Y.-Z., 2020. A new coronavirus associated with human respiratory disease in China. Nature 579, 265–269.

Xu, S., Li, Y., 2020. Beware of the second wave of COVID-19. Lancet (London, England) 395, 1321–1322.

Yan, W.X., Chong, S., Zhang, H., Makarova, K.S., Koonin, E.V., Cheng, D.R., Scott, D.A., Yan, W.X., Chong, S., Zhang, H., Makarova, K.S., Koonin, E.V., Cheng, D.R., 2018. Cas13d is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain- article Cas13d is a compact. RNA-Targeting Type VI CRISPR Effector Positively Modulated by a WYL-Domain-Containing Accessory Protein 327–339.

Zhao, Y., Chen, F., Li, Q., Wang, L., Fan, C., 2015. Isothermal amplification of nucleic acids. Chem. Rev. 115, 12491–12545.