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(54) Title: CAS13-BASED COMPOSITIONS AND METHODS OF USE THEREOF

(57) Abstract:

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CAS13-BASED COMPOSITIONS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of and priority to U.S.S.N.
63/194,099, filed May 27, 2021, and PCT/IB2021/054664, filed May 27,
2021, which claims the benefit of U.S. Provisional Application No.
63/030,796 filed May 27, 2020, each of which is hereby incorporated by
reference herein in its entirety.

FIELD OF THE INVENTION

10 The field of the invention is generally related compositions and
methods for targeting and editing nucleic acids, and in particular, CRISPR-
Cas13 based compositions and methods of use thereof.

BACKGROUND OF THE INVENTION

15 The rapid spread of SARS-CoV-2 poses significant challenges to the
health care systems and represents a great burden on economies around the
world. The causal agent of COVID-19, *Severe acute respiratory syndrome
coronavirus 2019* (SARS-CoV-2), belongs to the Coronaviridae family that
represents positive-stranded RNA viruses with some of the largest known
20 RNA genomes [1]. Members of this family pose a continuous threat to the
global community due to their propensity for causing disease outbreaks as
witnessed for the third time in the 21st century. Rapid mutation in their
genomic RNA along with frequent recombination events facilitates inter-
species transmission that often leads to unexpected and uncontrollable
25 epidemic outbreaks. Therefore, early detection and identification of viral
transmission is necessary for implementation of control measures that ensure
low transmission rates and prevent the loss of human life during present and
future pandemics.

30 The advancements in synthetic biology in synergy with COVID-19
pandemic spurred a wave of innovations in the field of molecular diagnostics
[2]. Point-of-care (POC) or at-home testing kits capable of detecting the
presence of a pathogen's nucleic acid or infectious markers at low cost
present a particularly desirable end goal. In order to be viable for POC

application, any developed technology must meet the ASSURED criteria (Accurate, Specific, Sensitive, User-friendly, Rapid, Equipment-free, and Deliverable to end-users) as defined by the WHO for effective POC test to control and manage infectious diseases [3]. Therefore, significant efforts are

5 directed toward developing an easy to use, point-of-care module that would enable large scale, high turnover SARS-CoV-2 screening on a massive scale. Promising approaches towards this goal involve CRISPR/Cas type V and VI systems [4, 5]. These modalities paved the way for developing the next

10 generation of diagnostic platforms that exploit highly specific target recognition and cleavage by Cas enzymes followed by *in trans* cleavage of reporters. For example, RT-LAMP and RT-RPA were coupled with CRISPR Cas9, Cas12, and Cas13 enzymes for sensitive and specific detection of nucleic acids and viruses, including SARS-CoV2 [32-38]. The Cas12 and Cas13 enzymes complex with their corresponding single-guide RNA

15 (sgRNA) and scan a DNA or RNA template for a complementary sequence. Once a complementary sequence is recognized, the enzyme cleaves the nucleic acid in cis and, once activated by the initial recognition, exhibits collateral trans cleavage activities, cleaving single-stranded (ss) DNA or RNA molecules present in the reaction [39, 40]. This collateral cleavage

20 activity of Cas12 and Cas13 has been harnessed for nucleic acid detection. For example, the CRISPR-Cas13 system coupled with RPA was used for virus detection via SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) [41-43]. To bypass the need for nucleic acid purification, HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases) was developed and coupled with SHERLOCK [44].

25 When coupled to isothermal amplification methods such as recombinase polymerase amplification (RPA) or loop-mediated isothermal amplification (LAMP), CRISPR/Cas detection assays facilitate target nucleic acid detection at attomolar levels [6, 7].

30 There is a need to identify and characterize new Cas effectors to increase utility and develop new tools for *in vivo* and *in vitro* applications. In particular, there is a need to identify and characterize the known class II/type

VI Cas proteins that exclusively target ssRNA substrates to expand the Cas13-based toolbox for diagnostic and other applications.

Thus, it is an object of the invention to provide Cas13-based compositions and methods of use thereof.

5

SUMMARY OF THE INVENTION

Compositions of class II, type VI CRISPR/Cas effector proteins and methods of preparation and use thereof are disclosed. The compositions and methods are especially applicable to rapid and facile detection of nucleic acids, perturbation of gene expression and RNA modification.

10

In particular, a polynucleotide containing a nucleotide sequence encoding a class II, type VI CRISPR/Cas effector protein (Cas13) and optionally including a heterologous sequence is disclosed. In some embodiments, the Cas effector protein includes the amino acid sequence encoded by SEQ ID NO:63, SEQ ID NO:65, or SEQ ID NO:67 (i.e., SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68), or a sequence with at least 70% sequence identity thereto. In some embodiments, the sequence encoding the Cas effector protein is or includes SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67, or a sequence with at least 70% sequence identity to SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67. In some embodiments, the nucleotide sequence does not include SEQ ID NO:63, 65 or 67. In some embodiments, the nucleotide sequence does not include SEQ ID NO:63, 65 or 67 even when it encodes the same protein encoded by SEQ ID NO:63, 65 or 67. In some embodiments, the sequence encoding the Cas effector protein is codon optimized for expression in a prokaryotic or eukaryotic cell.

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The heterologous sequence can be, for example, an expression control sequence, and can include a promoter, transcription terminator, multiple cloning site, drug resistance marker(s), one or more protease recognition sites, one or more epitope tags, or a combination thereof. Typically, the heterologous sequence is operably linked to the sequence encoding the Cas effector protein.

25

In some embodiments, the polynucleotide further includes a sequence encoding a crRNA. In preferred embodiments, the crRNA is capable of complexing with the Cas effector protein and hybridizing to a target RNA

sequence. In some embodiments, the Cas effector protein can originate or be derived from *Thermoclostridium caenicola* or a Proteobacteria bacterium.

In some embodiments, the polynucleotide is or is contained in a vector, such as an expression vector. Non-limiting examples of suitable
5 vectors include viral vectors or plasmids.

Cells harboring the polynucleotides and/or vectors are also provided. For example, prokaryotic or eukaryotic cells containing the polynucleotide and/or vector are disclosed.

Also provided are methods of producing a class II, type VI
10 CRISPR/Cas effector protein. In some embodiments, the method involves introducing the disclosed vector into a prokaryotic or eukaryotic cell under conditions suitable for expression of the sequence encoding the Cas effector protein. In some embodiments, the method further involves isolating and/or purifying the Cas effector protein.

15 Isolated class II, type VI CRISPR/Cas effector proteins produced by the foregoing method are also provided. In some embodiments, disclosed is an isolated class II, type VI CRISPR/Cas effector protein having the amino acid sequence of SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68, or a sequence with at least 70% sequence identity to SEQ ID NO:64, SEQ ID
20 NO:66, or SEQ ID NO:68.

In some embodiments, the Cas effector protein is associated with a crRNA. Thus, ribonucleoprotein complexes containing the Cas effector protein complexed with a crRNA, optionally wherein the crRNA is capable of hybridizing to a target RNA sequence are disclosed.

25 Also provided are compositions including a Cas13 protein having or including the amino acid sequence of SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68, or a sequence with at least 70% sequence identity to SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68. In preferred embodiments, the Cas13 protein contains one or more Higher Eukaryotes and Prokaryotes
30 Nucleotide-binding (HEPN) domains, preferably two HEPN domains. An exemplary HEPN domain is a RxxxxH (SEQ ID NO:192) motif sequence, wherein X represents any amino acid.

In some embodiments, the Cas13 protein is complexed with a crRNA, which optionally, but not necessarily, is capable of hybridizing to a target RNA sequence. The crRNA can include a spacer sequence that is capable of hybridizing to the target RNA sequence and a direct repeat
5 sequence.

In some preferred embodiments, the crRNA comprises a spacer of about 20-30 nucleotides, preferably 24-28 nucleotides.

In some embodiments, the Cas13 protein can cleave the target RNA sequence at a temperature of about 37-70 °C, about 50-70 °C, about 47-60
10 °C, or about 60 °C. In some embodiments, the Cas13 protein can cleave the target RNA sequence at a temperature of about 37 °C-42 °C, preferably about 37 °C.

The composition may be present in a cell, such as a prokaryotic or eukaryotic cell.

15 Methods of using the disclosed polynucleotides, vectors, proteins, protein complexes, and compositions are also provided. In particular, disclosed is a method of performing targeted knockdown of an RNA transcript involving introducing the disclosed Cas13 compositions to a cell, wherein the crRNA complexed to the Cas13 hybridizes to the RNA
20 transcript, thereby inducing cleavage of the RNA transcript by the Cas13 protein. The RNA transcript can be any ssRNA sequence including coding (e.g., an mRNA) or non-coding (e.g., lincRNA) RNA. In some embodiments, the RNA transcript is derived from a viral gene, such as a bacteriophage. Thus, the compositions can be used in methods of inhibiting viral gene
25 expression or viral replication.

Methods of using the compositions to detect target nucleic acids are also provided. For example, disclosed is a method of detecting the presence of an RNA transcript in a nucleic acid sample by contacting the sample with the Cas13 composition in the presence of an activatable single stranded RNA
30 (ssRNA) oligonucleotide that includes a reporter moiety. In some embodiments, the reporter moiety includes a fluorophore linked to a quencher via the ssRNA. Fluorescence can be emitted upon cleavage of the ssRNA oligonucleotide since the quencher is no longer in proximity to the

fluorophore. Preferably, the crRNA is designed to hybridize to the RNA transcript and the Cas13 cleaves the ssRNA oligonucleotide upon binding of the Cas13 crRNA complex to the RNA transcript. Detecting cleavage of the ssRNA oligonucleotide indicates the presence of the RNA transcript.

- 5 The RNA transcript could have been generated by transcription from a dsDNA molecule, and in some embodiments, the dsDNA molecule could have been generated by reverse transcription coupled (isothermal) amplification of a target RNA.

- Methods of monitoring RNA trafficking are also provided. For
10 example, described herein is a method of determining the localization of an RNA transcript in a cell by introducing the Cas13 composition to the cell. In such embodiments, the Cas13 is preferably catalytically inactive (e.g., the Cas13 functions as an RNA binding protein without nuclease activity). In such embodiments, the Cas13 preferably further includes a detectable
15 marker. The crRNA is designed to hybridize to the RNA transcript, and the Cas13 crRNA complex binds to the RNA transcript, thereby indicating the location of the RNA transcript. Exemplary detectable markers include GFP, eGFP, RFP, YFP, BFP, CFP, mNeonGreen, mCherry, mOrange, mRaspberry, mPlum, mKO, mRFP, mRFPPruby, mRuby, tagRFP, mKate2,
20 and DsRed.

- Also disclosed is a method for performing targeted editing of an RNA transcript by introducing a composition of a catalytically inactive Cas13 that further includes a deaminase domain of an RNA-dependent Adenosine Deaminase (ADAR) to a cell. The crRNA complexed to the
25 Cas13 is capable of hybridizing with a region in the RNA transcript that contains an A nucleotide to be edited. The crRNA forms an RNA duplex with the RNA transcript that contains an A-C mismatch at the target A nucleotide resulting in the target A nucleotide being deaminated by the deaminase domain (resulting in A-I).

- 30 Suitable deaminase domains include deaminase domains from ADAR1, ADAR2, and ADAR3, such as human ADAR1, ADAR2, and ADAR3.

 Additional advantages of the disclosed methods and compositions will be set forth in part in the description which follows, and in part will be

understood from the description, or can be learned by practice of the disclosed methods and compositions. The advantages of the disclosed method and compositions will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

- 5 It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

- The accompanying drawings, which are incorporated in and
10 constitute a part of this specification, illustrate several embodiments of the disclosed methods and compositions and together with the description, serve to explain the principles of the disclosed method and compositions.

- Figures 1A-1B** are heatmaps showing the screening and selection of the optimal ssRNA reporter for TccCas13a and HheCas13a *in-trans*
15 activity. Reactions containing Cas13 and two different crRNAs targeting the SARS-CoV-2 *N* gene or non-specific crRNA (NS) control were performed in the presence of one of three FAM-reporters with different RNA sequences. Data are shown as the mean of three replicates. **Figure 1C** is a maximum-likelihood phylogenetic tree of Cas13 proteins from different
20 organisms. The tree was generated using MEGA X software. Most selected proteins were isolated from mesophilic bacteria, although several have been cultivated as thermophiles, and thus offer an interesting collection of high-temperature stable proteins. TccCas13a and HheCas13a were selected as potentially thermophilic Cas13 proteins.
25 **Figure 1D** is a series of plots showing differential scanning fluorimetry (DSF) profiles of protein melting point using a conventional real-time PCR instrument. The peak in the left graph indicates protein denaturation. The right-side graph is the derivative of the left-side graph. **Figure 1E** is a plot showing denaturation temperature of TccCas13a, HheCas13a, and
30 LwaCas13a proteins, as determined by DSF in Fig. 1D. Data are shown as mean $\pm$ standard deviation (SD) ($n = 3$). **Figure 1F** is a heat map showing TccCas13a collateral cleavage preference for the ssRNA reporter. Reactions consisting of TccCas13a and its respective cognate crRNAs

or non-specific crRNA (NS) control were performed in the presence of ssRNA target and one of ten ssRNA reporters. NS: non-specific crRNA. Data are shown as mean ($n = 3$). Reactions were incubated at 56°C and the endpoint fluorescent signal was measured after 30 min. ssRNA reporter sequences are shown on top of the heatmap. A: Poly A reporter, C: Poly C reporter, G: Poly G reporter, U: Poly U reporter, UA: LwaCas13a reporter, AC: 3(AC) reporter, AG: 3(AG) reporter, UG: 3(UG) reporter, CG: 3(CG) reporter, Mix: Mix reporter (UGACGU). Figure 1G is a heatmap showing HheCas13a collateral cleavage preference for the ssRNA reporter. Reactions consisting of HheCas13a and its respective cognate crRNAs or non-specific crRNA (NS) control were performed in the presence of ssRNA target and one of six ssRNA reporters. NS: non-specific crRNA. Data are shown as mean ($n = 3$). Reactions were incubated at 56 °C and endpoint fluorescence signal detection was measured after 30 min. ssRNA reporter sequences are shown on top of the panel, A: Poly A reporter, U: Poly U reporter, G: Poly 6G reporter, UG: 3(UG) reporter, CG: 3(CG) reporter, Mix: Mix reporter.

Figures 2A-2B are graphs showing the *in-trans* activity of HheCas13a and TccCas13a assessed at varying target concentrations and two different temperatures. **Figure 2C** is a heatmap depicting the *in-trans* activity of HheCas13a and TccCas13a assessed at different temperatures with two crRNAs targeting the *N* SARS-CoV-2 gene and one non-specific (NS) crRNA. Reactions were run for 30 mins. Data are shown as mean with $n = 3$. **Figure 2D** is a bar graph showing the *in-trans* activity of TccCas13a assessed at 60 °C with two crRNAs of different spacer lengths targeting the *N* SARS-CoV-2 gene and one non-specific (NS) crRNA. Reactions were run for 30 mins. Values shown as mean $\pm$ SD ($n = 3$). **Figure 2E** is a plot showing end-point activity of LwaCas13a, HheCas13a and TccCas13a at different temperatures using their preferred reporter. One crRNA and a non-specific crRNA were tested for each. Values are shown as mean $\pm$ S.D and represent endpoint fluorescence at 30 min. **Figure 2F** is predicted secondary RNA structure of the direct repeat sequence of TccCas13a crRNA. RNAfold ran.tbi.univie.ac.at/ was used to predict the crRNA secondary structure.

Figures 3A-3D are bar graphs showing the results of the screening of the indicated HheCas13a and TccCas13a crRNAs paired with various primers in the two-pot assay. crRNAs designed to target the RNA substrate produced from T7-mediated *in vitro* transcription of the RT-LAMP amplification products using STOPCovid (SC) primer sets with T7 promoter appended to the FIP primer (T7-FIP) or to the BIP primer (T7-BIP). RT-LAMP amplification was performed first and the RT-LAMP product was added to the T7-*in vitro* transcription and Cas13-based detection reaction (two-pots). The *in-trans* activity of HheCas13a and TccCas13a were assessed at 56 °C. NS: non-specific crRNA. Reactions were run for 30-60 mins.

Figures 4A-4D are bar graphs showing the results of the screening of the indicated HheCas13a and TccCas13a crRNAs paired with various primers in the one-pot assay. crRNAs designed to target the RNA substrate produced from T7-mediated *in vitro* transcription of the RT-LAMP amplification using STOPCovid (SC) primer sets with T7 promoter appended to the FIP primer (T7-FIP) or to the BIP primer (T7-BIP). RT-LAMP amplification, T7-*in vitro* transcription and Cas13-based detection was performed in single step at single temperature, 55 °C (one-pot). NS: non-specific crRNA. Reactions were run for 30-60 mins. Values shown as mean $\pm$ SD (n = 3).

Figures 5A-5E are graphs showing the results from a second round of screening of the indicated HheCas13a and TccCas13a crRNAs and different primer sets for the establishment of one-pot assay. RT-LAMP amplification, T7-*in vitro* transcription and Cas13-based detection was performed in single step at single temperature, 55 °C (one-pot). NS: non-specific crRNA. Reactions were run for 80 mins. Values shown as mean (n = 3).

Figure 6 is a heatmap showing the *in-trans* activity of the one-pot detection assay with synthetic RNA using different *Bst* DNA polymerases from different vendors. NTC: non-template control. Reactions were run for 80 mins at 55 °C. Values shown as mean (n = 3).

Figures 7A-7D are bar graphs showing the cleavage activity towards the reporter probe in the one-pot assay with different concentrations of Bst DNA polymerase (Fig. 7A), MgSO₄ (Fig. 7B), Hi-T7 RNA polymerase (Fig. 7C), and TccCas13a and crRNA (RNP) concentrations (Fig. 7D). NTC: non-template control. Reactions were run for 80 mins at 55 °C. Values shown as mean $\pm$ SD (n = 3).

Figure 8 is a bar graph quantifying the limit of detection (LoD) of the thermophilic Cas13-based one pot detection assay using synthetic SARS-CoV-2 RNA as an input. Reactions were run for 80 mins at 56 °C. Values shown as mean $\pm$ SD (n = 3).

Figure 9 is a graph quantifying the performance of the thermophilic Cas13-based one-pot detection assay on SARS-CoV-2 patient samples. RNA extracted from clinical samples from 8 patients with SARS-CoV-2 infection (Table 6) were analyzed with the one-pot detection assay. NTC: no template control. Reactions were run for 80 mins at 56 °C.

Figure 10A is a schematic of specific Cas13-based detection. Specific target recognition by Cas13 RNP triggers non-specific, collateral activity that cleaves the reporters, resulting in a detectable fluorescent signal. **Figures 10B-10F** are bar graphs showing cleavage activity across various reporter and crRNA sequences. Reactions containing mCas13 and four different crRNAs targeting the SARS-CoV-2 *N* gene or non-specific crRNA (NS) control were performed in the presence of one of five reporters. F-NNNNN-Q represents the RNaseAlert v2 reporter (Thermofisher). NS: non-specific (not targeting SARS-CoV-2) crRNA. Values shown as mean $\pm$ SD (n = 3). **Figure 10G** is a bar graph quantifying the effect of mismatches between mCas13 crRNA and target RNA on mCas13 activity. Left, crRNA nucleotide sequence with the positions of mismatches (red) on the crRNA. Right, the fluorescence intensity, relative to the no-template control (NTC, gray) or crRNA with no mismatches (purple), resulting from mCas13 collateral cleavage activity on each tested crRNA. **Figure 10H** is a schematic illustration of the sequence similarity searching strategies for the identification of miniature Cas13 effectors. BLASTp analysis was performed before January 2021. **Figure 10I** is a bar graph showing titration of mCas13

and crRNA concentrations for optimal performance. 100 nM of *in vitro* transcribed RNA of SARS-CoV-2 N gene was used as the target sequence in mCas13 reactions. Values represent endpoint fluorescence at 30 mins. Values shown as mean $\pm$ SD (n = 3). **Figure 10J** is a bar graph quantifying mCas13 collateral activity at the indicated temperatures. Collateral activity was measured as end point fluorescence after incubation with 100 nM of *in vitro* transcribed RNA of SARS-CoV-2 N gene for 45 mins. Values shown as mean $\pm$ SD (n = 3). **Figure 10K** shows multiple sequence alignment of mCas13 and compact Cas13e and Cas13f orthologues. Protein sequences of mCas13, Cas13e.1, Cas13e.2, Cas13f.1, Cas13f.2, Cas13f.3, Cas13f.4 and Cas13f.5 were aligned using ClustalW in MEGAX and ESPript was used to generate the alignment visualization. Strictly conserved residues (%Strict) are shown in red text within blue rectangles. Conserved RxxxxH (SEQ ID NO:192) motifs of the HEPN domains are highlighted with yellow background. **Figure 10L** a bar graph showing activity assessment of mCas13 with different crRNA sequences. Reactions containing mCas13 and one of the different crRNAs targeting the SARS-CoV-2 N gene were preferred in the presence of the F-UUAUU-Q reporters. NS: non-specific (not SARS-CoV-2 targeting) crRNA. Values shown as mean $\pm$ SD (n=3). **Figure 10M** is an illustration of the sequence of crRNA 4 and the position of synthetically introduced single mismatches. **Figure 10N** is a graph showing real-time fluorescence measurements of the collateral cleavage activity of mCas13 with different crRNAs with different mismatches relative to no template control (NTC) and crRNA with no mismatch (No Mis). Values shown as mean $\pm$ SD (n=3).

Figure 11A is a schematic of assay workflow of mCas13-based detection. Following sample collection and RNA extraction, pathogen RNA is reverse transcribed and amplified via RT-LAMP isothermal reaction. RT-LAMP primer sets (in which the FIP contains a T7 promoter sequence) are used, resulting in amplicons containing the T7-promoter sequence, which serve as templates for *in vitro* transcription of target RNA. Upon target recognition, mCas13 cleaves specially designed reporters *in trans*, leading to fluorescent signal output. **Figure 11B** is a graph showing measurement of

real time fluorescence output of T7-mediated *in vitro* transcription and mCas13-based detection. Synthetic SARS-CoV-2 RNA was reverse transcribed and LAMP amplified with STOPCovid (SC) and DETECTR (DT) primer sets. crRNA 4: targeting crRNA # 4 identified in Figure 10. NS: non-specific crRNA. Values shown as mean (n = 3) **Figures 11C-11D** are bar graphs showing the limit of detection (LoD) of mCas13-based detection assay. LoD was determined using synthetic SARS-CoV-2 RNA, which was reverse transcribed and LAMP-amplified with STOPCovid and DETECTR primer sets. LAMP product was subsequently used for T7 *in vitro* transcription and concurrent mCas13 detection. Values shown as mean $\pm$ SD (n = 3). **Figure 11E** is a graph showing results of the mCas13-based detection assay across different viruses. Detection of non-specific viral targets including SARS-CoV-1, MERS-CoV, as well as two plant viruses, tobacco mosaic virus (TMV) and turnip mosaic virus (TuMV) was attempted using the STOPCovid primer set. Values shown as mean $\pm$ SD (n = 3).

Figure 12A is a graph showing validation of mCas13-based detection assay on RT-qPCR-validated SARS-CoV-2 clinical samples with different Ct values. Pink bars represent mCas13-based detection fluorescence output. FAM: RNA reporter labeled with FAM fluorophore used in mCas13 detection assays. Blue dots represent *N* gene Ct values. **Figure 12B** is a schematic representation of mCas13-based visual detection with a handheld fluorescence visualizer. **Figures 12C-12D** are images showing visual readouts of the limit of detection (LoD) of mCas13-based detection assay. LoD was determined using synthetic SARS-CoV-2 RNA (Fig. 12C) or RT-qPCR-validated SARS-CoV-2 clinical samples with different Ct values (Fig. 12D), which were reverse transcribed and LAMP-amplified with the STOPCovid primer set. LAMP product was used for T7 *in vitro* transcription and concurrent mCas13 detection. **Figure 12E** is a series of images showing RT-LAMP mCas13 visual detection reactions of 24 SARS-CoV-2 RT-qPCR positive samples and no template control (NTC) reactions used to generate the heatmap in Fig. 12F. Left, reaction tubes with fluorescence readouts are shown with the clinical sample ID. Right, reaction tubes with fluorescence readouts are shown with RT-qPCR Ct values indicated. **Figure 12F** is a heat

map displaying the validation of the mCas13-based visual detection assay of 24 SARS-CoV-2 RT-qPCR positive samples. RFU: random fluorescence units, showing the signal intensity that was obtained by the TECAN plate reader for the mCas13 reactions. **Figure 12G** is an image showing

5 fluorescent reporter (HEX reporter) concentration for clear visual detection of mCas13 collateral activity. mCas13 was incubated with crRNA 4 or non-specific (NS) crRNA and 100 nM of in vitro transcribed RNA of SARS-CoV-2 N gene using different concentrations of RNA reporter molecule with the sequence UUAUU that is conjugated to 5' HEX for 45 mins. Reactions

10 were visualized with p51 fluorescence visualizer and photo was taken with a smart phone. **Figure 12H** is an image showing a different repeat of the experiment done in Figure 3D. Picture was taken with different smart phone used in the other figures. NTC: non-template control. Figures 12I and 12J are images of RT-LAMP mCas13 visual detection reactions of 24 SARS-CoV-2

15 RT-qPCR positive samples and no template control (NTC) reactions used to generate the heatmap in Figure 12F. **Figure 12I** shows reaction tubes with fluorescence readouts are shown with the clinical sample ID. **Figure 12J** reaction tubes with fluorescence readouts are shown with RT-qPCR Ct values indicated.

20 **Figure 13** is schematic illustrating identification of mCas13 and development of its use in a nucleic acid detection assay.

Figures 14A-14D show *In vitro* characterization of thermostable Cas13a crRNAs sequence requirements. Evaluating the effect of single (14A), double (14B), and stretches of 4 mismatches (14C) between crRNA

25 and target RNA on TccCas13a activity. Left: crRNA nucleotide sequence with the positions of mismatches on the crRNA spacer. Right: the fluorescence intensity, relative to the non-specific crRNA control (NS) or crRNA with no mismatches (green), resulting from TccCas13a collateral cleavage activity on each tested crRNA. Reactions were incubated

30 at 56°C and the endpoint fluorescent signal was measured after 30 min.

Figure 14D shows TccCas13a RNA detection activity with different crRNA spacer lengths. Left: crRNA spacer nucleotide sequences with the length of each spacer shown on the left of the sequence. Right: the

fluorescence intensity, relative to the non-specific crRNA control (NS) resulting from TccCas13a collateral cleavage activity on each tested crRNA. Reactions were incubated at 56°C and the endpoint fluorescent signal was measured after 30 min. **Figure 14E** is a plot showing

5 representative Michaelis-Menten plot for TccCas13a-catalyzed ssRNA *trans* cleavage activity. Enzyme kinetic data and the measured K_{cat} , K_m , and K_{cat}/K_m are shown on the top of plot. Values are shown as mean $\pm$ S.D ($n = 3$). **Figure 14F-14I** are plots showing: 14F) single mismatches; 14G) double mismatches; 14H) 4 mismatches; 14I) crRNA spacer truncations. Left: crRNA

10 nucleotide sequence with the positions of mismatches on the crRNA spacer. Right: the fluorescence intensity, relative to the non-specific crRNA control (NS) or crRNA with no mismatches, resulting from HheCas13a collateral cleavage activity on each tested crRNA. Reactions were incubated at 56 °C and endpoint fluorescence signal detection was measured after 30 min.

15 Values are shown as mean $\pm$ S.D.

Figures 15A-15D illustrate exemplary one-pot SARS-CoV-2 detection using the thermophilic TccCas13a protein. **Figure 15A** is a schematic representation of the SARS-CoV-2 genome showing the region targeted by RT- LAMP amplification and the crRNA target sequence. The

20 small arrow on the T7-FIP primer indicates the T7 promoter sequence. SC region: genomic region of SARS-CoV-2 *N* gene targeted with STOPCovid primers. **Figure 15B** is an overview of an exemplary assay workflow. The detection protocol includes three distinct steps, all carried out in the same tube and at the same temperature (56°C). Following the extraction of

25 viral RNA, specific target sequences within the viral RNA are reverse-transcribed (RT) into cDNA and amplified with RT-LAMP isothermal amplification using LAMP primers containing the T7 promoter sequence (small red arrow on the T7-FIP primer). The resulting RT-LAMP amplicons are used for *in vitro* transcription using the thermostable Hi-

30 T7 RNA polymerase, producing RNA transcripts that are recognized and targeted simultaneously by the thermophilic TccCas13a protein. Recognition of the RNA transcripts by TccCas13a triggers Cas13 collateral cleavage activity, resulting in *trans*-cleavage of the reporter probe

conjugated to the HEX or FAM fluorophores. **Figure 15C and 15D** are plots showing performance of one-pot detection assay at different temperatures: as determined by real-time fluorescence at a given target RNA concentration (100 cp/μL), data are shown as mean ($n = 3$)

5 (15C); and endpoint fluorescent signal measured after 30 min, values are shown as mean $\pm$ S.D. (15D) The best performance was achieved at 56°C.

Figures 16A-16D illustrate evaluation of OPTIMA-dx for the detection of SARS-CoV-2. **Figure 16A** a schematic representation of
 10 SARS-CoV-2 RNA detection in one-pot assays and visual detection using the P51 Molecular Fluorescence Viewer. As the test is performed in a single pot, there is no need to open the reaction tube so it can be discarded without opening, thus avoiding the possibility of contamination at the point of care site. **Figure 16B** is a series of images showing assessment of the
 15 sensitivity of OPTIMA-dx and the effect of reaction incubation time on performance using fluorescence-based visual detection. Fluorescence rises above background after 45 min with little improvement as time increases. Three replicates were performed for each treatment. **Figure 16C** is a plot showing SARS-CoV-2 detection from 100 clinical COVID-19 samples
 20 with one-pot RT-LAMP- TccCas13a detection assay. RT-qPCR cycle threshold (C_T) plotted against fluorescent readout from the detection of SARS-CoV-2 positive samples ($n=73$) and SARS-CoV-2 negative samples ($n=27$). Detection reactions were incubated at 56°C and the endpoint fluorescent signal was measured after 1 hour. Light blue data points in
 25 negative samples represent no template controls (NTC). **Figure 16D** is a plot showing detection of RNase P internal control with one-pot RT-LAMP- TccCas13a detection assay. All 100 clinical samples in Fig. 16A were tested for the detection of RNase P gene. Detection reactions were incubated at 56°C and the endpoint fluorescent signal was measured after 1 hour. NTC:
 30 no template control.

Figures 17A-17D illustrate multiplexed OPTIMA-dx detection with TccCas13a and AapCas12b thermostable Cas enzymes. **Figure 17A** is a schematic representation of one-pot multiplexed OPTIMA-dx detection

reaction. The unique collateral activity of Cas12 and Cas13 orthologues enables the use of different reporter molecules with different fluorophore, RNA reporter with FAM fluorophore for Cas13 and DNA reporter with HEX fluorophore for Cas12, and multi-channel detection.

- 5 **Figure 17B** is a plot showing the performance of multiplexed detection of SARS-CoV-2 (at 400 cp/μL) and isolated human RNA (for RNase P detection) as measured by real-time fluorescence. Data are shown as means $\pm$ SD ($n = 3$). **Figure 17C** is a bar graph showing multiplexed detection of SARS-CoV-2 and the human internal control (RNase P) in RNA
- 10 extracted from 14 clinical COVID-19 samples. Detection reactions were incubated at 56°C and the endpoint fluorescent signal was measured with FAM and HEX channels after 1 hour. SARS- CoV-2: synthetic SARS-CoV-2 RNA used at 400 cp/μL, RNase P: isolated total human RNA. NTC: no template control. **Figure 17D** is a heat map showing multiplexed detection
- 15 of SARS-CoV-2 and the human internal control (RNase P) from 16 clinical oropharyngeal swabs processed with the quick extraction method. Detection reactions were incubated at 56°C and the endpoint fluorescent signal was measured with FAM and HEX channels after 1 hour. -Ve: SARS-CoV-2 negative samples as determined with RT-qPCR. NTC no
- 20 template control.

- Figures 18A-18D** are bar graphs showing HheCas13a and TccCas13a crRNA screening in two-pot detection reaction. Trans cleavage activity of HheCas13a and TccCas13a using different crRNAs when incubated with RT-LAMP product of amplified SARS-CoV-2 genomic standards. Assay
- 25 was performed as described in material and methods section. Endpoint fluorescence signal detection was measured after 1 hour. NS: non-specific crRNA. T7-FIP: RT-LAMP primers with modified FIP primer carrying T7 promoter sequence. T7-BIP: RT-LAMP primers with modified BIP primer carrying T7 promoter sequence. The location of the targeted sequence of
- 30 each crRNA (orange) relative to the RT-LAMP primers (F3, FIP-T7, BIP-T7, B3) is depicted on top of each graph. FIP/BIP-T7: primers containing T7 promoter sequence.

Figure 19 is a heat map showing establishment of one-pot SARS-CoV-2 detection using the thermophilic Cas13 proteins. Trans cleavage activity of HheCas13a and TccCas13a using different crRNAs in one-pot reactions using SARS-CoV-2 genomic standards as input. The assay was performed as described in material and methods. End-point fluorescence signal detection was carried out after 80 min. NTC: No template control. T7-FIP: modified RT-LAMP FIP primer with T7 promoter sequence. T7-BIP: modified RT-LAMP BIP primer with T7 promoter sequence. Data are shown as mean (n = 3).

Figures 20A-20D are plots showing establishment of one-pot SARS-CoV-2 detection using the thermophilic Cas13 proteins. Trans cleavage activity of HheCas13a and TccCas13a using different crRNAs in one-pot reactions using SARS-CoV-2 genomic standards as input. The assay was performed as described in material and methods. End-point fluorescence signal detection was carried out after 80 min. NTC: No template control. T7-FIP: modified RT-LAMP FIP primer with T7 promoter sequence. T7-BIP: modified RT-LAMP BIP primer with T7 promoter sequence. Data are shown as mean (n = 3).

Figure 21A is a plot showing activity screening of three different AapCas12b sgRNAs in One-pot RT-LAMP Cas12b detection of RNase P template as measured by real-time fluorescence signal produced from HEX reporter cleavage with AapCas12b collateral activity. Data are shown as means $\pm$ SD (n = 3). RNase P: total human RNA. NTC: No template control.

Figure 21B is a heat map showing analysis of the activity of Cas12 and Cas13 with different reporter molecules and different targets for one-pot OPTIMA-dx multiplex detection. Endpoint fluorescent signal measured after 60 min, values are shown as mean (n=3).

Figures 22A-22C illustrate the adaptability of OPTIMA-dx for specific detection of different pathogens. **Figure 22A** is a bar graph showing detection of major HCV genotypes with OPTIMA-dx. In vitro transcribed RNA was used as RNA template in the OPTIMA-dx detection reactions at concentrations of 500 pM. Values are shown as mean $\pm$ S.D. and represent endpoint fluorescence at 60 min. **Figure 22B** is a bar graph showing

detection of TYLCV DNA virus with OPTIMA-dx. DNA isolated from two different TYLCV infected plants and one healthy (not infected) plant was diluted 1:10 or 1:100 in water and used as template in the OPTIMA-dx detection reactions. A plasmid containing TYLCV genome was used as a control at concentrations of 1 ng/reaction. NTC: no template control. Values are shown as mean $\pm$ S.D and represent endpoint fluorescence at 60 min. **Figure 22C** is a plot showing performance of multiplexed detection of HCV at concentrations of 500 pM and isolated human RNA (for RNaseP detection) as measured by real-time fluorescence. Data are shown as means $\pm$ SD (n = 3).

DETAILED DESCRIPTION OF THE INVENTION

The disclosed methods and compositions can be understood more readily by reference to the following detailed description of particular embodiments and the Examples included therein and to the Figures and their previous and following description.

Robust, sensitive and specific diagnostic platforms for early SARS-CoV-2 detection are important to facilitate early identification of afflicted individuals, thus supporting the efforts to curb the ongoing COVID-19 pandemic. CRISPR systems have been repurposed for biosensing applications including virus detection. Different two-pot modalities have been developed for SARS-CoV-2 detection including Cas13. Despite many existing diagnostic strategies involving CRISPR/Cas, robust one-pot detection assays that involves minimal equipment and handling steps are still elusive. The working Examples demonstrate identification and characterization of a thermophilic Cas13a enzyme and show its trans and collateral activities at temperatures between 60-70 °C. The thermostability features of this enzyme was harnessed to build an assay for sensitive and specific detection of SARS-CoV2. The one-pot, isothermal assay couples RT-LAMP, *in vitro* transcription by thermostable Hi-T7 RNA polymerase, and specific amplicon recognition by thermostable Cas13a effector. This method facilitates handling by eliminating the need for separating amplification and detection steps utilizing only minimal equipment, thereby

allowing practical POC application. The assay can detect down to 20 copies per microliter of synthetic SARS-CoV-2 genomic RNA. Upon evaluating 8 clinical samples from COVID-19 patients, the assay shows 100% agreement with RT-qPCR. Hence, RT-LAMP CRISPR/Cas13a assay is a practical,
5 sensitive and robust platform with POC capacity for SARS-CoV2 detection, and for pathogen detection in general.

The working Examples also describe identification of a miniature Cas13 (mCas13) variant and characterization of its catalytic activity. The mCas13 was employed to design, build, and test a SARS-CoV-2 detection
10 module coupling reverse transcription loop-mediated isothermal amplification (RT-LAMP) with the mCas13 system to detect SARS-CoV-2 in synthetic and clinical samples. The mCas13 system exhibited sensitivity and specificity comparable to other CRISPR systems.

Also provided are thermostable orthologues of the Cas13a family
15 from the thermophilic organism *Thermoclostridium caenicola* (TccCas13a) and *Herbinix hemicellulosilytica* (HheCas13a). These Cas13 proteins share several properties such as thermostability and inability to process its own pre-crRNA. Use of the proteins in various capacities, including SARS-CoV-2 diagnostic assays, are exemplified.

20 This work expands the repertoire and application of Cas13 enzymes in diagnostics and for potential *in vivo* applications, including RNA knockdown and editing. The mCas13, TccCas13a, and HheCas13a systems can be adapted and used in large-scale testing for diverse pathogens, including RNA and DNA viruses, and bacteria.

25 I. Definitions

“Introduce” in the context of genome modification refers to bringing into contact. For example, to introduce a vector (e.g., encoding an Cas13 effector) to a cell is to provide contact between the cell and the composition. The term encompasses penetration of the contacted composition to the
30 interior of the cell by any suitable means, e.g., via transfection, electroporation, transduction, gene gun, nanoparticle delivery, etc.

The term “operably linked” or “operationally linked” refers to functional linkage between a regulatory sequence and a heterologous nucleic

acid sequence permitting them to function in their intended manner (e.g., resulting in expression of the latter). The term encompasses positioning of a regulatory region and a sequence to be transcribed in a nucleic acid so as to influence transcription or translation of such a sequence. For example, to
5 bring a coding sequence under the control of a promoter, the translation initiation site of the translational reading frame of the polypeptide is typically positioned between one and about fifty nucleotides downstream of the promoter. A promoter can, however, be positioned as much as about 5,000 nucleotides upstream of the translation initiation site or about 2,000
10 nucleotides upstream of the transcription start site. A promoter typically comprises at least a core (basal) promoter.

The term “expression” encompasses the transcription and/or translation of a particular nucleotide sequence driven by a promoter. “Expression vector” or “expression cassette” refers to a vector containing a
15 recombinant polynucleotide having expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector contains sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as
20 cosmids, plasmids (e.g., naked or contained in liposomes), phagemids, BACs, YACs, and viral vectors (e.g., vectors derived from lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

As used here, the term “heterologous” refers to elements occurring
25 where they are not normally found. For example, a promoter may be linked to a heterologous nucleic acid sequence, e.g., a sequence that is not normally found operably linked to the promoter. When used herein to describe a promoter element, heterologous means a promoter element that differs from that normally found in the native promoter, either in sequence, species, or
30 number. For example, a heterologous control element in a promoter sequence may be a control/ regulatory element of a different promoter added to enhance promoter control, or an additional control element of the same

promoter. The term “heterologous” thus can also encompass “exogenous” and “non-native” elements.

As used herein, the term “polynucleotide” generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified
5 RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be
10 single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. The term “nucleic acid” or “nucleic acid sequence” also encompasses a polynucleotide as defined above.

A “mutation” refers to a change in a nucleotide (e.g., DNA) or amino acid sequence resulting in an alteration from a given reference sequence. The
15 mutation can be a deletion, insertion, duplication, and/or substitution of at least one deoxyribonucleic acid base such as a purine (adenine and/or guanine) and/or a pyrimidine (thymine, uracil and/or cytosine). Mutations may or may not produce discernible changes in the observable characteristics (phenotype) of an subject.

20 “Encoding” or “encode” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (e.g., rRNA, tRNA and mRNA) or a defined sequence of amino
25 acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence, and the non-coding strand, used as the template for transcription of a gene or
30 cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is

not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell. An

5 “isolated nucleic acid” refers to a nucleic acid segment or fragment which has been separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been

10 substantially purified from other components which naturally accompany the nucleic acid (e.g., RNA or DNA or proteins, which naturally accompany it in the cell). The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or

15 which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences.

In the context of cells, the term “isolated” refers to a cell altered or removed from its natural state. An isolated cell is thus in an environment

20 different from that in which the cell naturally occurs, e.g., separated from its natural milieu such as by concentrating to a concentration at which it is not found in nature. “Isolated cell” is meant to include cells that are within samples that are substantially enriched for the cell of interest and/or in which the cell of interest is partially or substantially purified.

25 The terms “transformed,” “transduced,” and “transfected” encompass the introduction of a nucleic acid or other material into a cell by one of a number of techniques known in the art.

A “vector” is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the

30 interior of a cell. Examples of vectors include but are not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term is also construed to

include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral
5 vectors, and the like.

The term “percent (%) sequence identity” describes the percentage of nucleotides or amino acids in a candidate sequence that are identical with the nucleotides or amino acids in a reference nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the
10 maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Appropriate parameters for measuring alignment, including any
15 algorithms needed to achieve maximal alignment over the full-length of the sequences being compared can be determined by known methods.

“Identity” can be readily calculated by known methods, including, but not limited to, those described in Computational Molecular Biology, *Lesk, A. M.*, Ed., Oxford University Press, New York, 1988; Biocomputing:
20 Informatics and Genome Projects, *Smith, D. W.*, Ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, *Griffin, A. M., and Griffin, H. G.*, Eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, *von Heinje, G.*, Academic Press, 1987; and Sequence Analysis Primer, *Gribskov, M. and Devereux, J.*, Eds., M Stockton Press,
25 New York, 1991; and *Carillo, H., and Lipman, D.*, SIAM J Applied Math., 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. The percent identity between two sequences can be determined by using
30 analysis software (*i.e.*, Sequence Analysis Software Package of the Genetics Computer Group, Madison Wis.) that incorporates the *Needelman and Wunsch*, (J. Mol. Biol., 48: 443-453, 1970) algorithm (*e.g.*, NBLAST, and

XBLAST). In some forms, the default parameters can be used to determine the identity for the polynucleotides or polypeptides of the present disclosure.

In some forms, the % sequence identity of a given nucleic acid or amino acid sequence C to, with, or against a given nucleic acid or amino acid sequence D (which can alternatively be phrased as a given sequence C that has or includes a certain % sequence identity to, with, or against a given sequence D) is calculated as follows:

100 times the fraction W/Z ,

where W is the number of nucleotides or amino acids scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides or amino acids in D. It will be appreciated that where the length of sequence C is not equal to the length of sequence D, the % sequence identity of C to D will not equal the % sequence identity of D to C.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

Use of the term "about" is intended to describe values either above or below the stated value in a range of approximately +/- 10%; in other forms the values may range in value either above or below the stated value in a range of approximately +/- 5%; in other forms the values may range in value either above or below the stated value in a range of approximately +/- 2%; in other forms the values may range in value either above or below the stated value in a range of approximately +/- 1%. The preceding ranges are intended to be made clear by context, and no further limitation is implied.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed method and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and

permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a ligand is disclosed and discussed and a number of modifications that can be made to a number of molecules including the ligand are discussed, each and every combination and permutation of ligand and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, in this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Further, each of the materials, compositions, components, etc. contemplated and disclosed as above can also be specifically and independently included or excluded from any group, subgroup, list, set, etc. of such materials.

These concepts apply to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods, including, but not limited to those provided in the exemplary experiments, and that each such combination is specifically contemplated and should be considered disclosed. All methods described herein can be performed in any suitable order unless otherwise indicated or otherwise clearly contradicted by context. The use of any and all examples and experiments, and exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the embodiments and does not pose a

limitation on the scope of the embodiments unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

II. Compositions

- 5 Compositions of class II, type VI CRISPR/Cas effector proteins or nucleic acids encoding thereof are provided. The compositions can include polynucleotides, vectors (e.g., expression vectors), isolated proteins, and/or ribonucleoprotein complexes related to class II, type VI CRISPR/Cas effectors.

10 *Class II, type VI CRISPR/Cas System*

- Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–CRISPR-associated (Cas) (CRISPR–Cas) systems originate from Prokaryotes, where they serve primarily as a defensive mechanism against mobile genetic elements like phages and plasmids. These systems contain
- 15 two components. The first is a genomic locus called CRISPR that contains a series of short sequences of foreign origin called spacers that enable recognition of specific mobile genetic elements that were previously encountered. Spacers are separated by repetitive regulatory sequences named repeats. Together with the leader sequence, these spacers and repeats
- 20 constitute the CRISPR array. The second component of CRISPR–Cas systems are Cas proteins., which are encoded by cas genes, usually located in the proximity of a CRISPR array. See Burmistrz, M., et al., Int J Mol Sci., 21(3):1122 (2020).

- The mechanism of CRISPR–Cas systems includes three phases:
- 25 adaptation, maturation, and interference. During the adaptation phase, new spacers are incorporated into the CRISPR array into its leader end. During the maturation phase the CRISPR array is transcribed. The resulting transcript called pre-CRISPR RNA (pre-crRNA) is further processed into a set of CRISPR RNA (crRNA) molecules, each containing a single spacer
- 30 flanked by fragments of a repeat sequence. Subsequently, crRNAs are incorporated into ribonucleoprotein (RNP) complexes together with Cas proteins. RNP complexes scan nucleic acids searching for a sequence complementary to that encoded by crRNA.

These CRISPR systems are classified based on the structure of CRISPR-associated (Cas) genes that are typically adjacent to the CRISPR arrays. Generally, there are two classes of CRISPR systems, each containing multiple CRISPR types. Class 1 contains type I and type III CRISPR systems that are commonly found in Archaea. Class 2 contains type II, IV, V, and VI CRISPR systems (Adli, M., Nat Commun., 9:1911 (2018)).

The type VI CRISPR–Cas systems present a relatively simple structure, as they require only one Cas13 protein and crRNA molecule for activity. To date, four subtypes have been distinguished: VI-A (that uses Cas13a variant, alternatively known as C2c2), VI-B (Cas13b/C2c6), VI-C (Cas13c/C2c7), and VI-D (Cas13d). Although the Cas13 of these subtypes differ in size and sequence, they all share a common feature, which is the presence of two HEPN domains. These domains are responsible for RNA-targeted nucleolytic activity. HEPN domains are usually located close to different terminal ends of the Cas13 protein.

In general, processing of pre-crRNA into crRNA is performed by Cas13 itself in a metal-independent manner (with the exception of type VI-D) without the help of other host factors. In subtypes VI-A, VI-C, and VI-D crRNAs contain a repeat-derived handle on their 5' end. On the contrary, subtype VI-B generates crRNAs with handle on the 3' end. Secondary processing is presumably performed by other host nucleases. crRNA maturation is not necessary for type VI activity, and even unprocessed pre-crRNA is sufficient for recognition of targeted RNA. The complex of Cas13 and crRNA presents no nucleolytic activity until it binds to targeted ssRNA. Binding between crRNA and targeted RNA triggers the conformation change of the RNP complex. Both HEPN domains are moved closer to each other, creating a single catalytic site. As this site is located at some distance to crRNA-targeted RNA duplex, it is expected to cleave not only the targeted RNA but also any other ssRNA, including the host's own RNA, in close proximity of a RNP complex. This is sometimes referred to as 'collateral damage' or 'collateral cleavage'. Burmistrz, M., et al., 2020.

As used herein, CRISPR/Cas composition refers to the elements of a CRISPR system needed to carry out CRISPR/Cas-mediated activity.

CRISPR/Cas-mediated compositions typically include one or more nucleic acids encoding a crRNA, a tracrRNA (or chimeric thereof also referred to a guide RNA or single guide RNA) and a Cas enzyme, preferably Cas13.

The methods of delivery disclosed herein are suitable for use with numerous variations on the CRISPR/Cas system. In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence (also referred to as a protospacer in the context of an endogenous CRISPR system). In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target sequence and a guide sequence promotes the formation of a CRISPR complex. A target sequence can be any polynucleotide, such as DNA or RNA polynucleotides. In some embodiments, a target sequence is located in the nucleus or cytoplasm of a cell.

All type VI systems require a so called Protospacer Flanking Sequence (PFS) located in the direct vicinity of a protospacer sequence. This sequence varies between different subtypes. For example: LshCas13a uses a non-G PFS located at 3' end, whereas BzCas13b requires the double-sided PFS of non-C upstream of the target site and NAN or NNA downstream of the target site. The tracrRNA duplex directs Cas to the target consisting of the protospacer and the requisite PSF via heteroduplex formation between the spacer region of the crRNA and the protospacer.

Typically, formation of a CRISPR complex (including a guide sequence hybridized to a target sequence and complexed with one or more Cas proteins) results in cleavage of the RNA strands in or near (e.g., within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence.

There are many resources available for helping practitioners determine suitable target sites once a desired target sequence is identified, e.g., SnapGene.

A. Polynucleotides and expression vectors

Isolated nucleic acid sequences encoding the disclosed Cas13 polypeptides, variants thereof and fusion proteins thereof are disclosed.

Nucleic acids can be in sense or antisense orientation, or can be complementary to a reference sequence encoding a Cas13 or variant thereof such as those discussed below.

- Nucleic acids can be DNA, RNA, or nucleic acid analogs. Nucleic acid analogs can be modified at the base moiety, sugar moiety, or phosphate backbone. Such modification can improve, for example, stability, hybridization, or solubility of the nucleic acid. Modifications at the base moiety can include deoxyuridine for deoxythymidine, and 5-methyl-2'-deoxycytidine or 5-bromo-2'-deoxycytidine for deoxycytidine.
- Modifications of the sugar moiety can include modification of the 2' hydroxyl of the ribose sugar to form 2'-O-methyl or 2'-O-allyl sugars. The deoxyribose phosphate backbone can be modified to produce morpholino nucleic acids, in which each base moiety is linked to a six membered, morpholino ring, or peptide nucleic acids, in which the deoxyphosphate backbone is replaced by a pseudopeptide backbone and the four bases are retained.

- Particularly, polynucleotides containing a nucleotide sequence encoding a class II, type VI CRISPR/Cas effector protein (Cas13) and a heterologous sequence are disclosed. In some embodiments, the CRISPR/Cas effector is an RNA editing enzyme (e.g., RNA endonuclease) with RNA (e.g., ssRNA) cleavage activity. In a preferred embodiment, the Cas13 enzyme is a Cas13a protein derived from thermophilic bacteria, preferably from *Thermoclostridium caenicola* (TccCas13a) or *Herbinix hemicellulosilytica* (HheCas13a).

- DNA sequences for HheCas13a are known in the art. See e.g., Addgene plasmid No.: 91871 and East-Seletsky, et al., Mol Cell, 2017, which are hereby incorporated by reference in their entirety.

An exemplary DNA sequence encoding HheCas13a is:

- ATGAAGTTGACGCGTCGCCGCATCAGTGGCAATTCAGTCGACCAGAAGATT
ACAGCTGCGTTTTATCGTGATATGTCGCAAGGGTTATTATATTATGATTCCG
GAGGACAATGACTGTACGGACAAGGTCATCGAAAGTATGGATTTCTGAACGC
TCTTGCGTGACGCATTCTGAAGAACGGCGAGGATGATAAGAATCCGTTT
TACATGTTTCGTTAAAGGTTTAGTTGGTTCCAACGATAAGATCGTGTGCGAA

CCTATCGACGTCGACTCAGACCCTGATAACTTAGATATCTTGATTAACAAA
AATCTGACGGGGTTCGGACGCAACTTGAAAGCACCTGACAGTAACGATACC
TTAGAAAATCTTATCCGCAAAATCCAGGCCGGCATTCCCGAAGAAGAGGTT
CTTCCGGAGCTGAAGAAAATTAAAGAGATGATCCAGAAAGACATCGTTAAC
5 CGCAAAGAGCAGTTGCTTAAATCCATCAAAAAACAACCGTATTCCTTTTTCA
TTGGAAGGTTCCAAGCTGGTCCCTTCCACCAAAAAGATGAAGTGGCTTTTC
AAGTTGATTGACGTTCTAATAAAACCTTCAATGAAAAAATGTTGGAGAAG
TACTGGGAGATCTACGACTATGACAAGCTTAAGGCGAATATCACGAACCGC
CTGGACAAAACGGATAAGAAGGCACGTTCAATTTGCGGTGCGGTAAGCGAG
10 GAGTTACGTGAGTACCATAAAAACTTGCGCACAACTATAATCGCTTCGTA
TCTGGAGACCGTCCAGCAGCCGGGCTTGACAATGGGGGGAGTGCTAAATAT
AATCCCGATAAGGAGGAGTTCTTATTGTTTCTTAAAGAAGTTGAGCAGTAC
TTCAAGAAATACTTCCCAGTCAAGAGCAAGCATTCGAACAAGTCCAAAGAC
AAATCATTGGTTGATAAGTACAAAAATTATTGCAGTTACAAAGTGGTCAAG
15 AAAGAGGTCAACCGCAGCATCATCAATCAATTGGTGGCTGGACTTATTCAG
CAGGGTAAGCTTCTGTATTACTTCTACTATAACGATACCTGGCAGGAGGAT
TTCCTGAATTCCCTACGGTCTTAGTTATATTCAGGTTGAGGAGGCATTTAAA
AAGAGTGTAATGACCAGTCTTTCGTGGGGAATCAACCGTCTGACCTCGTTC
TTTATCGACGACTCGAACACTGTGAAGTTTGATGATATCACAACGAAAAAG
20 GCTAAGGAGGCGATTGAGTCTAATTATTTCAACAAATTACGCACATGCTCC
CGTATGCAGGACCACTTCAAAGAGAAATTGGCCTTCTTTTACCCGGTGTAT
GTGAAGGACAAGAAGGATCGCCCGGATGATGATATCGAAAACCTTAATTGTA
CTGGTTAAGAACGCCATTGAGAGCGTGAGCTATTTGCGCAATCGCACGTTT
CACTTTAAAGAGAGCTCCTTATTGGAGCTGCTTAAAGAGTTAGACGATAAA
25 AATTCTGGTCAAAAATAAAATCGACTATAGCGTAGCCGCTGAATTTATTTAAA
CGCGATATCGAGAACTTGTATGACGTCTTCCGTGAACAGATCCGCTCACTT
GGTATTGCTGAGTACTACAAGGCGGACATGATTTCGGACTGTTTTAAGACA
TGCGGGTTAGAATTCGCGCTTTATTGCGCAAAAAAATTCGTTGATGCCCGCT
TTTAAGAACGTTTATAAACGCGGAGCAAATCTTAACAAGGCGTATATTCGC
30 GATAAAGGGCCAAAGGAAACAGGAGACCAGGGTCAGAACTCGTACAAAGCT
TTGGAGGAATATCGTGAGCTTACATGGTATATTGAGGTGAAGAATAACGAC
CAGTCGTACAACGCCTACAAGAATTTGCTTCAACTGATTTATTATCATGCG
TTTCTTCCGGAGGTTTCGTGAAAATGAGGCTCTTATCACTGACTTCATTAAC

CGCACTAAAGAGTGGAATCGTAAGGAAACAGAGGAGCGTCTGAACACTAAA
AACATAAAAAACACAAGAATTTGACGAGAATGACGATATTACGGTAAAT
ACGTATCGTTACGAATCTATTCCGGATTACCAGGGGGAATCCCTTGATGAT
TATCTTAAGGTGTTACAGCGCAAGCAAATGGCGCGTGCGAAGGAGGTAAAT
5 GAGAAGGAGGAGGGCAACAATAACTATATCCAATTCATTTCGTGACGTCGTA
GTATGGGCGTTTCGGGGCCTACTTGGAACAAGCTGAAGAATTATAAGAAC
GAACTGCAACCACCGTTGAGCAAAGAGAACATTGGCTTAAATGATACCTTA
AAGGAATTGTTTCCCGAAGAAAAAGTAAAGTCACCATTTAATATCAAGTGC
CGTTTCTCCATTAGTACATTTATCGACAACAAAGGTAAGTCCACCGATAAT
10 ACCTCGGCCGAGGCCGTCAAGACTGATGGCAAGGAGGACGAAAAGGACAAA
AAGAATATTAAACGCAAAGACTTGCTTTGCTTTTACCTGTTCTTACGCTTG
CTTGATGAAAACGAAATTTGCAAATTACAACACCAATTTATTAAATACCGT
TGCAGTTTAAAGAGCGTCGTTTCCAGGTAACCGCACCAAACTGGAGAAG
GAAACTGAACTTTTGGCCGAGCTGGAAGAACTGATGGAAGTGGTACGTTTT
15 ACGATGCCATCAATTCCTGAAATCTCTGCAAAGGCGGAATCAGGTTATGAC
ACAATGATTAAGAAGTATTTTAAAGACTTCATCGAGAAAAAAGTCTTTAAG
AATCCGAAGACGTCCAACCTGTACTACCACTCCGACTCTAAAACCTCCGGTC
ACCCGCAAATATATGGCTCTGCTGATGCGTTCTGCGCCTTTGCATCTCTAC
AAGGATATCTTCAAGGGCTACTATTTGATCACTAAGAAGGAGTGTGTTGGAG
20 TATATTAAATTAAGCAACATCATTAAGACTATCAAACTCATTGAATGAA
TTGCACGAGCAGCTGGAGCGCATTAATTAAGAGTGAGAAACAAAACGGG
AAAGACTCTCTGTATCTTGACAAAAAGGACTTCTATAAAGTAAAGGAGTAC
GTCGAAAATTTAGAACAAGTGGCGTGTTACAAGCACTTGCAGCACAAAATC
AACTTCGAAAGCTTATACCGTATTTTTTCGTATTCACGTGGACATCGCTGCG
25 CGTATGGTCGGTTATACCCAAGACTGGGAGCGTGATATGCACTTTCTTTTC
AAGGCGTTGGTCTATAATGGCGTACTTGAGGAGCGCCGTTTTGAAGCAATC
TTTAACAATAACGATGACAACAACGACGGGCGTATCGTAAAAAAGATCCAG
AATAACTTAAATAACAAGAACCGCGAATTAGTATCTATGTTATGTTGGAAT
AAAAAGCTGAACAAGAACGAATTTGGTGCTATTATTTGGAAGCGTAACCCCT
30 ATCGCCCATCTGAACCACTTTACACAAACGGAACAAAACCTCAAAGTCGAGC
CTTGAATCTCTGATCAACTCGCTGCGCATCCTGTTAGCGTACGACCGTAA
CGTCAAAACGCTGTGACAAAGACTATCAATGACCTTCTGTAAACGACTAT
CACATCCGCATTAAGTGGGAAGGGCGCGTGATGAAGGGCAGATCTACTTT

AATATCAAGGAAAAGGAAGACATCGAGAACGAGCCTATTATCCACTTGAAA
CACCTGCATAAAAAGGACTGCTACATTTACAAGAACAGCTATATGTTTCGAC
AAGCAAAAGGAGTGGATTTGTAATGGCATTAAAGAAGAAGTTTATGACAAG
TCCATTCTTAAGTGTATCGGAACTTGTTCAAGTTTGAAGTATGAGGACAAA
5 AACAAATCCTCAGCAAATCCTAAGCACACC (SEQ ID NO:67).

An exemplary DNA sequence encoding TccCas13a is:

ATGAAGATCACGAAAAGGAAATGGGGAGAGCATCATCCGCCGCTTTACTTC
TACCGGGATGAGGACTCCGGCAGGCTCCTGGCACAGAACGACAGAAAGCAG
GATTATACCGATACCCTGTTTAATGATATTGCGCAGGATACATTTGAAAGA
10 TCGCTGAGAAACCGGCTTTTGAAAACACCCGAAAAGGGAGACAAAAGATTC
TACAGCAACGAGATCGTCAAGCTGGTGGAGAACTGTGCCAGGGTGC GGAT
GTGGCGGAGATCATGAAAAGCATGGAGAGGAACGAAAAGCTGCGCCCCAAG
AATGAAAAGAGATTAAAAATCTGAAAAGCAATTGGACGGTACCCTTTTCC
GAATACGGTAAAAGGTATACCGCCCCAGAAGGCGCCATGACCCTCAACGAT
15 GCCTTGTTTTACCTGGTAGAAGGAAACCTTTAAAGCAGGCCATGGCCAAA
GCTGAACTGGGCAAAATCCGGGAGGCATTAATAAAGGAAAAGGAAAACCGC
ATCAACCGCGTCCGGTATTCCATCAAGAACAATAAAATACCGCTGAGAATC
CAAGAGGATGGCGGCATTACACCCAATAATGACCGTGCGGCCTGGCTGCTG
GGGCTCATGAAGCCGGCGGACCCGGCAAAAGGAATAACCGACTGCTACCCG
20 CTCTTAGGCGAGTTGGAAGAAGTATTCGACTTTGACAAGCTGTCCAAAACG
CTGCACGAAAAGATAAGCCGTTGCCAAGGCCGACCCCGTTCCATAGCCATG
GCGGTGCATGAGGCCCTGAAGCAATATCTCCGGGAGCTTTGGGAGAAGTCT
CCCTCGCGACAGCAGGATCTGAAGTATTACTTTCAGGCCGTACAGGAGTAC
TTTAAGGACAATTTTCCCATCCGGACAAAACGGATGGGCGCCCGCCTGCGG
25 CAGGAATTGCTCAAGGATAAGACGTCCCTTTCCCGTCTGCTGGAACCCAAG
CATATGGCTAATGCCGTTCCGCCGAGGCTGATCAACCAGAGTACCCAGATG
CACATCCTTTATGGAAGCTTTATGCATATTGCTGCGGGGAAGACGGCAGG
CTTTTGGTAAACAGCGAGACGCTGCAAAGGATACAGGTCCATGAAGCGGTA
AAAAAGCAGGCCATGACGGCCGTGCTGTGGTCCATATCCCGTCTGCGCTAT
30 TTTTACCAGTTTGAAGACGGCGATATCTTGAGCAATAAAAACCCGATTAAA
GATTTTCAGAGATAAATTTCTCAGAGACACGAATAAATATAACCCATGAAGAT
GTTGAGGCCTGCAAGGAAAAACTGCAGGACTTCTTCCCGCTGAAAGAATTG
CAGGAAAAGATTAAGGAGGATGCAAAGGATTACAGGAAACAGACAATAAG

CAGGCTGATACAACGGATTTCAAAGCGATCGGGCACATCGTCAGGGATGAT
CGGAAGCTCTGCAACCAGTTGCTGGCGGAGTGCGTTTCCTGCATCGGGGAG
CTGAGACATCATATCTTTCATTATAAAAATGTGACCTTGATACAGGCGCTC
AAAAGGATCGCCGATAAAGTGAAACCGGAGGATTTGTCTGTGCTCCGGGCC
5 ATTTACCTGTTGGACAGGAGAAAACCTCAAAAAGGCGTTTGCCAAAAGAATC
AGCAGCATGAACCTTCCGCTGTATTACAGGGAGGATCTATTATCCCGCATT
TTCAAAAAGAAGGGACGGCGTTTTTCCTGTACAGCGCCAAGATCCAGATG
ACACCGTCCTTTCAGAGGGTCTATGAGCGTGGTAAAAATCTGCGCCGGGAG
TTCGAGTGCGAACGCATGAAAGCCGAGGCATCCAACGGACAGAACGGGCAG
10 GATGGCGACCGGCTGAAATGGTTCCGACAGCTTGCGGCTGGGGATAGCGCC
GATACCCATTTCAACTGGGCTGTGGAGGCCTATGCGGAATCGGCAGCCGAT
GTGGAAAACAACGTTGAATTCGATACCGATGTGGATGCCCAGCGTGCCCTG
CGGAACCTTCTGCTGCTGATATATAGGCACCATTTCTTGCCGGAGGTGCAG
AAGGATGAAACCCTGGTGACCGGCAAGATCCATAAGGTTTTTGAAAGAAAC
15 AGGCAGCTGTCTGAAGGCCAAGGGCCAAATCAAGGTAAGGCCCATGGATAC
AGCGTTATAGAAGAGCTGTATCATGAAGGCATGCCGCTTTCCGATCTCATG
AAGCAATTGCAGCGGAGGATCTCCGAAACCGAAAGGGAGAGCCGGGAACTG
GCACAGGAAAAACGGATTATGCCAGCGCTTTATCCTCGATATCTTTGCC
GAGGCCTTTAACGATTTCTTGAGGGCGCACTATGGCGAAGAATACCTTGAG
20 ATCATGAGCCCCAGGAAAGATGCCGAAGCGGCGAAGAAATGGGTAAAAGAA
AGCAAAACGGTGGATTTGAAAACATCTATAGACGAAAAAGAACCGGAAGGG
CATCTCCTGGTGCTCTATCCCGTCCTGCGCCTGCTGGACGAAAGGGAACCTG
GGAGAGCTTCAGCAGCAGATGATCCGTTATCGGACATCCCTGGCCAGTTGG
CAGGGTGAGAGCAATTTAGTGAAGAAATAAGGATAGCCGGGCAGATTGAG
25 GAATTGACCGAACTGGTCAAGCTGACGGAACCGGAGCCCCAGTTTGCGGAG
GAAGTATGGGGGAAACGGGCTAAAGAAGCGTTTGAAGACTTTATTGAAGGA
AACATGAAAAATTATGAGGCTTTTTATCTTCAGAGCGACAACAACACGCCG
GTATATCGGAGGAACATGAGCCGGTTGCTGCGCTCGGGGCTTATGGGAGTG
TACCAAAAGGTGCTTGCCAGCCACAAGCAGGCGCTCAAAGAGATTACTTG
30 CTCTGGTCGGAAAAACATTGGAACGTGAAGGATGAGAATGGAGCGGATATC
TCTTCCGCTGAACAGGCCCAATGCCTTTTGCAGCGGCTCCATAGGAAGTAC
GCCGAATCCCCGTCCCGATTTACGGAGGAGGACTGCAAACGTATGAGAAG
GTTCTCCGGCGGCTTGAGGACTATAACCAGGCCGTGAAAAACCTGTCCTTC

AGTTCATTGTATGAAATATGCGTCCTCAATCTTGAAATCCTCTCCCGATGG
 GTGGGGTTTGTCCAGGACTGGGAGCGGGATATGTACTTCCTCCTTTTGGCG
 TGGGTCAGACAGGGCAAACCTGGACGGGATAAAGGAGGAAGATGTCAGAGAT
 ATCTTCTCTGAAGGCAACATTATTCGCAATCTGGTGGATACGCTGAAAGGC
 5 GAAAACATGAATGCCTTTGAAAGCGTTTATTTCCCTGAGAATAAGGGGTCT
 AAGTATTTAGGTGTGCGGAATGATGTTGCCCATCTGGATCTGATGCGGAAA
 AACGGATGGCGGTTGGAGGCTGGCAAAACCTGCAGCGTGATGGAAGATTAC
 ATCAACCGTTTGAGGTTTCTCCTGTCCTATGACCAGAAACGCATGAACGCC
 GTCACCAAGACCTTGCAGCAGATATTTGATAGGCATAAAGTCAAAATCCGG
 10 TTCACCGTGGAGAAAGGAGGAATGCTGAAGATAGAGGATGTGACTGCCGAC
 AAGATCGTGCATCTGAAAGGTTCCAGGTTGAGCGGCATTGAAATTCCAAGT
 CACGGGGAGAGGTTTATTGACACGTTGAAAGCGCTGATGGTATACCCGAGA
 GGATGA (SEQ ID NO:63).

In some preferred embodiments, the Cas13 effector is a Cas13
 15 protein derived from a bacterium in the Proteobacteria phylum. An
 exemplary Proteobacteria-derived Cas13 is mCas13 encoded by the
 following DNA sequence:

ATGGGTATTGATTATTCGCTTACAAGTGACTGTTATCGAGGCATCAACAAG
 TCTTGTTTTGCAGTTGCTTTGAATATTGCATATGATAACTGTGATCATAAA
 20 GGATGTAGGACTCTTCTGAGTGAGGTGCTGCGCAGCAAGGGAGGGATTTCT
 GATGAGCAAATAAAATCACAAAGTAGTGGATGGAATTCAGAAGCGACTTAAA
 GACATTCGCAATTATTTCTCGCATTATTACCACGCAGAAGACTGTCTGCGG
 TTTGGAGACCAAGATGCCGTCAAGGTTTTTCTGGAAGAAATATACAAGAAC
 GCGGAATCAAAGACTGTCGGAGCGACAAAAGAAAGCGACTATAAAGGCGTT
 25 GTGCCGCCATTATTTGAATTGCATAACGGTACATATATGATTACGGCGGCG
 GGGGTTATTTTTCTGGCATCATTTTTCTGCCATCGGAGTAATGTCTATCGG
 ATGCTGGGAGCGGTGAAAGGATTTAAACATAACGGAAAAGAGCAATTGAGC
 GATGGGCAGAAACGAGATTATGGTTTCACTCGCCGGCTGCTGGCTTATTAT
 GCGCTCCGGGACAGCTATTCTGTGGGGGCGGAAGACAAGACACGATGTTTC
 30 CGCGAGATATTAAGCTATTTGTCGAGAGTACCGCAATTGGCAGTAGATTGG
 CTGAATGAACAACAGCTGCTTACACCAGAAGAAAAAGAAGCTTTTTTTGAAT
 CAACCCGCCGAAGATGAGGGCGGGGATATTTTCGGACTCTTCGTCCAGTGAT
 AAAAATAAAAAAAGCAAAGAAAAGAGGCGTAGTCTCCGCAGAGATGAAAAA

TTCATTTTATTTGCTATCCAATTTATTGAGGGGTGGGCGGCTGAACAGGGA
TTAGATGTGACATTTGCACGCTACCAAAAAACAGTGGAAAAAGCGGAGAAT
AAGAATCAGGATGGCAAACAGGCCAGAGCGGTGCAATTAAAATACAGAAAC
CAAGGACTCAATCCGGATTTCAATAACGAATGGATGTATTACATTCAGAAT
5 GAACACGCAATTATCCAGATTAACTGAATAATAAAAAAGCCGTTGCTGCC
CGTATTTCTGAAAAATGAATTAAAATATCTGGTGCTGCTGATTTTTGAGGAG
AAGGGCAATGACGCCGTCCAAAAGCTGAATTGTTATATTTACAGTATGAGC
CAAAAAATCGAGGGCGAATGGAAACACAGGCCGAGGATGAGCGATGGATG
CCGTCGTTTACCAAGCGTGCCGACAGGACGGTTACGCCGGAGGCAGTGCAG
10 AGCCGGTTAAGCTATATTCGCAAACAACCTTCAGGAGACGATAGAGAAAATC
GGTCAGGAAGAGCCGCGGAATAATAAGTGGCTGATATACAAAGGCCAAAAA
ATATCAATGATACTGAAGTTTATCTCCGACAGCATTTCGCGATATTCAAAGG
CGGCCGAATGTGAAACAATATCATATTTTACGCGATGCGCTTCAGAGGCTC
GACTTTGATGGATTTTATAAGGAACTTCAAAATTACGTCAATGACGGCCGG
15 ATTGCGGTTTTATTATACGATCAGATCAAGGGTGTCAATGACATCAGCGGG
CTTTGTAAAAAAGTCTGCGAACTGACACTTGAAAGACTGGCTGGGCTGGAG
GCAAAGAATGGCTCCGAGCTGAGGCGTTATATTGGGCTTGAAGCGCAGGAA
AAACATCCGAAGTACGGAGAGTGGAACACACTGCAGGAAAAGGCCAAGCGA
TTTCTGGAGTCGCAGTTTTCTATCGGGAAAACTTTTTGCGGAAAATGTTT
20 TATGGCGATTGTTGTCAAAGCGGTGCTTTGACGAAGAAAAAGGTTACAAT
ACACAAGCGAAAGAGCGAAAAAGTCTGTATAGCATTGTGAAAGAAAACTC
AAGGACATCAAGCCATACACGATGACCGGTGGTATCTTATCGACAGGAAT
CCGAAGAACTATGACAATAAACACAGCAGGATTATCCGACAGATGTGCAAC
ACCTATATACAAGATGTTCTCTGTATGAAGATGGCGATGTGGCATTATGAG
25 AAATTAATCTCCGCGACTGAATTCAGGAATAAATTGGAATGGAATTGTATT
GGGCAGGGAAATATGGGTTATGAGCGATATTTCGCTGTGGTATAAGACAGGC
TGCGGGGTTGTACATACAATTTACGCCGGCGGATTTTTTGCGACTGGATATC
ATTGAAAAACCTGCGATGATAGAAAATATTTGCCAGTGTTTTGTGCTCGGA
AACAAAAAATCAATTCGGGTGCCGAAAAGAAGATAACCTGGGATAAATTC
30 AATAAAGACGGCATTGCAAAATACAGAAAGCGGCAGGCCGAGGCTGTGCGT
GCGATATTTGCGTTTGAGGAAGGTTTAAAAATACAAGAGGATAAATGGAGT
CATGAGAGATACTTTCCATTTTGCAATATTCTTGACGAGGCGGTAAACAA
GGTAAAATAAAAGATACAGGCAAAGACAAGGAGGCTCTGAATCGCGGCAGA

AATGATTTTTTTCATGAGGAATTTAAGTCAACAGAAGATCAGCAGGCGATT
 TTTTCAAGAAATACTTTCCGATTGTCTGAACGAAAAGACGACACAAAAAACGG
 CGAGATAAAAAGCAAAAGTGA (SEQ ID NO:65; GenBank accession No.:
 DSVK01000191.1).

5 In some embodiments, the sequence encodes the Cas13 effector
 protein of SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68. In some
 embodiments, the sequence encodes the Cas13 effector protein with at least
 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%
 sequence identity to Cas13 encoded by SEQ ID NO:63, SEQ ID NO:65, or
 10 SEQ ID NO:67.

 In some embodiments, the sequence encoding the Cas13 effector
 protein is or includes SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67. In
 some embodiments, the sequence encoding the Cas13 effector protein is or
 includes a sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%,
 15 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:63, SEQ ID
 NO:65 or SEQ ID NO:67.

 Preferably, the sequence encoding the Cas13 effector protein is codon
 optimized for expression in a prokaryotic or eukaryotic cell, such as a
 bacterial, yeast, or mammalian cell. The eukaryotic cells can be those of or
 20 derived from a particular organism, such as a mammal, including but not
 limited to human, mouse, rat, rabbit, cat, dog, horse, pig, or non-human
 primate. In general, codon optimization refers to a process of modifying a
 nucleic acid sequence for enhanced expression in the host cells of interest by
 replacing at least one codon (e.g., about or more than about 1, 2, 3, 4, 5, 10,
 25 15, 20, 25, 50, or more codons) of the native sequence with codons that are
 more frequently or most frequently used in the genes of that host cell while
 maintaining the native amino acid sequence. Various species exhibit
 particular bias for certain codons of a particular amino acid. Codon bias
 (differences in codon usage between organisms) often correlates with the
 30 efficiency of translation of messenger RNA (mRNA), which is in turn
 believed to be dependent on, among other things, the properties of the
 codons being translated and the availability of particular transfer RNA
 (tRNA) molecules.

The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily
5 available, for example, at the “Codon Usage Database”, and these tables can be adapted in a number of ways. See Nakamura, Y., et al., *Nucl. Acids Res.*, 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell, for example Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or
10 more codons (e.g., 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a CRISPR enzyme correspond to the most frequently used codon for a particular amino acid.

In some embodiments, the heterologous sequence includes one or more expression control sequences. In some embodiments, the heterologous
15 sequence includes a promoter, transcription terminator, multiple cloning site, drug resistance marker(s), one or more protease recognition sites, one or more epitope tags, or a combination thereof. Typically, the heterologous sequence, such as an expression control sequence, is operably linked to the sequence encoding the Cas13 effector protein.

20 In some embodiments, the polynucleotide further includes a sequence encoding one or more crRNAs or guide RNA (gRNA) or single guide (sgRNA). In preferred embodiments, the crRNAs are capable of complexing with the Cas13 effector protein and hybridizing to one or more target RNA sequences.

25 In some embodiments, the polynucleotide is or is contained in a vector, such as an expression vector. Nucleic acids, such as those described above, can be inserted into vectors for expression in cells. As used herein, a “vector” is a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of
30 the inserted segment. Vectors can be expression vectors. An “expression vector” is a vector that includes one or more expression control sequences, and an “expression control sequence” is a DNA sequence that controls and regulates the transcription and/or translation of another DNA sequence.

Nucleic acids in vectors can be operably linked to one or more expression control sequences. As used herein, “operably linked” means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest. Examples of expression control sequences include promoters, enhancers, and transcription terminating regions. A promoter is an expression control sequence composed of a region of a DNA molecule, typically within 100 nucleotides upstream of the point at which transcription starts (generally near the initiation site for RNA polymerase II). To bring a coding sequence under the control of a promoter, it is necessary to position the translation initiation site of the translational reading frame of the polypeptide between one and about fifty nucleotides downstream of the promoter. Enhancers provide expression specificity in terms of time, location, and level. Unlike promoters, enhancers can function when located at various distances from the transcription site. An enhancer also can be located downstream from the transcription initiation site. A coding sequence is “operably linked” and “under the control” of expression control sequences in a cell when RNA polymerase is able to transcribe the coding sequence into mRNA, which then can be translated into the protein encoded by the coding sequence.

Suitable expression vectors include, without limitation, plasmids and viral vectors derived from, for example, bacteriophage, baculoviruses, tobacco mosaic virus, herpes viruses, cytomegalo virus, retroviruses, vaccinia viruses, adenoviruses, and adeno-associated viruses. Numerous vectors and expression systems are commercially available from such corporations as Novagen (Madison, WI), Clontech (Palo Alto, CA), Stratagene (La Jolla, CA), and Invitrogen Life Technologies (Carlsbad, CA). In some preferred embodiments, suitable vectors include viral vectors (e.g., retroviral, lentiviral, adenoviral, adeno-associated or herpes simplex viral vectors) or plasmids.

An expression vector can include a tag sequence. Tag sequences, are typically expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino terminus. Examples of useful tags include, but are not limited to,

green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, Flag™ tag (Kodak, New Haven, CT), maltose E binding protein and protein A.

In some embodiments, one or more vectors driving expression of one or more elements of a CRISPR/Cas13 system are introduced into a target cell such that expression of the elements of the CRISPR system direct formation of a CRISPR complex at one or more target RNA sequences. For example, a Cas13 protein and one or more guide sequences could each be operably linked to separate regulatory elements on separate vectors. Alternatively, two or more of the elements expressed from the same or different regulatory elements may be combined in a single vector, with one or more additional vectors providing any components of the CRISPR/Cas13 system not included in the first vector. CRISPR system elements that are combined in a single vector may be arranged in any suitable orientation, such as one element located 5' with respect to ("upstream" of) or 3' with respect to ("downstream" of) a second element. The coding sequence of one element can be located on the same or opposite strand of the coding sequence of a second element, and oriented in the same or opposite direction.

In some embodiments, a single promoter drives expression of a transcript encoding a Cas13 protein and one or more of the crRNA sequences. In some embodiments, one or more of the elements of CRISPR/Cas13 system are under the control of an inducible promoter, which can include inducible Cas13.

In some embodiments, a vector includes one or more insertion sites, such as a restriction endonuclease recognition sequence (also referred to as a "cloning site"). In some embodiments, one or more insertion sites (e.g., about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more insertion sites) are located upstream and/or downstream of one or more sequence elements of one or more vectors. In some embodiments, a vector includes an insertion site upstream of a crRNA sequence, and optionally downstream of a regulatory element operably linked to the crRNA sequence, such that following insertion of a Cas13 sequence into the insertion site and upon expression the crRNA sequence directs sequence-specific binding of a Cas13

RNP complex to a target RNA sequence in a cell. In some embodiments, a vector includes two or more insertion sites. In such an arrangement, the two or more crRNA sequences can include two or more copies of a single crRNA sequence, two or more different crRNA sequences, or combinations of these.

- 5 When multiple different crRNA sequences are used, a single expression construct may be used to target Cas13 activity to multiple different, corresponding target RNA sequences within a cell. For example, a single vector can include about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 crRNA sequences. In some embodiments, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, such crRNA-sequence-containing vectors may be provided, and optionally delivered to a cell.

- In some embodiments, the relatively small size of mCas13 allows for easier packaging into commonly used delivery vectors such as adeno-associated viruses (AAV). Thus, delivery of mCas13 protein (or TccCas13 or HheCas13a) *in vivo* for various applications (e.g., RNA targeting and manipulation as well as therapeutic interventions) can be accomplished by use of an AAV vector. The AAV vector can provide one or more elements of the gene editing compositions (e.g., crRNA expression cassette, CAR expression cassette, homology arms).

- 20 AAV is a non-pathogenic, single-stranded DNA virus that has been actively employed over the years for delivering therapeutic genes in both *in vitro* and *in vivo* systems (Choi, et al., *Curr. Gene Ther.*, 5:299-310, (2005)). AAV belongs to the parvovirus family and is dependent on co-infection with other viruses, mainly adenoviruses, in order to replicate. Initially distinguished serologically, molecular cloning of AAV genes has identified hundreds of unique AAV strains in numerous species. Each end of the single-stranded DNA genome contains an inverted terminal repeat (ITR), which is the only cis-acting element required for genome replication and packaging. The single-stranded AAV genome contains three genes, Rep (Replication), Cap (Capsid), and aap (Assembly). These three genes give rise to at least nine gene products through the use of three promoters, alternative translation start sites, and differential splicing. These coding sequences are flanked by the ITRs. The Rep gene encodes four proteins (Rep78, Rep68,

Rep52, and Rep40), while Cap expression gives rise to the viral capsid proteins (VP; VP1/VP2/VP3), which form the outer capsid shell that protects the viral genome, as well as being actively involved in cell binding and internalization. It is estimated that the viral coat is comprised of 60 proteins
 5 arranged into an icosahedral structure with the capsid proteins in a molar ratio of 1:1:10 (VP1:VP2:VP3).

Suitable AAV vectors can be a naturally occurring serotype of AAV including, but not limited to, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, artificial variants such as
 10 AAV.rh10, AAV.rh32/33, AAV.rh43, AAV.rh64R1, rAAV2-retro, AAV-DJ, AAV-PHP.B, AAV-PHP.S, AAV-PHP.eB, or other natural or engineered versions of AAV.

Twelve natural serotypes of AAV have thus far been identified, with the best characterized and most commonly used being AAV2. These
 15 serotypes differ in their tropism, or the types of cells they infect, making AAV a very useful system for preferentially transducing specific cell types. For example, AAV serotypes 1, 2, 5 or a hybrid capsid AAV1, AAV2, AAV5 or any combination thereof can be used for targeting brain or neuronal cells; AAV4 can be selected for targeting cardiac cells. AAV8 is
 20 useful for delivery to the liver cells. Researchers have further refined the tropism of AAV through pseudotyping, or the mixing of a capsid and genome from different viral serotypes. These serotypes are denoted using a slash, so that AAV2/5 indicates a virus containing the genome of serotype 2 packaged in the capsid from serotype 5. Use of these pseudotyped viruses
 25 can improve transduction efficiency, as well as alter tropism. For example, AAV2/5 targets neurons that are not efficiently transduced by AAV2/2, and is distributed more widely in the brain, indicating improved transduction efficiency.

An exemplary sequence of an expression vector, a plasmid in
 30 particular, containing a nucleic acid sequence encoding TccCas13a is:
 tcatgacccaaaatcccttaacgtgagttttcggtccactgagcgtcagacc
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5 gacgatagttaccggataaggcgcagcggctcgggctgaacggggggttcgt
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 20 tgcagcactggggccagatggtaagccctcccgatcgtagttatctacac
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 tatacttttagattgattttaaacttcatttttaattttaaaggatctaggt
 gaagatcctttttgataatc (SEQ ID NO:69) .

25 An exemplary sequence of an expression vector, a plasmid in
 particular, containing a nucleic acid sequence encoding mCas13a is:
 tttcctgtttggtcactgatgcctccgtgtaagggggatttctgttcatgg
 gggtaatgataccgatgaaacgagagaggatgctcacgatacgggttactg
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 30 tatggatgcggcgggaccagagaaaaatcactcaggggtcaatgccagcgt
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ATCCGTCAGATGTGCAACACGTACATCCAAGATGTGTTATGCATGAAGATG
GCGATGTGGCACTACGAGAAATTAATCTCCGCGACTGAGTTCCGTAACAAG

TTGGAGTGGAACATGCATTGGTCAGGGTAACATGGGTTATGAACGTTACTCA
CTGTGGTACAAGACCGGTTGCGGTGTGGTGATCCAATTCACGCCAGCTGAT
TTCCTGCGGCTGGACATTATTGAAAAACCGGCAATGATCGAGAACATCTGC
CAGTGTTTCGTGCTGGGCAACAAAAAGCTAAACAGCGGCGCGGAAAAAGAAA
5 ATCACTTGGGATAAATTCAACAAGGACGGCATAGCGAAATACCGTAAGCGC
CAGGCCGAGGCGGTGCGTGCGATCTTCGCGTTTGAAGAGGGTCTCAAGATC
CAAGAAGACAAATGGTCACACGAACGTTACTTCCCGTTTTGTAATATCTTG
GATGAAGCCGTGAAGCAGGGGAAAATCAAAGATACCGGTAAGGACAAAGAA
GCCTTAAATCGTGGTCGTAACGACTTTTTTCATGAAGAATTTAAAAGCACC
10 GAAGACCAGCAAGCGATTTTTTCAAAAATACTTCCCGATTGTTGAACGGAAG
GACGACACCAAAAAACGTCGCGATAAGAAACAGAAGTAAGcgggccgcactc
gaggcccgaaaggaagctgagttggctgctgccaccgctgagcaataacta
gcataacccttggggcctctaaacgggtcttgaggggttttttgcgtgaaa
ggaggaactatatccggatatcccgcaagaggcccggcagtaccggcataa
15 ccaagcctatgcctacagcatccagggtgacggtgccgaggatgacgatga
gcgcattgttagatttcatacacgggtgcctgactgcgttagcaatttaact
gtgataaactaccgcattaaagcttatcgatgataagctgtcaaactatgag
aattcttgaagacgaaagggcctcgtgatacgccctatttttataggttaat
gtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaat
20 gtgcgcggaaccctatttggtttatttttctaaatacattcaaatatgtat
ccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaagg
aagagtatgagtatcaacatttcctgtgcgccttattcccttttttgcg
gcattttgccttcctgtttttgctcaccagaaacgctggtgaaagtaaaa
gatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctc
25 aacagcggtgaagatccttgagagttttcgccccgaagaacgttttccaatg
atgagcactttttaagttctgctatgtggcgcggtattatcccggtgttgac
gccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttg
gttgagtactcaccagtcacagaaaagcatcttacggatggcatgacagta
agagaattatgcagtgcgtgccataaccatgagtataaactgcggccaac
30 ttacttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcac
aacatgggggatcatgtaactgccttgatcgttggaaccggagctgaat
gaagccataccaaacgacgagcgtgacaccacgatgcctgcagcaatggca
acaacgttgcgcaaactattaactggcgaactacttactctagcttcccg

caacaattaatagactggatggaggcggataaagttgcaggaccacttctg
cgctcgcccttccggctggctggtttattgctgataaatctggagccggt
gagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccc
tcccgatatcgtagttatctacacgacggggagtcaggcaactatggatgaa
5 cgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaa
ctgtcagaccaagtttactcatatatacttttagattgatttaaaacttcat
ttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgacc
aaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaa
aagatcaaaggatcttcttgagatccttttttctgcgcgtaaatctgctgc
10 ttgcaaacaaaaaaccaccgctaccagcgggtggtttggttgccggatcaa
gagctaccaactctttttcgaaggtaactggcttcagcagagcgcagata
ccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaac
tctgtagcacccgctacatacctcgctctgctaactcctgttaccagtggct
gctgccagtggcgataagtcgtgtcttaccgggttggaactcaagacgatag
15 ttaccggataaaggcgcagcggctcgggctgaacggggggttcgtgcacacag
cccagcttggagcgaacgacacctacaccgaactgagataacctacagcgtgag
ctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccg
gtaagcggcagggctcggaacaggagagcgcacgaggagcttccaggggga
aacgcctggtatctttatagtctgtcgggtttcgccacctctgacttgag
20 cgtcgatttttgtgatgctcgtcagggggcgagcctatggaaaaacgcc
agcaacgcggcctttttacggttcctggccttttgctggccttttgctcac
atgttctttcctgcgttatccctgattctgtggataaccgtattaccgcc
tttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgag
tcagtgagcaggaagcggaagagcgctgatgcggtattttctccttacg
25 catctgtgcggtatttcacaccgcaatgggtgcactctcagtacaatctgct
ctgatgccgcatagttaagccagtatacactccgctatcgctacgtgactg
ggatcatggctgcgccccgacacccgccaacacccgctgacgcgccctgacg
ggcttgtctgctcccgcatccgcttacagacaagctgtgaccgtctccgg
gagctgcatgtgtcagaggttttcaccgtcatcaccgaaacgcgcgaggga
30 gctgcggtaaagctcatcagcgtggctcgtgaagcgattcacagatgtctgc
ctgttcatccgcgtccagctcgttgagttttctccagaagcgtaaatgtctg
gcttctgataaagcgggccatgttaagggcggttt (SEQ ID NO:70).

mCas13 coding sequence is in capital letters, preceded by vector sequence and sequence encoding 6x His affinity tag, Thrombin site, Strep-tag II, SUMO, and followed by additional vector sequence in lowercase letters.

Suitable expression vectors for expressing the disclosed Cas13 effectors include vectors having a nucleotide sequence with at least 60% sequence identity to SEQ ID NO:69 or 70.

While the specifics can be varied in different engineered CRISPR systems, the overall methodology is similar. A practitioner interested in using the CRISPR/Cas13 technology to target an RNA sequence (identified using one of the many available online tools) can insert a short DNA fragment containing the target sequence into a guide RNA expression plasmid. The expression plasmid contains the target sequence (or spacer), one or more direct repeat sequences, as well as a suitable promoter and necessary elements for proper processing in a cell of interest. Such vectors are commercially available (see, for example, Addgene). Many of the systems rely on custom, complementary oligos that are annealed to form a double stranded DNA and then cloned into the crRNA expression plasmid. Co-expression of the crRNA(s) and the appropriate Cas13 enzyme from the same or separate plasmids in transfected cells can result in a target RNA cleavage (depending of the activity of the Cas enzyme) at the desired target site.

B. Cells

Vectors containing nucleic acids to be expressed can be transferred into host cells. The term “host cell” is intended to include prokaryotic and eukaryotic cells into which a recombinant expression vector can be introduced. As used herein, “transformed” and “transfected” encompass the introduction of a nucleic acid molecule (e.g., a vector) into a cell by one of a number of techniques. Although not limited to a particular technique, a number of these techniques are well established within the art. Prokaryotic cells can be transformed with nucleic acids by, for example, electroporation or calcium chloride mediated transformation. Nucleic acids can be transfected into mammalian cells by techniques including, for example, calcium phosphate co-precipitation, DEAE-dextran-mediated transfection,

lipofection, electroporation, or microinjection. Host cells (e.g., a prokaryotic cell or a eukaryotic cell such as a CHO cell) can be used to, for example, produce the antigenic polypeptides described herein.

Cells harboring the polynucleotides, vectors, proteins, RNPs, and/or
5 other compositions disclosed herein are also provided. For example,
prokaryotic or eukaryotic cells containing the polynucleotide and/or vector
are disclosed. The cell can be a mammalian cell. The mammalian cell can be
human or non-human mammal, e.g., primate, bovine, ovine, porcine, canine,
rodent, monkey, rat, or mouse cell. The cell can be a non-mammalian
10 eukaryotic cell such as poultry bird (e.g., chicken), vertebrate fish (e.g.,
salmon) or shellfish (e.g., oyster, clam, lobster, shrimp) cell. The cell can
also be a plant cell.

In preferred embodiments, the cell is a human cell including, but not
limited to, skin cells, lung cells, heart cells, kidney cells, pancreatic cells,
15 muscle cells, neuronal cells, human embryonic stem cells, blood cells (e.g.,
white blood cells), and pluripotent stem cells. The cell can be T cells (e.g.,
CD8+ T cells such as effector T cells, memory T cells, central memory T
cells, and effector memory T cells; or CD4+ T cells such as Th1 cells, Th2
cells, Th3 cells, Th9 cells, Th17 cells, Tfh cells, and Treg cells; or gamma-
20 delta T cells / gdT cells), hematopoietic stem cells (HSC), macrophages,
natural killer cells (NK), B cells, dendritic cells (DC), or other immune cells.

In some embodiments, the cell can be from established cell lines or
they can be primary cells, where “primary cells,” refers to cells and cells
cultures that have been derived from a subject and allowed to grow *in vitro*
25 for a limited number of passages or splittings of the culture.

The Cas13 compositions can be introduced to the cell through a
variety of techniques, including viral or non-viral approaches. For example,
the Cas13 and/or crRNA can be introduced via a viral vector (e.g., a
retrovirus such as a lentivirus, adenovirus, poxvirus, Epstein-Barr virus, or
30 adeno-associated virus (AAV)). Non-viral approaches such as physical
and/or chemical methods can also be used, including, but not limited to
cationic liposomes and polymers, DNA nanoclew, gene gun, microinjection,
transfection, electroporation, nucleofection, particle bombardment,

ultrasound utilization, magnetofection, conjugation to cell penetrating peptides, and/or nanoparticle mediated delivery. Such methods are described for example, in Nayerossadat N., et al., *Adv. Biomed. Res.*, 1:27 (2012) and Lino CA, et al., *Drug Deliv.*, 25(1):1234-1257 (2018). In view of the
 5 respective advantages and disadvantages of each method, a skilled artisan would be able to determine an optimal method for introduction of the elements of the Crispr/Cas13 system.

C. Exemplary Cas13 proteins, modifications and RNP complexes

Isolated Cas13 effector proteins are also provided. In some
 10 embodiments, the cas13 protein is produced by expressing a disclosed polynucleotide or expression vector in a desired cell, and optionally purifying the protein from the cell.

Preferably, the Cas13 proteins are thermostable enzymes. The working examples demonstrate that HheCas13a exhibits robust activity at
 15 temperatures up to 60 °C while TccCas13a maintained a robust activity at temperatures as high as ~ 70 °C. Thus, in some embodiments, the Cas13 enzyme exhibits ssRNA cleavage activity at a temperature of about 37-70 °C. In some embodiments, the Cas13 enzyme exhibits ssRNA cleavage activity at a temperature of about 47-60 °C. In some embodiments, the Cas13 enzyme
 20 exhibits ssRNA cleavage activity at a temperature of about 37-42 °C. In some embodiments, the Cas13 enzyme exhibits ssRNA cleavage activity at a temperature of about 60 °C.

In preferred embodiments, the Cas13 protein contains one or more Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) domains. In
 25 a preferred embodiment, the Cas13 protein includes two HEPN domains. An exemplary HEPN domain is a RxxxxH (SEQ ID NO:192) motif sequence, wherein X represents any amino acid. A HEPN domain can include at least one RxxxxH (SEQ ID NO:192) motif having the sequence of R[N/H/K]X₁X₂X₃H. In some embodiments, an HEPN domain includes a
 30 RxxxxH (SEQ ID NO:192) motif having the sequence of R[N/H]X₁X₂X₃H (SEQ ID NO:193). In an embodiment, a HEPN domain has the sequence of R[N/K]X₁X₂X₃H (SEQ ID NO:194). In certain embodiments, X₁ is R, S, D,

E, Q, N, G, Y, or H. In certain embodiments, X₂ is I, S, T, V, or L. In certain embodiments, X₃ is L, F, N, Y, V, I, S, D, E, or A.

i. Exemplary sequences

Preferably, the Cas13 protein is a Cas13a protein derived from
 5 thermophilic bacteria, preferably from *Thermoclostridium caenicola*
 (TccCas13a) or *Herbinix hemicellulosilytica* (HheCas13a). An exemplary
 amino acid sequence for HheCas13a is:
 MKLTRRRISGNSVDQKITAAFYRDMSQGLLYDSEDNDCTDKVIESMDFER
 SWRGRILKNGEDDKNPFYMFVKGLVGSNDKIVCEPIDVSDPDNLDILINK
 10 NLTGFGRNLKAPDSNDTLENLIRKIQAGIPEEEVLPELKKIKEMIQKDIVN
 RKEQLLKSIIKNNRIPFSLEGSKLV PSTKKMKWLFKLIDVPNKT FNEKMLEK
 YWEIYDYDKL KANITNRLDKTDKKARSISR AVSEELREYHKNLRTNYNRFV
 SGDRPAAGLDNGGSAKYNPDKEEFLLFLKEVEQYFKKYFPVSKSHSNKSKD
 KSLVDKYKNYCSYKVVKKEVNRSIINQLVAGLIQQGKLLYFYNDTWQED
 15 FLNSYGLSYIQVEEAFKKSVM TSLSWG INRLTSFFIDDSNTVKFDDITTKK
 AKEAIESNYFNKLRTC SRMQDHFKEKLAFFYPVYVKDKKDRPDDDIENLIV
 LVKNAIESVSYLNRNRTFHFKESSLELLKELDDKNSGQNKIDYSVAAEFIK
 RD IENLYDVFREQIRSLGIAEYYKADMISDCFKTCGLEFALYSPKNSLMPA
 FKNVYKRGANLNKAYIRDKGPKETGDQGQNSYKALEEYREL TWYIEVKNN
 20 QSYNAYKNLLQLIYYHAF LPEVRENEALITDFINRTKEWNRKETEERLNTK
 NNKKHKNFDENDITVNTYRYESIPDYQGESLDDYLKVLQRKQMARAKEVN
 EKEEGNNNYIQFIRDVVWAFGAYLENKLKNYKNELQPPLSKENIGLNDTL
 KELFPEEKVKSPFNIKCRFSISTFIDNKGKSTDNTSAEAVKTDGKEDEKDK
 KNIKRKDLLCFYLFRLLDENEICKLQHQFIKYRCSLKERRFPGNRTKLEK
 25 ETELLAELEELMELVRFTMP SIPEISAKAESGYDTMIKKYFKDFIEKKVFK
 NPKTSNLYYHSDSKTPVTRKYMALLMRSAPLHLYKDIFKGYLITKKECLE
 YIKLSNIIKDYQNSLNELHEQLERIKLKSEKQNGKDSLYLDKKDFYKVKEY
 VENLEQVACYKHLQHKINFESLYRIFRIHVDIAARMVGYTQDWERDMHFLE
 KALVYNGVLEERRFEAIFNNNDNDNDGRIVKKIQNNLNNKNRELVSMLCWN
 30 KKLNKNEFGAI IWKRNP IAHLNHFTQTEQNSKSSLESLSLIRILLAYDRK
 RQNAVTKTINDLLLNDYHIRIKWEGRVDEGQIYFNIKEKEDIENEP I IHLK
 HLHKKDCYIYKNSYMFDKQKEWICNGIKEEVYDKSILKCIGNLKF KDYEDK
 NKSSANPKHT (SEQ ID NO:68).

An exemplary amino acid sequence for TccCas13a is:

MKITKRKWGEHHPPLYFYRDEDSGRLLAQNDRKQDYDTLFDNDIAQDTFER
 SLRNRLKLTPEKGDGRFYSNEIVKLVEKLCQGADVAEIMKSMERNEKLRPK
 NEKEIKNLKKQLDGTLSYEGKRYTAPEGAMTLNDALFYLVVEGNPLKQAMAK
 5 AELGKIREALIKEKENRINRVYSIKNNKIPLRIQEDGGITPNNDRAAWLL
 GLMKPADPAKGITDCYPLLGELEEVDFDKLSKTLHEKISRCQGRPRSIAM
 AVDEALKQYLRELWEKSPSRQQDLKYFQAVQYFQKDNFP IRTKRMGARLR
 QELLKDKTSLSRLLPEKHMNAVRRRLINQSTQMHILYGKLYAYCCGEDGR
 LLVNSETLQRIQVHEAVKKQAMTAVLWSISRLRYFYQFEDGDILSNKNPIK
 10 DFRDKFLRDTNKYTHEDVEACKEKLQDFFPLKELQEKIKEDAKGLQETDNK
 QADTTDFKAIGHIVRDDRKLCLNQLLAECVSCIGELRHHIFHYKNVTLIQAL
 KRIADKVKPEDLSVLRAIYLLDRNLKKAFAKRISMNPLYYREDLLSRI
 FKKEGTAFFLYSAKIQTSPSFQRVYERGKNLRREFECERMKAEEASNGQNGQ
 DGDRLKWFRQLAAGDSADTHFNWAVEAYAESAADVENNVEFDTDVDAQRAL
 15 RNLLLLLIYRHHFLPEVQKDETLVTGKIHKVLEERNRQLSEGQGPNGKAHGY
 SVIEELYHEGMPLSDLMKQLQRRISETERESRELAQEKTDYAQRFIELDIFA
 EAFNDFLEAHYGEEYLEIMSPRKDAEAAKKWVKESKTVDLKTSIDEKEPEG
 HLLVLYPVLRLLDERELGELQQQMIRYRTSLASWQGESNFSEEIRIAGQIE
 ELTELVKLTEPEPQFAEEVWGKRAKEAFEDFIEGNMKNYEAFYLQSDNNTF
 20 VYRRNMSRLLRSGLMGVYQKVLASHKQALKRDYLLWSEKHWNVVDENGADI
 SSAEQAQCLLQRLHRKYAESPSRFTEEDCKLYEKVLRRLLEDYNQAVKNLSF
 SSLYEICVLNLEILSRWVGFDWVERDYMFLLLAWVRQKLDGIKEEDVRD
 IFSEGNIIRNLVDTLKGEMNAFESVYFPENKGSKYLGVNRNDVAHLDLMRK
 NGWRLEAGKTCVMEDYINRLRFLSYDQKRMNAVTKTLQQIFDRHKVKIR
 25 FTVEKGGMLKIEDVTADKIVHLKGSRLSGIEIPSHGERFIDTLKALMVYPR
 G (SEQ ID NO:64; Genbank Accession No.: WP_149678719.1).

In some preferred embodiments, the Cas13 protein is a Cas13 protein
 originated, isolated or derived from a bacterium in the Proteobacteria
 phylum. An exemplary Proteobacteria-derived Cas13 is mCas13 having the
 30 following amino acid sequence:

MGIDYSLTSDCYRGINKSCFAVALNIAVDNCDHKGCRLLSEVLRSGGGIS
 DEQIKSQVVDGIQKRLKDIRNYFSHYHAEDCLRFQDQDAVKVFLEEIYKN
 AESKTVGATKESDYKGVVPLFELHNGTYMITAAGVIFLASFFCHRSNVYR

MLGAVKGFKHTGKEQLSDGQKRDYGFTRRLAYYALRDSYSVGAEDKTRCF
 REILSYLSRVPQLAVDWLNEQQLLTPEEKEAFLNQPAEDEGGDISDSSSSD
 KNKKSKEKRRSLRRDEKFILFAIQFIEGWAAEQGLDVTFARYQKTVEKAEN
 KNQDGKQARAVQLKYRNQGLNPDPFNNEWMYYIQNEHAI IQIKLNNKKAVAA
 5 RISENELKYLVL LIFEEKGNDAVQKLNCYIYSMSQKIEGEWKHRPEDERWM
 PSFTKRADRTVTPEAVQSRLSYIRKQLQETIEKIGQEEPRNNKWL IYKGGK
 ISMILKFISDSIRDIQRRPNVKQYHILRDALQRLDFDGFYKELQNYVNDGR
 IAVSLYDQIKGVNDISGLCKKVCELTLERLAGLEAKNGSELRRYIGLEAQE
 KHPKYGEWNTLQEKAKRFLESQFSIGKNFLRKM FYGDCCQKRCFDEEKGYN
 10 TQAKERKSLYSIVKEKLKDIKPIHDDRWYLIDRNPKNYDNKHSRIIRQMCN
 TYIQDVLCKMMAMWHYEKLISATEFRNKLEWNCIGQGNMGYERYSLWYKTG
 CGVVIQFTPADFLRLDIIEKPAMIENICQCFVLGNKKLNSGAEKKITWDKF
 NKDGIAYRKRQAEAVRAIFAEEGLKIQEDKWSHERYFPFCNILDEAVKQ
 GKIKDTGKDKEALNRGRNDDFFHEEFKSTEDQQAIFQKYFPIVERKDDTKKR
 15 RDKKQK (SEQ ID NO:66; GenBank accession No.: HFH51004.1).

Thus, in some embodiments, the Cas13 protein includes the amino acid sequence of SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68. In some embodiments, the Cas13 protein includes an amino acid sequence with at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or
 20 99% sequence identity to SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68 or Uniprot accession numbers disclosed herein, and including nucleic acid sequences encoding amino acid sequences thereof.

ii. Cas13 variants and modifications

It should be appreciated that also disclosed are Cas13 effector protein
 25 variants including one or more mutations (e.g., conservative or non-conservative mutations) relative to any of the Cas effector proteins disclosed herein. For example, it is also contemplated that other Cas13 variants can be evolved from those disclosed herein, for example, by targeted mutation of one or more amino acid residues in specific regions of the enzyme. Such
 30 mutation(s) may alter substrate binding, alter conformation of bound substrate, alter substrate accessibility to the active site, alter tolerance to non-optimal presentation of a target sequence to the active site, and/or alter target sequence specificity (recognition). In some embodiments, a suitable Cas13

effector protein has an amino acid sequence that has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 21, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more mutations compared to any one of the amino acid sequences set forth in SEQ ID NO:64, 66 or 68.

In some embodiments, a disclosed Cas13 protein (e.g., encoded by a polynucleotide or vector, contained in an RNP, or expressed in a cell) is mutated with respect to a corresponding wild-type protein. In certain embodiments, the Cas13 is engineered and can include one or more mutations that reduce or eliminate a nuclease activity. Mutations can also be made at neighboring residues, e.g., at amino acids near those that participate in the nuclease activity. In some embodiments, one HEPN domain is inactivated. In other embodiments, a second HEPN domain is inactivated.

The mutated Cas13 enzyme can have abolished or reduced ability to cleave a target polynucleotide containing a target sequence. In some embodiments, a Cas13 enzyme is considered to substantially lack all cleavage activity when the RNA cleavage activity of the mutated enzyme is less than about 25%, 10%, 5%, 1%, 0.1%, 0.01%, or lower with respect to its non-mutated form.

RNA cleavage is mediated by catalytic residues in the two conserved HEPN domains of Cas13. Therefore, catalytically dead or inactive Cas13 mutants can be generated by mutating one or more catalytic residues within the HEPN domains (e.g., via Alanine substitution). Such mutations can render the Cas13 as a catalytically inactive RNA-binding protein. In particular embodiments, the one or more mutated residues are one or more residues in the disclosed Cas13 proteins that correspond to R597, H602, R1278 and H1283 of *Leptotrichia shahii* Cas 13 (LshCas13), such as mutations R597A, H602A, R1278A and H1283A.

The Cas13 proteins may be modified in various ways. In some embodiments, the modification(s) may render the protein more stable (e.g., resistant to degradation *in vivo*) or more capable of penetrating into cells or subcellular compartments, or other desirable characteristic as will be appreciated by one skilled in the art. Such modifications include, without

limitation, chemical modification, N terminus modification, C terminus modification, peptide bond modification, backbone modifications, residue modification, D-amino acids, or non-natural amino acids or others. In some embodiments, one or more modifications may be used simultaneously.

5 The modifications may or may not cause an altered functionality. By means of example, modifications which do not result in an altered functionality include for instance codon optimization for expression into a particular host, or providing the Cas13 with a particular marker or epitope tag (e.g. for visualization and/or isolation or purification).

10 In some embodiments, a disclosed Cas13 protein can be fused to or operably linked to domains which include but are not limited to a transcriptional activator, a transcriptional repressor, a recombinase, a transposase, a histone remodeler, a DNA methyltransferase, a cryptochrome, a light inducible/controllable domain, a chemically inducible/controllable domain, a detectable marker, or a deaminase domain. Exemplary detectable markers include fluorescent proteins, such as, GFP, eGFP, RFP, YFP, BFP, CFP, mNeonGreen, mCherry, mOrange, mRaspberry, mPlum, mKO, mRFP, mRFPruby, mRuby, tagRFP, mKate2, and DsRed.

20 In some embodiments, the Cas13 is covalently linked to the one or more domains. In some embodiments, the Cas13 is covalently linked to the one or more domains via one or more linkers (e.g., peptide linkers). In some embodiments, the Cas13 protein is a fusion protein including the Cas13 effector and one or more domains, such as those described herein.

25 Exemplary deaminase domains include deaminase domains of the RNA-dependent Adenosine Deaminases (ADAR) such as ADAR1, ADAR2, and ADAR3, e.g., human ADAR1, ADAR2, and ADAR3. The ADARs catalyze the hydrolytic deamination of adenosine to inosine in double-stranded RNA (dsRNA) in a process generally referred to as A-to-I RNA editing. Modified ADAR deaminase domains in which the adenosine deaminase activity is converted to cytidine deaminase activity are known and are contemplated herein. See for example Published PCT Application No.: WO 2019/071048, which is hereby incorporated by reference in its entirety.

In some embodiments, the Cas13 protein includes one or more nuclear localization sequences (NLSs). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. In general, the one or more NLSs are of sufficient strength to drive accumulation of the Cas13 enzyme in a detectable amount in the nucleus of a eukaryotic cell. In general, strength of nuclear localization activity may derive from the number of NLSs in the CRISPR enzyme, the particular NLS(s) used, or a combination of these factors.

In some embodiments, the Cas13 protein can be modified with one or more (e.g., two or more, three or more, or four or more) mitochondrial localization sequences (MLSs). Any convenient mitochondrial localization sequence can be used. Examples of mitochondrial localization sequences include a mitochondrial localization sequence of SDHB, mono/di/triphenylphosphonium or other phosphoniums, VAMP 1A, VAMP 1B, the 67 N-terminal amino acids of DGAT2, and the 20 N-terminal amino acids of Bax. The MLS(s) can be placed at the N- or C-termini of the Cas13 protein.

iii. Cas13 RNPs

In some embodiments, the Cas13 protein is complexed with an RNA including or consisting of a crRNA sequence (e.g., a gRNA, sgRNA, etc.), thereby forming a ribonucleoprotein (RNP) complex. The crRNA may be capable of hybridizing to a target RNA sequence, such as an mRNA, lincRNA, or viral RNA (e.g., SARS-Cov-2 N or E gene transcript). The crRNA can include a spacer sequence that is capable of hybridizing to the target RNA sequence and a direct repeat sequence.

In certain embodiments, the guide RNA or crRNA is or includes a direct repeat sequence linked to a guide sequence or spacer sequence. In certain embodiments the guide RNA or mature crRNA includes about 19

nucleotides of partial direct repeat followed by 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides of guide or spacer sequence, such as 18-30, 20-25, 25-30, 21-25, 22-25, 23-25, or 25-28 nucleotides of guide or spacer sequence. In some preferred embodiments, the crRNA includes a
 5 spacer of about 20-30 nucleotides. In some preferred embodiments, the crRNA includes a spacer of about 24-28 nucleotides. In some embodiments, the crRNA contains a stem loop or other secondary structure. In preferred embodiments the crRNA contains a stem loop or other secondary structure in the direct repeat sequence, wherein the stem loop or secondary structure is
 10 important for cleavage activity.

Exemplary crRNAs that can be included in the Cas13 RNP complex are crRNAs encoded by the nucleic acid sequence of SEQ ID NOs:20-37 and 41-61, sequences having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity thereto, nucleic acid sequences having
 15 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleic acid substitution(s), addition(s), deletion(s), or a combination thereof relative thereto, or the reverse complement of any of the foregoing.

III. Methods of Manufacture

A. *Methods for Producing Cas13 Proteins*

20 Isolated Cas13 polypeptides and fusions proteins thereof can be obtained by, for example, chemical synthesis or by recombinant production in a host cell. To recombinantly produce the protein, a nucleic acid containing a nucleotide sequence encoding the polypeptide can be used to transform, transduce, or transfect a bacterial or eukaryotic host cell (e.g., an
 25 insect, yeast, or mammalian cell). In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleotide sequence encoding a Cas13 polypeptide. Regulatory sequences (also referred to herein as expression control sequences) typically do not encode a gene product, but instead affect the expression of the nucleic acid sequences to which they are
 30 operably linked.

Useful prokaryotic and eukaryotic systems for expressing and producing polypeptides are well known in the art and include, for example,

Escherichia coli strains such as BL-21, and cultured mammalian cells such as CHO cells.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express antigenic polypeptides or fusions proteins. Viral based expression systems are well known in the art and include, but are not limited to, baculoviral, SV40, retroviral, or vaccinia based viral vectors.

Mammalian cell lines that stably express Cas13 polypeptides or fusions proteins can be produced using expression vectors with appropriate control elements and a selectable marker. For example, the eukaryotic expression vectors pCR3.1 (Invitrogen Life Technologies) and p91023(B) (see Wong *et al.* (1985) *Science* 228:810-815) are suitable for expression of variant costimulatory polypeptides in, for example, Chinese hamster ovary (CHO) cells, COS-1 cells, human embryonic kidney 293 cells, NIH3T3 cells, BHK21 cells, MDCK cells, and human vascular endothelial cells (HUVEC). Following introduction of an expression vector by electroporation, lipofection, calcium phosphate, or calcium chloride co-precipitation, DEAE dextran, or other suitable transfection method, stable cell lines can be selected (e.g., by antibiotic resistance to G418, kanamycin, or hygromycin). The transfected cells can be cultured such that the polypeptide of interest is expressed, and the polypeptide can be recovered from, for example, the cell culture supernatant or from lysed cells. Alternatively, a antigenic polypeptides and fusion proteins can be produced by (a) ligating amplified sequences into a mammalian expression vector such as pcDNA3 (Invitrogen Life Technologies), and (b) transcribing and translating *in vitro* using wheat germ extract or rabbit reticulocyte lysate.

Cas13 polypeptides and fusion proteins can be isolated using, for example, chromatographic methods such as DEAE ion exchange, gel filtration, and hydroxylapatite chromatography. For example, Cas13 proteins in a cell culture supernatant or a cytoplasmic extract can be isolated using a protein G column. In some embodiments, Cas13 proteins are “engineered” to contain an amino acid sequence that allows the polypeptides to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, or FlagTM (Kodak) can be used to aid

polypeptide purification. Such tags are typically inserted at either the carboxyl or amino terminus. Other fusions that can be useful include enzymes that aid in the detection of the polypeptide, such as alkaline phosphatase. Immunoaffinity chromatography also can be used to purify antigenic polypeptides and fusions proteins.

B. Methods for Producing Isolated Nucleic Acid Molecules

Isolated nucleic acid molecules encoding Cas13 polypeptides and fusion proteins can be produced by standard techniques, including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, polymerase chain reaction (PCR) techniques can be used to obtain an isolated nucleic acid encoding an Cas13 polypeptide. PCR is a technique in which target nucleic acids are enzymatically amplified. Typically, sequence information from the ends of the region of interest or beyond can be employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers typically are 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, ed. by Dieffenbach and Dveksler, Cold Spring Harbor Laboratory Press, 1995. When using RNA as a source of template, reverse transcriptase can be used to synthesize a complementary DNA (cDNA) strand. Ligase chain reaction, strand displacement amplification, self-sustained sequence replication or nucleic acid sequence-based amplification also can be used to obtain isolated nucleic acids. See, for example, Lewis (1992) *Genetic Engineering News* 12:1; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878; and Weiss (1991) *Science* 254:1292-1293.

Isolated nucleic acids can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides (e.g., using phosphoramidite technology for automated DNA synthesis in the 3' to 5' direction). For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with

each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase can be used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per

5 oligonucleotide pair, which then can be ligated into a vector.

IV. Methods of Use

Disclosed herein are various methods related to the disclosed compositions and reagents (including Cas13 proteins, Cas13 fusion proteins and other engineered variants, crRNAs, etc.) and their use. For example,
10 disclosed are methods of perturbing (e.g., reducing or increasing) gene expression, deaminating a target nucleic acid (RNA editing), monitoring trafficking of RNA molecules in cells, etc.

Disclosed is a method of modifying a target locus of interest, in particular in eukaryotic cells, tissues, organs, or organisms, more in
15 particular in mammalian cells, tissues, organs, or organisms. The method involves delivering to said locus a disclosed Cas13 effector protein and one or more crRNA components (i.e., an RNA containing a crRNA), wherein the Cas13 protein forms an RNP complex with the one or more crRNAs components. Upon binding of the complex to the locus of interest, the Cas13
20 effector protein induces the modification of the target locus of interest depending on the Cas13's activity. In a preferred embodiment, the modification is the introduction of a strand break (e.g., cleavage). In some embodiments, the modification is a deamination of a target nucleotide. The complex can be formed *in vitro* or *ex vivo* and introduced into a cell or
25 contacted with RNA; or can be formed *in vivo*.

Cas13 is capable of robust RNA detection. In certain embodiments, Cas13 is converted to an RNA binding protein ("dead Cas13") by inactivation of its nuclease activity. Converted to an RNA binding protein, Cas13 is useful for localizing other functional components to RNA in a
30 sequence dependent manner. The components can be natural or synthetic. Dead Cas13 can be used to (i) bring effector modules to specific transcripts to modulate the function or translation, which could be used for large-scale screening, construction of synthetic regulatory circuits and other purposes;

(ii) fluorescently tag specific RNAs to visualize their trafficking and/or localization; (iii) alter RNA localization through domains with affinity for specific subcellular compartments; and (iv) capture specific transcripts (through direct pull down of dead Cas13 or use of dead Cas13 to localize
5 biotin ligase activity to specific transcripts) to enrich for proximal molecular partners, including RNAs and proteins.

It will be appreciated that the small size of the mCas13 protein can allow easier packaging into commonly used delivery vectors, such as adeno-associated viruses (AAV) and thus easier delivery of this protein *in vivo* for
10 various RNA targeting and manipulation as well as therapeutic applications. Such applications include, without limitation, RNA knockdown, RNA editing, antiviral activity, and RNA tagging and imaging.

Furthermore, the discovery of the thermostable enzymes has allowed the development of various biotechnological applications at elevated
15 temperature, such as, the development of nucleic acid detection methods. Besides the one-pot assay described herein, the thermophilic TccCas13a is useful in developing different detection modules for nucleic acids, and possibly other molecules. In particular, the disclosed TccCas13a protein allows for various RNA targeting and manipulation applications in various
20 prokaryotic and eukaryotic organisms, such as RNA knockdown, RNA editing, antiviral activity, and RNA tagging and imaging. The robust thermostability of TccCas13a can also allow all of the aforementioned applications in thermophilic organisms. This is an advantage of TccCas13a over all other known Cas13 enzymes.

25 Besides SARS-CoV-2 detection, both mCas13 and TccCas13 nucleic acid detection modules can be developed for the detection of other pathogens, including any DNA and RNA viruses, bacteria and others.

A. *Targeted RNA knockdown*

The disclosed Cas13 based compositions can be used to reduce or
30 inhibit expression of an RNA transcript. RNA knockdown with Cas13 can be applied to perturbing RNAs in multiple biological contexts, including genome-wide pooled knockdown screening, interrogation of LincRNA and

nascent transcript function, allele-specific knockdown, and RNA viral therapeutics.

Such inhibitory effects (i.e., targeted knockdown of an RNA transcript) can be achieved by introducing a Cas13 to a cell, wherein the

5 Cas13 is complexed with a crRNA that targets (e.g., is complementary to or hybridizes to) the RNA transcript, thereby inducing cleavage of the RNA transcript by the Cas13 protein. Any RNA can be targeted for cleavage. For example, in some embodiments, the RNA transcript can be endogenous or exogenous to a cell. In some embodiments, the RNA transcript can be coding

10 (e.g., an mRNA) or non-coding (e.g., lincRNA). In some embodiments, the RNA transcript is derived from a viral gene, such as a bacteriophage. Thus, the Cas13 compositions can be used to inhibit or reduce viral gene expression or viral replication.

Current RNA knockdown strategies such as siRNA have the

15 disadvantage that they are mostly limited to targeting cytosolic transcripts since the protein machinery is cytosolic. An advantage of a Cas13 mediated knockdown is that it is an exogenous system that is not essential to cell function, and it can be used in any compartment in the cell. By fusing a NLS signal to the Cas13, it can be guided to the nucleus, allowing nuclear RNAs

20 to be targeted, e.g., to probe the function of lincRNAs. Long intergenic non-coding RNAs (lincRNAs) are a vastly underexplored area of research. Most lincRNAs have as of yet unknown functions which could be studied using the RNA targeting effector protein of the invention. Knockdown of RNA transcript relies on cleavage of the targeted transcripts by the endogenous

25 RNase activity of the dual HEPN domains of the Cas13 protein, the efficiency of which varies between different orthologs and subtypes of Cas13.

In certain embodiments, modulation of cleavage efficiency can be exploited by introduction of mismatches, e.g., 1 or more mismatches, such as

30 1 or 2 mismatches between the spacer sequence and target sequence, including the position of the mismatch along the spacer/target. In some embodiments, the more central (i.e., not 3' or 5') for instance a mismatch is, the more cleavage efficiency is affected. In some embodiments, the more

terminal (i.e., 3' or 5') a mismatch is, the more cleavage efficiency is affected. Accordingly, by choosing a mismatch position along the spacer, cleavage efficiency can be modulated. By means of example, if less than 100 % cleavage of targets is desired (e.g. in a cell population), 1 or more, such as
 5 2 mismatches between spacer and target sequence may be introduced in the spacer sequences.

B. RNA imaging and/or localization

Methods to monitor RNA trafficking are also provided. For example, the Cas13 proteins can be used to effectively fluorescently tag specific RNAs
 10 to visualize their trafficking and/or localization. Typically, described herein is a method of determining the localization of an RNA transcript in a cell by introducing the Cas13 composition to the cell. In such embodiments, the Cas13 is preferably catalytically inactive (e.g., the Cas13 functions as an RNA binding protein without nuclease activity). The Cas13 can be linked
 15 (e.g., as a fusion protein with or without a linker) to a detectable marker. The Cas13-crRNA RNP targets and hybridizes to the targeted RNA transcript, thereby indicating the location and/or movement of the RNA transcript within the cell. Exemplary detectable markers that can be used to visualize the targeted RNA transcript include GFP, eGFP, RFP, YFP, BFP, CFP,
 20 mNeonGreen, mCherry, mOrange, mRaspberry, mPlum, mKO, mRFP, mRFPPruby, mRuby, tagRFP, mKate2, and DsRed.

C. RNA editing

Also disclosed is a method for performing targeted editing of an RNA transcript by introducing a catalytically inactive Cas13 that further
 25 includes a deaminase domain of an ADAR to a cell. The Cas13-crRNA RNP is capable of hybridizing with a region in an RNA transcript that contains an A nucleotide to be edited. The crRNA forms an RNA duplex with the RNA transcript wherein the duplex contains an A-C mismatch at the target A nucleotide resulting in the target A nucleotide being deaminated by the
 30 deaminase domain (resulting in A-I). Suitable deaminase domains include deaminase domains from ADAR1, ADAR2, and ADAR3, such as human ADAR1, ADAR2, and ADAR3. Modified ADAR deaminase domains in which the adenosine deaminase activity is converted to cytidine deaminase

activity are known and are contemplated herein. Transitions in the RNA bases of a given transcript can be directly induced by using adenine (ADAR1/2), APOBEC) or cytosine deaminases (AID) which convert A to I or C to U, respectively.

5 D. *RNA isolation or purification, enrichment or depletion*

The Cas13 can be used as an RNA targeting effector protein when complexed to a target RNA to isolate and/or purify the target RNA. The Cas13 protein can for instance be fused to an affinity tag that can be used to isolate and/or purify the RNA-Cas13 protein complex. Such applications are
10 for instance useful in the analysis of gene expression profiles in cells. In particular embodiments, the Cas13 proteins can be used to target a specific noncoding RNA (ncRNA) thereby blocking its activity, providing a useful functional probe. In certain embodiments, the Cas13 protein may be used to specifically enrich for a particular RNA (including but not limited to
15 increasing stability, etc.), or alternatively to specifically deplete a particular RNA (such as without limitation for instance particular splice variants, isoforms, etc.).

 E. *Identification of RNA binding proteins*

Identifying proteins bound to specific RNAs can be useful for
20 understanding the roles of many RNAs. For instance, many lincRNAs associate with transcriptional and epigenetic regulators to control transcription. Understanding what proteins bind to a given lincRNA can help elucidate the components in a given regulatory pathway. A Cas13 effector protein can be designed to recruit a biotin ligase to a specific transcript in
25 order to label locally bound proteins with biotin. The proteins can then be pulled down and analyzed by mass spectrometry to identify them.

 F. *Detection of nucleic acids*

The Cas13 proteins can be used for detection of nucleic acids or proteins in a biological sample. The samples can be cellular or cell-free. For
30 example, northern blotting involves the use of electrophoresis to separate RNA samples by size. The Cas13 protein can be used to specifically bind and detect the target RNA sequence.

Cas13 protein complexes with the guide RNA via recognition of a short hairpin in the crRNA, and target specificity is encoded by the spacer that is complementary to the target region. In addition to programmable RNase activity, all Cas13s exhibit “collateral activity” after recognition and
5 cleavage of a target transcript, leading to non-specific degradation of any nearby transcripts regardless of complementarity to the spacer. This collateral activity can be referred to as trans activity because the Cas13 is activated to cleave other RNA species in trans without sequence specificity. Conversely, the activity toward the targeted RNA transcript (e.g., having
10 some complementarity to the spacer sequence of the crRNA) can be referred to as cis activity. The disclosed Cas13 based detection assay take advantage of this cis and trans Cas13 activity.

Cas13 proteins such as mCas13, TccCas13a, and HheCas13a can cleave non-targeted single stranded RNA (ssRNA) once activated by
15 detection of a target RNA. Once a type VI CRISPR/Cas effector protein (e.g., mCas13, TccCas13a, or HheCas13a) is activated by a crRNA, which occurs when the crRNA hybridizes to a target sequence of a target RNA (i.e., the sample includes the targeted RNA), the protein becomes a nuclease that cleaves non-target ssRNAs (i.e., ssRNAs to which the spacer sequence of the
20 crRNA does not hybridize). Thus, when the target DNA or RNA transcribed therefrom is present in the sample (e.g., in some cases above a threshold amount), the result is cleavage of ssRNAs in the sample, which can be detected using any convenient detection method (e.g., using a labeled ssRNA oligonucleotide).

25 Disclosed is a method of detecting the presence of an RNA transcript in a nucleic acid sample by contacting the sample with the Cas13 composition in the presence of an activatable single stranded RNA (ssRNA) oligonucleotide that includes a reporter moiety. In some embodiments, the reporter moiety includes a fluorophore linked to a quencher via the ssRNA.
30 Fluorescence can be emitted upon cleavage of the ssRNA oligonucleotide since the quencher is no longer in proximity to the fluorophore. Preferably, the crRNA is designed to hybridize to the RNA transcript and the Cas13 cleaves the ssRNA oligonucleotide upon binding of the Cas13 crRNA

complex to the RNA transcript. Detecting cleavage of the ssRNA oligonucleotide indicates the presence of the RNA transcript. The RNA transcript could have been generated by transcription from a dsDNA molecule, and in some embodiments, the dsDNA molecule could have been
5 generated by reverse transcription coupled amplification of a target RNA.

In a specific embodiment, a Cas13-based method of detecting the presence of a nucleic acid in a sample involves (a) performing an amplification reaction (e.g., LAMP, RPA) on nucleic acids derived from the sample to generate a specific amplification product; (b) transcribing the
10 amplification product to generate an RNA transcript; (c) contacting the RNA transcript with a Cas13-crRNA RNP complex having a crRNA complementary to the RNA transcript; in the presence of an activatable ssRNA oligonucleotide; and (d) detecting cleavage of the ssRNA oligonucleotide by the Cas13 enzyme. In some embodiments, transcription
15 of the amplification product in step (b) is mediated by a T7 RNA polymerase *in vitro*. Preferably, cleavage of the ssRNA oligonucleotide is dependent on or subsequent to binding of the RNP to the RNA transcript. In some embodiments, cleavage of the ssRNA oligonucleotide results in release of a previously quenched fluorescent signal, thereby indicating the presence of
20 the nucleic acid.

The Cas13-based detection assay can be performed as a one-pot or two-pot assay depending on the Cas13 enzyme that is used. In some embodiments when TccCas13a or HheCas13a is used, the assay can be performed as a one-pot assay in which isothermal amplification (e.g., LAMP,
25 optionally coupled with reverse transcription (i.e., RT-LAMP)) of the target nucleic acids, coupled with T7-mediated *in vitro* transcription and Cas13-based detection of the amplified and *in vitro*-transcribed target RNA is carried out in the same tube.

In some embodiments, because of its incompatibility with the
30 elevated temperatures required for the isothermal amplification (e.g., RT-LAMP) reaction, mCas13 can be preferably used in a two-pot assay. In the two-pot assay, the RT-LAMP reaction is performed in a first tube while T7-

mediated *in vitro* transcription and mCas13-based detection of the amplified and *in vitro*-transcribed target RNA is performed in a second tube.

In preferred embodiments, the sample is or the nucleic acids are derived from mucus, sputum (processed or unprocessed), bronchial alveolar lavage (BAL), bronchial wash (BW), bodily fluids, cerebrospinal fluid (CSF), urine, tissue (e.g., biopsy material), rectal swab, nasopharyngeal aspirate, nasopharyngeal swab, throat swab, sputa/deep throat saliva, feces, mucosal excretions, plasma, serum, or whole blood. In some embodiments, the sample is a nucleic acid isolated and/or derived from any of the foregoing biological samples.

The methods may be used for any purpose for which detection of viral, bacterial or other nucleic acids is desirable, including diagnostic and prognostic applications, such as in laboratory and clinical settings. In some embodiments, the methods may be used for detection of a nucleic acid for genotyping. In some embodiments, the nucleic acid to be detected is diagnostic for a disease state. The disease state can be an infection, an organ disease, a blood disease, an immune system disease, a cancer, a brain and nervous system disease, an endocrine disease, a pregnancy or childbirth-related disease, an inherited disease, or an environmentally-acquired disease, cancer, or a fungal infection, a bacterial infection, a parasite infection, or a viral infection. Thus, in some embodiments, the method is useful for detecting a nucleic acid (e.g., DNA or RNA) from a bacterium, fungus, virus, or parasite.

Exemplary viruses that can be detected include, without limitation, Myoviridae, Podoviridae, Siphoviridae, Alloherpesviridae, Herpesviridae (including human herpes virus, and Varicella Zoster virus), Malcoherpesviridae, Lipothrixviridae, Rudiviridae, Adenoviridae, Ampullaviridae, Ascoviridae, Asfarviridae (including African swine fever virus), Baculoviridae, Cicaudaviridae, Clavaviridae, Corticoviridae, Fuselloviridae, Globuloviridae, Guttaviridae, Hytrosaviridae, Iridoviridae, Marseilleviridae, Mimiviridae, Nudiviridae, Nimaviridae, Pandoraviridae, Papillomaviridae, Phycodnaviridae, Plasmaviridae, Polydnaviruses, Polyomaviridae (including Simian virus 40, JC virus, BK virus), Poxviridae

(including Cowpox and smallpox), Sphaerolipoviridae, Tectiviridae, Turriviridae, Dinodnavirus, Salterprovirus, Rhizidovirus, a Coronaviridae virus, a Picornaviridae virus, a Caliciviridae virus, a Flaviviridae virus, a Togaviridae virus, a Bornaviridae, a Filoviridae, a Paramyxoviridae, a

5 Pneumoviridae, a Rhabdoviridae, an Arenaviridae, a Bunyaviridae, an Orthomyxoviridae, or a Deltavirus. In some embodiments, the virus is coronavirus (e.g., SARS-Cov-2), SARS, Poliovirus, Rhinovirus, Hepatitis A, Norwalk virus, Yellow fever virus, West Nile virus, Hepatitis C virus, Dengue fever virus, Zika virus, Rubella virus, Ross River virus, Sindbis

10 virus, Chikungunya virus, Borna disease virus, Ebola virus, Marburg virus, Measles virus, Mumps virus, Nipah virus, Hendra virus, Newcastle disease virus, Human respiratory syncytial virus, Rabies virus, Lassa virus, Hantavirus, Crimean-Congo hemorrhagic fever virus, Influenza, or Hepatitis D virus.

15 In some embodiments, the nucleic acid to be detected can be associated with a pathogen, including pathogenic bacteria such as, *E. faecalis*, *E. faecium*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus aureus* (e.g., MRSA), *E. coli* O157:H7, *Borrelia burgdorferi*, *Helicobacter pylori*, *Ehrlichia chaffeensis*, *Clostridium difficile*, *Vibrio*

20 *cholerae* O139, *Salmonella enterica*, *Bartonella henselae*, *Streptococcus pyogenes*, *Chlamydia pneumoniae*, *Clostridium botulinum*, *Corynebacterium amycolatum*, *Klebsiella pneumoniae*, *Vibrio vulnificus*, and *Parachlamydia*.

The disclosures compositions and methods can also be understood through the following numbered paragraphs:

- 25 1. A polynucleotide comprising a nucleotide sequence encoding a class II, type VI CRISPR/Cas effector protein (Cas13) and optionally a heterologous sequence, wherein the Cas effector protein comprises an amino acid sequence encoded by SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67, or a sequence with at least 70% sequence identity
- 30 thereto;
- optionally wherein the sequence encoding the Cas effector protein comprises SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67, or a sequence

with at least 70% sequence identity to SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67.

2. The polynucleotide of paragraph 1, wherein the sequence encoding the Cas effector protein is codon optimized for expression in a prokaryotic or eukaryotic cell.

3. The polynucleotide of paragraph 1 or 2, comprising a heterologous sequence, wherein the heterologous sequence comprises a promoter, transcription terminator, multiple cloning site, drug resistance marker, one or more protease recognition sites, one or more epitope tags, or a combination thereof.

4. The polynucleotide of paragraph 3, wherein the heterologous sequence is operably linked to the sequence encoding the Cas effector protein.

5. The polynucleotide of any one of paragraphs 1-4 further comprising a sequence encoding an RNA comprising a crRNA sequence, wherein the RNA is capable of complexing with the Cas effector protein and hybridizing to a target RNA sequence.

6. The polynucleotide of any one of paragraphs 1-5, wherein the Cas effector protein is derived from *Thermoclostridium caenicola* or a Proteobacteria bacterium.

7. A vector comprising the polynucleotide of any one of paragraphs 1-6, optionally wherein the vector comprises the nucleotide sequence of SEQ ID NO:69 or SEQ ID NO:70, or a sequence having at least 60% sequence identity to SEQ ID NO:69 or SEQ ID NO:70.

8. The vector of paragraph 7, wherein the vector is a viral vector or plasmid.

9. A prokaryotic or eukaryotic cell comprising the vector of paragraph 7 or 8.

10. A method of producing a class II, type VI CRISPR/Cas effector protein comprising contacting the vector of paragraph 7 or 8 with a prokaryotic or eukaryotic cell under conditions suitable for expression of the sequence encoding the Cas effector protein.

11. The method of paragraph 10 further comprising isolating and/or purifying the Cas effector protein.
12. An isolated class II, type VI CRISPR/Cas effector protein, wherein the Cas effector protein is produced by the method of paragraph 10 or 11.
13. An isolated class II, type VI CRISPR/Cas effector protein comprising the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:66, or a sequence with at least 70% sequence identity to SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68.
14. A ribonucleoprotein complex comprising the Cas effector protein of paragraph 12 or 13 complexed with an RNA comprising crRNA sequence, optionally wherein the RNA is capable of hybridizing to a target RNA sequence.
15. A composition comprising a Cas13 protein, wherein the Cas13 protein comprises the amino acid sequence of SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68, or a sequence with at least 70% sequence identity to SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68.
16. The composition of paragraph 15, wherein the Cas13 protein comprises one or more HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains, preferably two HEPN domains.
17. The composition of paragraph 16, wherein the HEPN domain comprises a RxxxxH (SEQ ID NO:192) motif sequence, wherein X represents any amino acid.
18. The composition of any one of paragraphs 15-17, wherein the Cas13 protein is complexed with and RNA comprising a crRNA sequence, optionally wherein the RNA is capable of hybridizing to a target RNA sequence.
19. The composition of paragraph 18, wherein the crRNA sequence comprises a spacer sequence that is capable of hybridizing to the target RNA sequence, and a direct repeat sequence.
20. The composition of paragraph 18 or 19, wherein the crRNA sequence comprises a spacer of about 20-30 nucleotides, preferably 24-28 nucleotides.

21. The composition of any one of paragraphs 18-20, wherein the Cas13 protein can cleave the target RNA sequence at a temperature of about 37-70 °C, about 50-70 °C, about 47-60 °C, or about 60 °C.
22. The composition of any one of paragraphs 18-20, wherein the
5 Cas13 protein can cleave the target RNA sequence at a temperature of about 37 °C-42 °C, preferably about 37 °C.
23. The composition of any one of paragraphs 18-22 comprised in a prokaryotic or eukaryotic cell.
24. A method of performing targeted knockdown of an RNA
10 transcript comprising introducing the composition of any one of paragraphs 18-22 to a cell, wherein the crRNA sequence hybridizes to the RNA transcript, thereby inducing cleavage of the RNA transcript by the Cas13 protein.
25. The method of paragraph 24, wherein RNA transcript is a
15 mRNA or lincRNA.
26. The method of paragraph 24, wherein RNA transcript is derived from a viral gene, preferably a bacteriophage.
27. A method of detecting the presence of an RNA transcript in a
20 nucleic acid sample comprising contacting the sample with the composition of any one of paragraphs 18-22 in the presence of an activatable single stranded RNA (ssRNA) oligonucleotide comprising a reporter moiety,
wherein the crRNA is designed to hybridize to the RNA transcript,
wherein the Cas13 cleaves the ssRNA oligonucleotide upon binding of the Cas13 crRNA complex to the RNA transcript, wherein detection of the
25 cleavage of the ssRNA oligonucleotide indicates the presence of the RNA transcript.
28. The method of paragraph 27, wherein the RNA transcript is generated by transcription from a dsDNA molecule, optionally wherein the dsDNA molecule is generated by reverse transcription coupled isothermal
30 amplification of a target RNA.
29. The method of paragraph 27 or 28, wherein the reporter moiety comprises a fluorophore linked to a quencher via the ssRNA, wherein fluorescence is emitted upon cleavage of the ssRNA oligonucleotide.

30. A method of determining the localization of an RNA transcript in a cell comprising introducing the composition of any one of paragraphs 18-22 to the cell, wherein the Cas13 is catalytically inactive and further comprises a detectable marker,

5 wherein the crRNA is designed to hybridize to the RNA transcript, and wherein the Cas13 crRNA complex binds to the RNA transcript, thereby indicating the location of the RNA transcript.

31. The method of paragraph 30, wherein the detectable marker is selected from GFP, eGFP, RFP, YFP, BFP, CFP, mNeonGreen, mCherry, mOrange, mRaspberry, mPlum, mKO, mRFP, mRFPruby, mRuby, tagRFP, mKate2, and DsRed.

32. A method for performing targeted editing of an RNA transcript comprising introducing the composition of any one of paragraphs 18-22 to a cell,

15 wherein the Cas13 is catalytically inactive and further comprises a deaminase domain of an RNA-dependent Adenosine Deaminase (ADAR),

wherein the crRNA is capable of hybridizing with a region in the RNA transcript comprising a target A nucleotide to form an RNA duplex, wherein the duplex comprises an A-C mismatch at the target A nucleotide,

20 wherein the target A nucleotide is deaminated by the deaminase domain.

33. A composition or methods as described herein including but not limited to the text and figures, and including combinations thereof include that associated with the working examples.

25 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the disclosure.

30

Examples

Example 1: Development of a Cas13-based, one-pot assay for SARS-CoV-2 detection.

This Example reports the identification and characterization of a
 5 thermostable Cas13a ortholog from *Thermoclostridium caenicola*
 (TccCas13a) that is highly active at high temperatures required for some
 amplification methods, such as RT-LAMP. Besides TccCas13a, other
 thermophilic Cas13a orthologues, including Cas13a from *Herbinix*
hemicellulosilytica (HheCas13a) also exhibited strong activity at elevated
 10 temperatures. These Cas13s were characterized with regards to temperature,
 reporter cleavage preference and optimal reaction conditions. RT-LAMP
 utilizing a SARS-CoV-2 *N*-gene specific primer set with FIP primer carrying
 a T7 promoter was coupled to *in vitro* transcription mediated by the
 thermostable Hi-T7 RNA polymerase and the reaction conditions were
 15 optimized for one-pot detection assay. The data described below
 demonstrates that this protocol can robustly detect SARS-CoV-2 in clinical
 samples derived from COVID-19 patients. The data indicate that the
 thermophilic Cas13-based one-pot detection assay holds great promise for
 use as a prospective POC modality to help address increased demand for
 20 SARS-CoV-2 testing.

Materials and Methods

Computational identification of a thermophilic CRISPR/Cas13a

Various existing Cas13 enzymes and their bacterial hosts were
 manually interrogated to identify potential thermophilic Cas13s originating
 25 from thermophilic organisms. After the identification of HheCas13a as a
 potential thermophilic Cas13 protein, the protein sequence of HheCas13a
 was used as a query in the Basic Local Alignment Search Tool (BLAST)
 against the NCBI non-redundant (nr) protein database using default
 settings. Only subject sequences with query coverage (Query cover)
 30 above 80% were considered for second round of host interrogation
 (analyzing growth conditions using BacDive data base and other
 resources). TccCas13a protein (accession# WP_149678719.1) from

Thermoclostridium caenicola was identified as a potential thermophilic Cas13 protein.

Phylogenetic tree was reconstructed using protein sequences of different Cas13s belonging to different families/subtypes of Class II/type VI CRISPR-Cas systems. All protein sequences were organized in a single .txt file and aligned using MUSCLE in MEGAX software with default settings. The phylogenetic reconstruction was based on the Maximum-Likelihood method using MEGAX with WAG+G+F model and 1000 bootstrap samplings. The generated output file (.nwk) was visualized using TreeGraph_2.

CRISPRCasFinder [1] was performed on the genomic DNA sequence (GenBank# NZ_FQZP01000023.1) to identify the associated CRISPR array. CRISPRDetect [2] was then used to predict the orientation of the direct repeat in the TccCas13a CRISPR array.

Cas13 protein expression and purification

The expression vector for HheCas13a “p2CT-His-MBP-Hhe_Cas13a_WT” was obtained from Addgene (plasmid #91871), and the purification of HheCas13a was performed following a previously published protocol [10]. To produce the expression plasmid for TccCas13a expression and purification, the *E. coli* codon-optimized TccCas13 coding sequence was synthesized (GenScript) *de novo* and subcloned in frame with His and SUMO tags on the N-terminus into the His6-TwinStrep-SUMO bacterial expression vector (Addgene #115267) using BamHI and NotI (Table 4).

Purification of TccCas13a protein was performed following the protocol of Kellner *et al.* (2019) [20] with a few modifications. Briefly, the TccCas13a expression vector was transformed into BL21 *E. coli* cells. Starter cultures were prepared by growing single colonies in LB broth supplemented with 100 µg/mL ampicillin for around 12 h at 37 °C. Next, 25 mL of starter culture was used to inoculate 1 L of Terrific Broth medium (TB) (IBI scientific) supplemented with 100 µg/mL ampicillin, and the 1 L cultures (4 L total) were incubated at 37 °C until an OD₆₀₀ of 0.5. Cells were incubated at 4°C 30 mins, and the expression was induced with 0.5 mM IPTG. Cultures were then incubated at 16 °C at 180 rpm for overnight

expression. Next, cells were harvested by centrifugation for 20 min at 4 °C at 4000 rpm. Cell pellets were resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 5% glycerol, 1 mM TCEP, 4.5 mM MgCl₂, 1 mM PMSF, EDTA-free protease inhibitor (Roche)) and supplemented with 1 mg/mL lysozyme (L6876, Sigma). Cells were then lysed by sonication and clarified by centrifugation at 12,000 rpm for 60 min. The soluble 6xHis-SUMO-TccCas13a in cleared lysate was then purified with an affinity chromatography column (HiTrap Q HP, 5 mL GE Healthcare) (AKTA PURE, GE Healthcare) followed by concurrent removal of the 6xHis-SUMO tag by SUMO protease and overnight dialysis in dialysis buffer (50 mM Tris-HCL pH 7.5, 200 mM KCL, 5% glycerol, 1 mM TCEP). Cleaved protein was concentrated to 1.5 mL by Amicon Ultra-15 Centrifugal Filter Units (100 kDa NMWL, UFC905024, Millipore) and further purified via size-exclusion chromatography on a S200 column (GE Healthcare) in gel filtration buffer (50 mM Tris-HCl, 200 mM KCL, 10% glycerol, 1 mM TCEP, pH 7.5). The protein-containing fractions resulting from the gel filtration were pooled, snap frozen, and stored at -80 °C.

Nucleic acid preparation

A short region of the SARS-CoV-2 *N* gene sequence was used as the target sequence in all preliminary thermophilic Cas13 characterization and optimization experiments to screen collateral reporters and assess thermostability of Cas13 proteins. The *N* gene target RNA sequence was prepared by *in vitro* transcription of PCR amplicons containing the T7 promoter sequence using the 2019-nCoV_N_Positive Control plasmid as a PCR template (10006625, IDT) (primers used are listed in table 2). Purified PCR amplicons (QIAquick PCR Purification Kit, QIAGEN) were transcribed *in vitro* using HiScribe T7 Quick High Yield RNA Synthesis Kit (E2050, NEB). The transcripts were then purified by using Direct-zol RNA Miniprep Kits (R2050, Zymo Research) following the manufacturer's instructions, and the purified RNA was stored at -80 °C.

HheCas13a and TccCas13a crRNAs were designed to target the *N* gene sequence of the SARS-CoV-2 genome. For crRNA production, templates for *in vitro* transcription were generated using single-stranded

DNA oligos containing a T7 promoter, scaffold, and spacer in reverse complement orientation (IDT), and were then annealed to T7 forward primer in Taq DNA polymerase buffer (Invitrogen) (Table 5). The annealed oligos were then used as templates for the subsequent *in vitro* transcription as described above.

To establish the thermophilic Cas13-based one-pot assay, control synthetic SARS-CoV-2 viral genomic sequences were ordered as synthetic RNA from Twist Bioscience, and were diluted to 10,000 RNA copies/ μ L and used at indicated concentrations. For RT-LAMP amplification (described below), previously published LAMP primers designed to amplify the SARS-CoV-2 *N* gene (Joung *et al.*, 2020 [12], Broughton *et al.*, 2020 [4]) were used, with the following modifications. The FIP or BIP primers were designed with the T7 promoter sequence appended at the 5' end of the first half of the primers (Table 1).

Differential scanning fluorimetry

DSF was performed using 5 to 15 μ M of the purified Cas13 proteins in gel filtration buffer (with 5% glycerol) containing 10% SYPRO Orange fluorescent dye (ThermoFisher, S6650) in final reaction volume of 35 μ L. Proteins were tested in triplicates and the fluorescence was monitored using a 96-well Real-Time PCR detection system (CFX96 qPCR machine, Bio-Rad), from 25 to 95 $^{\circ}$ C, with a gradual temperature increase of 1 $^{\circ}$ C every 10 s.

Protein thermostability assay

LwaCas13a, HheCas13a and TccCas13a proteins were diluted to approximately 0.2 mg/mL in protein storage buffer (50 mM Tris-HCL pH 7.5, 600 mM NaCl, 5% glycerol, 2 mM DTT) and incubated at a range of temperatures (37, 60, 70 and 90 $^{\circ}$ C) for 30 minutes. Samples were spun down in a microcentrifuge at 14200 rpm for 25 minutes. A total of 5 μ L of the supernatant was mixed with the same volume of protein sample loading buffer and heated at 95 $^{\circ}$ C for 10 minutes. The samples were cooled on ice for 3 minutes and run on NuPAGE (10%) Bis-Tris polyacrylamide gel (ThermoFisher, NP0301BOX).

Protein thermostability assay for HheCas13a and TccCas13a RNPs was performed in the same way with the exception of incubating the aforementioned proteins with 1 μ M of their cognate crRNAs for 5 minutes at 37°C in order to assemble the RNP before subjecting them to a range of different temperatures.

In vitro cis cleavage assays

HheCas13a and TccCas13a cleavage reactions were performed at 37°C and 60°C with synthetic, *in vitro* transcribed RNA target. Briefly, for both HheCas13a or TccCas13a cleavage assays, cleavage reactions were carried out in 20 μ L reaction volume with 50 nM of Cas13a protein, 50 nM of their cognate crRNAs, and 100 nM of target RNA in 1x isothermal buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Tween 20, pH 8.8) supplemented with additional 6 mM MgSO₄ (final of 8 mM MgSO₂), and the reactions were then incubated at the indicated temperatures for 1 hr (no pre-assembly of Cas13a protein and crRNA to form RNP was performed). The samples were then boiled at 70°C for 3 minutes in 2X RNA Loading Dye (NEB, B0363S) and cooled on ice for 3 minutes before loading into 6% polyacrylamide-urea denaturing gel. The electrophoresis was run for 45 minutes at 25W. The gel was stained with SYBRTM Gold Nucleic Acid Gel Stain (ThermoFisher, S11494) for 10 minutes, briefly washed with 1X TBE buffer and visualized using Bio-Rad Molecular Imager[®] Gel DocTM system.

Fluorescent ssRNA cleavage assays

For all reporter screening and thermostability assays, 50 nM of HheCas13a or TccCas13a proteins were incubated with 50 nM of the respective crRNAs, 250 nM of ssRNA reporter (either poly A, poly U, or mixed sequence reporter) in 1X isothermal buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Tween 20, pH 8.8) supplemented with additional 6 mM MgSO₄ (final of 8 mM MgSO₂), 0.8 U/uL RNaseOUT (10777019, Invitrogen) and 2 uL of (1-100 nM) of target RNA in 20 uL reaction volume. These reactions were incubated in a 96 well-plate (BioRad) at different temperatures for 1 hour in a PCR (C1000 touch

thermal cycler, BioRad) machine using the FAM channel, with fluorescence measurements taken every 2 min.

One-pot detection reactions

For Bst DNA polymerase screening and other optimization reactions,
 5 reverse transcription and LAMP isothermal amplification of the target
 nucleic acids, coupled with T7-mediated *in vitro* transcription and Cas13-
 based detection of the amplified and *in vitro*-transcribed target RNA was
 carried out in the same tube. The reaction was performed using RT-LAMP
 primers with final concentrations of 1.6 μ M FIP/BIP primers (with the T7
 10 promoter sequence fused to either the FIP or BIP primer), 0.2 μ M F3/B3
 primers, and 0.4 μ M LF/LB primers, 1X Isothermal Amplification Buffer
 (from different vendors in Bst DNA polymerase screening reactions) or from
 Lucigen (30027, Lucigen) in other optimization experiments), 1.4 mM
 dNTPs, 0.32 U/uL Bst DNA Polymerase (from different vendors in Bst DNA
 15 polymerase screening reactions) or from Lucigen (30027, Lucigen)), 0.45
 U/uL of WarmStart RTx Reverse Transcriptase (M0380, NEB) or 2 U/ uL
 SuperScript IV reverse transcriptase (18090010, Invitrogen), 6 mM MgSO₄,
 0.1 U/uL thermostable RNaseH (M0523S, NEB), 0.8 U/uL RNaseOUT or
 RNasin plus (N2611, Promega), 0.5 mM NTPS, 4 U/ μ L Hi-T7 RNA
 20 polymerase (M0658S, NEB), 0.4 U/ μ L thermostable inorganic
 pyrophosphatase (M0296, NEB), 250 nM RNA reporter, 50 nM Cas13, 50
 nM crRNA, and 2 μ L of template RNA in 25- μ L reactions. These reactions
 were incubated in a 96 well-plate (BioRad) at 56 °C for 1-2 hours in a PCR
 (C1000 touch thermal cycler, BioRad) machine using the FAM channel, with
 25 fluorescence measurements taken every 2 min.

*Nucleotide and Amino acid sequences used***Table 1: RT-LAMP primers used in Examples 1 and 2.**

Name	Sequence	Note
DE-F3	GAAATTTGGATCTTTGTCATCC (SEQ ID NO:1)	Original and modified DETECTR primers. Modified part (T7 promoter sequence) shown in underline.
DE-B3	GAAATTTGGATCTTTGTCATCC (SEQ ID NO:2)	
DE-FIP	TGCGGCCAATGTTTGTAAATCAGCCAAGGAAATTT TGGGGAC (SEQ ID NO:3)	
DE-BIP	CGCATTGGCATGGAAGTCACTTTGATGGCACCTG TG TAG (SEQ ID NO:4)	
DE-LF	TTCCTTGTCTGATTAGTTC (SEQ ID NO:5)	
DE-LB	ACCTTCGGGAACGTGGTT (SEQ ID NO:6)	
DE-FIP-T7	TGCGGCCAATGTTTGTAAATCAG <u>TAATACGACTCA</u> <u>CTATAGGCCAAGGAAATTTGGGGAC</u> (SEQ ID NO:7)	
DE-BIP-T7	CGCATTGGCATGGAAGT <u>TAATACGACTCACTAT</u> <u>AGGCACTTTGATGGCACCTGTGTAG</u> (SEQ ID NO:8)	
SC-F3	GCTGCTGAGGCTTCTAAG (SEQ ID NO:9)	Original and modified STOPCovid primers. Modified part (T7 promoter sequence) shown in underline.
SC-B3	GCGTCAATATGCTTATTCAGC (SEQ ID NO:10)	
SC-FIP	GCGGCCAATGTTTGTAAATCAGTAGACGTGGTCC AGAACAA (SEQ ID NO:11)	
SC-BIP	TCAGCGTTCTTCGGAATGTCGCTGTGTAGGTCAA CCACG (SEQ ID NO:12)	
SC-LF	CCTTGTCTGATTAGTTCCTGGT (SEQ ID NO:13)	
SC-LB	TGGCATGGAAGTCACACC (SEQ ID NO:14)	

SC-T7-FIP	GCGGCCAATGTTTGTAATCAGT <u>TAATACGACTCA</u> <u>CTATAGGG</u> GAGACGTGGTCCAGAACAA (SEQ ID NO:15)	
SC-T7-BIP	TCAGCGTTCTTCGGAATGTCGCTAATACGACTCA <u>CTATAGGG</u> TGTGTAGGTCAACCACG (SEQ ID NO:16)	

SEQ ID NOs:9-16 were used in Example 1. SEQ ID NOs:1-16 were used in Example 2.

RNase P- F3	TTGATGAGCTGGAGCCA (SEQ ID NO:71)	Original and modified RNase P POP primers. Modified part (T7 promoter sequence) is underlined.
RNase P- B3	CACCCTCAATGCAGAGTC (SEQ ID NO:72)	
RNase P- FIP	GTGTGACCCTGAAGACTCGGTTTTAGCCACTG ACTCGGATC (SEQ ID NO:73)	
RNase P- BIP	CCTCCGTGATATGGCTCTTCGTTTTTTCTTAC ATGGCTCTGGTC (SEQ ID NO:74)	
RNase P- LF	ATGTGGATGGCTGAGTTGTT (SEQ ID NO:75)	
RNase P- LB	CATGCTGAGTACTGGACCTC (SEQ ID NO:76)	
RNase P- T7-FIP	GTGTGACCCTGAAGACTCGGTTTTTAATACGA <u>CTCACTATAGGG</u> AGCCACTGACTCGGATC (SEQ ID NO:77)	

HCV-F3	TGTCTTCACGCAGAAAGCG (SEQ ID NO:78)	HCV RT-LAMP primers. Modified part (T7 promoter sequence)
HCV-B3	TACCACAAGGCCTTTCGC (SEQ ID NO:79)	
HCV-T7-FIP	<u>TCCGCAGACCACTATGGCTCTCTAATACGAC</u> <u>T CACTATAGGGCCATGGCGTTAGTATGAGT</u> (SEQ ID NO:80)	
HCV-BIP	AGGACGACCGGGTCCTTTCTACTACTCGGCT A GCAGTCTT (SEQ ID NO:81)	
HCV-LF	GGTCCTGGAGGCTGCACGAC (SEQ ID NO:82)	
HCV-LB	GAGATTTGGGCGTGCCCCCGC (SEQ ID NO:83)	
TYLCV-F3	GGTAAAGTCTGGATGGATGA (SEQ ID NO:84)	TYLCV LAMP primers. Modified part (T7 promoter sequence) is underlined. -Original primers [Mahas, et al. Viruses, 2021. 13(3)].
TYLCV-B3	TGTTCCCTTCATTCCAGAGG (SEQ ID NO:85)	
TYLCV-T7-FIP	<u>ACCTGTCCAAAATCCATTGTAATACGACTCA</u> <u>C TATAGGGCAGAATCACACTAATCAGGTC</u> (SEQ ID NO:86)	
TYLCV-BIP	CAGTACCGCAACCGTGAAGACAATAACTGTA GCATGAAATTCCT (SEQ ID NO:87)	
TYLCV-LF	CTATCACGGACCAAGAAGAAC (SEQ ID NO:88)	
TYLCV-LB	GATTTGCGGGATAGGTTTCAAG (SEQ ID NO:89)	

Table 2: Primers to PCR amplify N gene regions for IVT in Examples 1 and 2.

N gene-1-T7-F	GAAATTAATACGACTCACTATAGGGAGGCTTCTAAGAAG CCTCGGC (SEQ ID NO:17)
N gene-1-R_2	CTGTCTCTGCGGTAAGGCTTGAG (SEQ ID NO:18)
N gene-target 2-T7-F	GAAATTAATACGACTCACTATAGGGTTGCAACTGAG GGAGCCTTG (SEQ ID NO:39)
N gene-target- 2-R	GCTTCTTAGAAGCCTCAGCAGCAG (SEQ ID NO:40)

ORF1-R	GACATACTTATCGGCAATTTTGTACC (SEQ ID NO:189)
M13-R	CAGGAAACAGCTATGAC (SEQ ID NO:190)

Table 3: RNA reporter designs and sequences used in in Examples 1 and 2.

UA reporter-FAM	5'-/56-FAM/UUAUU/3IABKFQ/-3'
UA reporter-HEX	5'-/HEX/UUAUU/3IABKFQ/-3'
Poly U reporter-FAM	5'-/56-FAM/UUUUU/3IABKFQ/-3'
Poly A reporter-FAM	5'-/56-FAM/AAAAA/3IABKFQ/-3'
Mix reporter-FAM	5'-/56-FAM/UGACGU/3IABKFQ/-3'
Mix reporter-HEX	5'-/56-HEX/UGACGU/3IABKFQ/-3'
Poly G reporter-FAM	5'-/56-FAM/rGrGrGrGrG/3IABkFQ/-3'
Poly C reporter-FAM	5'-/56-FAM/rCrCrCrCrC/3IABkFQ/-3'
3(AG) reporter-FAM	5'-/56-FAM/rArGrArGrArG/3IABkFQ/-3'
3(AC) reporter-FAM	5'-/56-FAM/rArCrArCrArC/3IABkFQ/-3'
3(CG) reporter-FAM	5'-/56-FAM/rCrGrCrGrG/3IABkFQ/-3'
3(UG) reporter-FAM	5'-/56-FAM/rUrGrUrGrUrG/3IABkFQ/-3'
LwaCas13a reporter	5'-/56-FAM/rUrUrArUrU/3IABKFQ/-3'
AapCas12b reporter-HEX	5'-/HEX/TTTTTTT/3IABKFQ/-3'

Table 4: Cas13 protein sequence used in this study.

Name	Protein sequence
TccCas13a	MGSSHHHHHHSSGLVPRGSHMASWSHPQFEKGGGSGGGSGGS AWSHPQFEKMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEI FFKIKKTTPLRRLMEAFKRQKEMDSLRFYDGIQADQTPE DLDMEDNDIIEAHREQIGGSMKITKRKWGEHHPPLYFYRDEDSG RLLAQNDRKQDYDTLFDIAQDTFERSLRNRLKTPEKGDKRF YSNEIVKLVEKLCQGADVAEIMKSMERNEKLRPKNEKEIKNLK KQLDGTLSSEYGKRYTAPEGAMTLNDALFYLVGNPLKQAMAK AELGKIREALIKEKENRINRVYSIKNNKIPLRIQEDGGITPNNDR AAWLLGLMKPADPAKGITDCYPLLGELEEVDKLSKTLHEKI SRCQGRPSIAMAVDEALKQYLRELWEKSPSRQQDLKYFQAV QEYFKDNFPIRTKRMGARLRQELLKDKTSLSRLEPKHMANAV RRRLINQSTQMHILYGKLYAYCCGEDGRLLVNSETLQRIQVHEA VKKQAMTAVLWSISRLRYFYQFEDGDILSNKNPIKDFRDKFLRD TNKYTHEDVEACKEKLQDFFPLKELQEKIKEDAKGLQETDNKQ ADTTDFKAIGHIVRDDRKLCNQLLAECVSCIGELRHHIFHYKNV TLIQALKRIADKVKPEDLSVLRAIYLLDRRLKKAFAKRISMNL PLYYREDLLSRIFKKEGTAFFLYSAKIQMTPSFQRVYERGKNLRR EFECERMKAEEASNGQNGQDGDRLKWFRQLAAGDSADTHFNW AVEAYAESAADVENNVEFDTDVDAQRALRNLLLLIYRHHFLPE VQKDETLVTGKIHKVLERNRQLSEGQGNQGAHGYSVIEELY HEGMPLSDLMKQLQRRISETERESRELAQEKTDYAQRFILDFAE AFNDFLEAHYGEEYLEIMSPRKDAEAAKKWVKESKTVDLKTSI DEKEPEGHLLVLYPVLRLDERELGELQQMIRYRTSLASWQG ESNFSEEIRIAGQIEELTELVKLTEPEPQFAEEVWGKRAKEAFEDF IEGNMKNYEAFYLQSDNNTPVYRRNMSRLLRSGLMGVYQKVL ASHKQALKRDYLLWSEKHWNVKDENGADISSAEQAQCLLQRL HRKYAESPSRFTEEDCKLYEKVLRRLLEDYNQAVKNLSFSSLYEI CVLNLEILSRWVGQVQDWERDMYFLLLAWVRQGKLDGIKEED VRDIFSEGNIIRNLVDTLKGEMNMFESVYFPENKGSKYLGVRN DVAHLDLMRKNGWRLEAGKTCVSMEDYINRLRFLSYDQKRM NAVTKTLQQIFDRHKVKIRFTVEKGGMLKIEDVTADKIVHLKGS RLSGIEIPSHGERFIDTLKALMVYPRG* (SEQ ID NO:19)

6x His affinity tag: residues 5-10; Thrombin site: residues 14-19; Strep-tag II: residues 24-31 and 44-51; SUMO: residues 52-148; TccCas13a protein: residues 151-1375. For HheCas13 sequence (Ref [10]).

Table 5: crRNA sequences used in this study.

crRNA #	crRNA Name	crRNA sequence
1101	Hhe-Sense-28-1	ATCAGACAAGGAACTGATTACAAACATTGTT GCAGTTCCCCTGTCTACGGGGATTGTTACCCT ATAGTGAGTCGTATTAATTTC (SEQ ID NO:20)
1102	Hhe-Sense-28-2	CCGCAAATTGCACAATTTGCCCCAGCGGTTG CAGTTCCCCTGTCTACGGGGATTGTTACCCTA TAGTGAGTCGTATTAATTTC (SEQ ID NO:21)
1103	Hhe-Sense-28-3	CGCTTCAGCGTTCCTTCGGAATGTCGCGCGTTG CAGTTCCCCTGTCTACGGGGATTGTTACCCTA TAGTGAGTCGTATTAATTTC (SEQ ID NO:22)
1104	Hhe-Sense-28-4	CATTGGCATGGAAGTCACACCTTCGGGAGTTG CAGTTCCCCTGTCTACGGGGATTGTTACCCTA TAGTGAGTCGTATTAATTTC (SEQ ID NO:23)
1105	Hhe-Sense-28-5	GAACGTGGTTGACCTACACAGGTGCCATGTTG CAGTTCCCCTGTCTACGGGGATTGTTACCCTA TAGTGAGTCGTATTAATTTC (SEQ ID NO:24)
1106	Hhe-Sense-28-6	ATCAAATTGGATGACAAAGATCCAAATTGTT GCAGTTCCCCTGTCTACGGGGATTGTTACCCT ATAGTGAGTCGTATTAATTTC (SEQ ID NO:25)
NS	Hhe-NS	ctccgtgatatggctcttcgcatgctgaGTTGCAGTTCCCCTG TCTACGGGGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:26)
1087	Hhe-AntiSense-28-4	TCCCGAAGGTGTGACTTCCATGCCAATGGTTG CAGTTCCCCTGTCTACGGGGATTGTTACCCTA TAGTGAGTCGTATTAATTTC (SEQ ID NO:27)
1088	Hhe-AntiSense-28-6	AATTTGGATCTTTGTCATCCAATTTGATGTTG CAGTTCCCCTGTCTACGGGGATTGTTACCCTA TAGTGAGTCGTATTAATTTC (SEQ ID NO:28)
1125	Tcc-Sense-24-1	CAGACAAGGAACTGATTACAAACAGTTGCAG TCTCCGCCTACATGGGAGTTGTGACCCTATAG TGAGTCGTATTAATTTC (SEQ ID NO:29)

1126	Tcc-Sense-24-3	CTTCAGCGTTCTTCGGAATGTCGCGTTGCAGT CTCCGCCTACATGGGAGTTGTGACCCTATAGT GAGTCGTATTAATTTTC (SEQ ID NO:30)
1127	Tcc-Sense-24-6	CAAATTGGATGACAAAGATCCAAAGTTGCAG TCTCCGCCTACATGGGAGTTGTGACCCTATAG TGAGTCGTATTAATTTTC (SEQ ID NO:31)
1171	Tcc-Sense-24-2	GCAAATTGCACAATTTGCCCCAGGTTGCAGT CTCCGCCTACATGGGAGTTGTGACCCTATAGT GAGTCGTATTAATTTTC (SEQ ID NO:32)
1172	Tcc-Sense-24-4	TTGGCATGGAAGTCACACCTTCGGGTTGCAGT CTCCGCCTACATGGGAGTTGTGACCCTATAGT GAGTCGTATTAATTTTC (SEQ ID NO:33)
1173	Tcc-Sense-24-5	ACGTGGTTGACCTACACAGGTGCCGTTGCAGT CTCCGCCTACATGGGAGTTGTGACCCTATAGT GAGTCGTATTAATTTTC (SEQ ID NO:34)
NS	Tcc-NS	actcagccatccacatccgagtcttcagGTTGCAGTCTCCGCC TACATGGGAGTTGTGACCCTATAGTGAGTCGT ATTAATTTTC (SEQ ID NO:35)
1174	Tcc-AntiSense- 24-1	TGTTTGTAATCAGTTCCTTGTCTGGTTGCAGTC TCCGCCTACATGGGAGTTGTGACCCTATAGTG AGTCGTATTAATTTTC (SEQ ID NO:36)
1175	Tcc-AntiSense- 24-3	GCGACATTCCGAAGAACGCTGAAGGTTGCAG TCTCCGCCTACATGGGAGTTGTGACCCTATAG TGAGTCGTATTAATTTTC (SEQ ID NO:37)
T7 oligo	T7 oligo for IVT	GAAATTAATACGACTCACTATAGGG (SEQ ID NO:38)

1243	Tcc-RNase P-24-1	TCAGCCATCCACATCCGAGTCTTC GTTGCAGTCTCCGCCTACATGGGA GTTGTGACCCTATAGTGAGTCGTA TTAATTTC (SEQ ID NO:71)	(crRNA1) Selected crRNA for detection
1244	Tcc-RNase P-24-2	CCGTGATATGGCTCTTCGCATGCT GTTGCAGTCTCCGCCTACATGGGA GTTGTGACCCTATAGTGAGTCGTA TTAATTTC (SEQ ID NO:72)	(crRNA2)
1246	1104-Mis 1-1	GATTGGCATGGAAGTCACACCTT CGGGAGTTGCAGTTCCCCTGTCTA CGGGGATTGTTACCCTATAGTGA GTCGTATTAATTTC (SEQ ID NO:73)	Figs. 14F-14I
1247	1104-Mis 1-2	CTTTGGCATGGAAGTCACACCTTC GGGAGTTGCAGTTCCCCTGTCTAC GGGGATTGTTACCCTATAGTGAG TCGTATTAATTTC (SEQ ID NO:74)	Figs. 14F-14I
1248	1104-Mis 1-3	CAATGGCATGGAAGTCACACCTT CGGGAGTTGCAGTTCCCCTGTCTA CGGGGATTGTTACCCTATAGTGA GTCGTATTAATTTC (SEQ ID NO:75)	Figs. 14F-14I
1249	1104-Mis 1-4	CATAGGCATGGAAGTCACACCTT CGGGAGTTGCAGTTCCCCTGTCTA CGGGGATTGTTACCCTATAGTGA GTCGTATTAATTTC (SEQ ID NO:76)	Figs. 14F-14I
1250	1104-Mis 1-5	CATTCGCATGGAAGTCACACCTTC GGGAGTTGCAGTTCCCCTGTCTAC GGGGATTGTTACCCTATAGTGAG TCGTATTAATTTC (SEQ ID NO:77)	Figs. 14F-14I

1251	1104-Mis 1-6	CATTGCCATGGAAGTCACACCTTC GGGAGTTGCAGTTCCCCTGTCTAC GGGGATTGTTACCCTATAGTGAG TCGTATTAATTTC (SEQ ID NO:78)	Figs. 14F-14I
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1252	1104-Mis 1-7	CATTGGGATGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:79)	Figs. 14F-14I
1253	1104-Mis 1-8	CATTGGCTTGGAAGTCACACCTTCGG GAGTTGCAGTTCCCCTGTCTACGGGG ATTGTTACCCTATAGTGAGTCGTATT AATTTC (SEQ ID NO:80)	Figs. 14F-14I
1254	1104-Mis 1-9	CATTGGCAAGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:81)	Figs. 14F-14I
1255	1104-Mis 1-10	CATTGGCATCGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:82)	Figs. 14F-14I
1256	1104-Mis 1-11	CATTGGCATGCAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:83)	Figs. 14F-14I
1257	1104-Mis 1-12	CATTGGCATGGTAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:84)	Figs. 14F-14I

1258	1104-Mis 1-13	CATTGGCATGGATGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:85)	Figs. 14F-14I
1259	1104-Mis 1-14	CATTGGCATGGAACTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:86)	Figs. 14F-14I
1260	1104-Mis 1-15	CATTGGCATGGAAGACACACCTTC GGGAGTTGCAGTTCCCCTGTCTACG GGGATTGTTACCCTATAGTGAGTCG TATTAATTTC (SEQ ID NO:87)	Figs. 14F-14I
1261	1104-Mis 1-16	CATTGGCATGGAAGTGACACCTTC GGGAGTTGCAGTTCCCCTGTCTACG GGGATTGTTACCCTATAGTGAGTCG TATTAATTTC (SEQ ID NO:88)	Figs. 14F-14I
1262	1104-Mis 1-17	CATTGGCATGGAAGTCTCACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:89)	Figs. 14F-14I
1263	1104-Mis 1-18	CATTGGCATGGAAGTCAGACCTTC GGGAGTTGCAGTTCCCCTGTCTACG GGGATTGTTACCCTATAGTGAGTCG TATTAATTTC (SEQ ID NO:90)	Figs. 14F-14I
1264	1104-Mis 1-19	CATTGGCATGGAAGTCACTCCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:91)	Figs. 14F-14I

1265	1104-Mis 1-20	CATTGGCATGGAAGTCACAGCTTC GGGAGTTGCAGTTCCCCTGTCTACG GGGATTGTTACCCTATAGTGAGTCG TATTAATTTC (SEQ ID NO:92)	Figs. 14F-14I
1266	1104-Mis 1-21	CATTGGCATGGAAGTCACACGTTTC GGGAGTTGCAGTTCCCCTGTCTACG GGGATTGTTACCCTATAGTGAGTCG TATTAATTTC (SEQ ID NO:93)	Figs. 14F-14I
1267	1104-Mis 1-22	CATTGGCATGGAAGTCACACCATC GGGAGTTGCAGTTCCCCTGTCTACG GGGATTGTTACCCTATAGTGAGTCG TATTAATTTC (SEQ ID NO:94)	Figs. 14F-14I

1268	1104-Mis 1-23	CATTGGCATGGAAGTCACACCTACG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:95)	Figs. 14F-14I
1269	1104-Mis 1-24	CATTGGCATGGAAGTCACACCTTGG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:96)	Figs. 14F-14I
1270	1104-Mis 1-25	CATTGGCATGGAAGTCACACCTTCC GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:97)	Figs. 14F-14I
1271	1104-Mis 1-26	CATTGGCATGGAAGTCACACCTTCG CGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:98)	Figs. 14F-14I

1272	1104-Mis 1-27	CATTGGCATGGAAGTCACACCTTCG GCAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:99)	Figs. 14F-14I
1273	1104-Mis 1-28	CATTGGCATGGAAGTCACACCTTCG GGTGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:100)	Figs. 14F-14I
1274	1104-Mis-2-1	GTTTGGCATGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:101)	Figs. 14F-14I
1275	1104-Mis-2-2	CAAAGGCATGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:102)	Figs. 14F-14I
1276	1104-Mis-2-3	CATTCCCATGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:103)	Figs. 14F-14I
1277	1104-Mis-2-4	CATTGGGTTGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:104)	Figs. 14F-14I
1278	1104-Mis-2-5	CATTGGCAACGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:105)	Figs. 14F-14I

1279	1104-Mis-2-6	CATTGGCATGCTAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:106)	Figs. 14F-14I
1280	1104-Mis-2-7	CATTGGCATGGATCTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:107)	Figs. 14F-14I
1281	1104-Mis-2-8	CATTGGCATGGAAGAGACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:108)	Figs. 14F-14I
1282	1104-Mis-2-9	CATTGGCATGGAAGTCTGACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:109)	Figs. 14F-14I
1283	1104-Mis-2-10	CATTGGCATGGAAGTCACTGCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:110)	Figs. 14F-14I
1284	1104-Mis-2-11	CATTGGCATGGAAGTCACACGATCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:111)	Figs. 14F-14I
1285	1104-Mis-2-12	CATTGGCATGGAAGTCACACCTAGG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:112)	Figs. 14F-14I

1286	1104-Mis- 2-13	CATTGGCATGGAAGTCACACCTTCC CGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:113)	Figs. 14F- 14I
1287	1104-Mis- 2-14	CATTGGCATGGAAGTCACACCTTCG GCTGTTGCAGTTCCCCTGTCTACGGG GATTGTTACCCTATAGTGAGTCGTAT TAATTTC (SEQ ID NO:114)	Figs. 14F- 14I
1288	1104-Seed- 1	GTAAGGCATGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:115)	Figs. 14F- 14I
1289	1104-Seed- 2	CATTCCGTTGGAAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:116)	Figs. 14F- 14I
1290	1104-Seed- 3	CATTGGCAACCTAGTCACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:117)	Figs. 14F- 14I
1291	1104-Seed- 4	CATTGGCATGGATCAGACACCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:118)	Figs. 14F- 14I
1292	1104-Seed- 5	CATTGGCATGGAAGTCTGTGCTTCG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:119)	Figs. 14F- 14I

1293	1104-Seed-6	CATTGGCATGGAAGTCACACGAAGG GGAGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:120)	Figs. 14F- 14I
1294	1104-Seed-7	CATTGGCATGGAAGTCACACCTTCC CCTGTTGCAGTTCCCCTGTCTACGG GGATTGTTACCCTATAGTGAGTCGT ATTAATTTC (SEQ ID NO:121)	Figs. 14F- 14I
1295	1104-Spacer-16	CATGGAAGTCACACCTGTTGCAGTT CCCCTGTCTACGGGGATTGTTACCCT ATAGTGAGTCGTATTAATTTC (SEQ ID NO:122)	Figs. 14F- 14I
1296	1104-Spacer-18	GCATGGAAGTCACACCTTGTGTCAG TTCCCCTGTCTACGGGGATTGTTACC CTATAGTGAGTCGTATTAATTTC (SEQ ID NO:123)	Figs. 14F- 14I
1297	1104-Spacer-20	GGCATGGAAGTCACACCTTCGTTGC AGTTCCCCTGTCTACGGGGATTGTTA CCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:124)	Figs. 14F- 14I
1298	1104-Spacer-22	TGGCATGGAAGTCACACCTTCGGTT GCAGTTCCCCTGTCTACGGGGATTG TTACCCTATAGTGAGTCGTATTAAT TTC (SEQ ID NO:125)	Figs. 14F- 14I
1299	1104-Spacer-24	TTGGCATGGAAGTCACACCTTCGGGT TGCAGTTCCCCTGTCTACGGGGATTG TTACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:126)	Figs. 14F- 14I

1300	1104-Spacer-26	ATTGGCATGGAAGTCACACCTTCGGG GTTGCAGTTCCTGTCTACGGGGAT TGTTACCCTATAGTGAGTCGTATTAA TTTC (SEQ ID NO:127)	Figs. 14F- 14I
1301	1104-Spacer-30	gcattggcatggaagtcacaccttcgggaaGTTGCA GTTCCCTGTCTACGGGGATTGTTA CCCTATAGTGAGTCGTATTAAATTTC (SEQ ID NO:128)	Figs. 14F- 14I
1303	1172-Mis 1-1	ATGGCATGGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAAATT TC (SEQ ID NO:129)	Fig. 14A
1304	1172-Mis 1-2	TAGGCATGGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAAATT TC (SEQ ID NO:130)	Fig. 14A
1305	1172-Mis 1-3	TTCGCATGGAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTAA ATTTC (SEQ ID NO:131)	Fig. 14A
1306	1172-Mis 1-4	TTGCCATGGAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTAA ATTTC (SEQ ID NO:132)	Fig. 14A
1307	1172-Mis 1-5	TTGGGATGGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAAATT TC (SEQ ID NO:134)	Fig. 14A

1308	1172-Mis 1-6	TTGGCTTGGAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:135)	Fig. 14A
1309	1172-Mis 1-7	TTGGCAAGGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCT ATAGTGAGTCGTATTAATTTC (SEQ ID NO:136)	Fig. 14A
1310	1172-Mis 1-8	TTGGCATCGAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:137)	Fig. 14A
1311	1172-Mis 1-9	TTGGCATGCAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:138)	Fig. 14A
1312	1172-Mis 1-10	TTGGCATGGTAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:139)	Fig. 14A
1313	1172-Mis 1-11	TTGGCATGGATGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:140)	Fig. 14A
1314	1172-Mis 1-12	TTGGCATGGAACTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:141)	Fig. 14A

1315	1172-Mis 1-13	TTGGCATGGAAGACACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:142)	Fig. 14A
1316	1172-Mis 1-14	TTGGCATGGAAGTGACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:143)	Fig. 14A
1317	1172-Mis 1-15	TTGGCATGGAAGTCTCACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:144)	Fig. 14A
1318	1172-Mis 1-16	TTGGCATGGAAGTCAGACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:145)	Fig. 14A
1319	1172-Mis 1-17	TTGGCATGGAAGTCACTCCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:146)	Fig. 14A
1320	1172-Mis 1-18	TTGGCATGGAAGTCACAGCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:147)	Fig. 14A
1321	1172-Mis 1-19	TTGGCATGGAAGTCACACGTTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:148)	Fig. 14A

1322	1172-Mis 1-20	TTGGCATGGAAGTCACACCATCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:149)	Fig. 14A
1323	1172-Mis 1-21	TTGGCATGGAAGTCACACCTACGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:150)	Fig. 14A
1324	1172-Mis 1-22	TTGGCATGGAAGTCACACCTTGGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:151)	Fig. 14A
1325	1172-Mis 1-23	TTGGCATGGAAGTCACACCTTCCGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:152)	Fig. 14A
1326	1172-Mis 1-24	TTGGCATGGAAGTCACACCTTCGCG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:153)	Fig. 14A
1327	1172-Mis-2-1	AAGGCATGGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:154)	Fig. 14B
1328	1172-Mis-2-2	TTCCCATGGAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:155)	Fig. 14B

1329	1172-Mis- 2-3	TTGGGTTGGAAGTCACACCTTCGGG TTGCAGTCTCCGCCTACATGGGAGT TGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:156)	Fig. 14B
1330	1172-Mis- 2-4	TTGGCAACGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:157)	Fig. 14B
1331	1172-Mis- 2-5	TTGGCATGCTAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:158)	Fig. 14B
1332	1172-Mis- 2-6	TTGGCATGGATCTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:159)	Fig. 14B
1333	1172-Mis- 2-7	TTGGCATGGAAGAGACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:160)	Fig. 14B
1334	1172-Mis- 2-8	TTGGCATGGAAGTCTGACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:161)	Fig. 14B
1335	1172-Mis- 2-9	TTGGCATGGAAGTCACTGCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTG TGACCCTATAGTGAGTCGTATTAATT TC (SEQ ID NO:162)	Fig. 14B

1336	1172-Mis- 2-10	TTGGCATGGAAGTCACACGATCGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:163)	Fig. 14B
1337	1172-Mis- 2-11	TTGGCATGGAAGTCACACCTAGGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:164)	Fig. 14B
1338	1172-Mis- 2-12	TTGGCATGGAAGTCACACCTTCCCGTT GCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:165)	Fig. 14B
1339	1172-Seed- 1	AACCCATGGAAGTCACACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:166)	Fig. 14C
1340	1172-Seed- 2	TTGGGTACGAAGTCACACCTTCGGGT GCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:167)	Fig. 14C
1341	1172-Seed- 3	TTGGCATGCTTCTCACACCTTCGGGT GCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:168)	Fig. 14C
1342	1172-Seed- 4	TTGGCATGGAAGAGTGACCTTCGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:169)	Fig. 14C

1343	1172-Seed-5	TTGGCATGGAAGTCACTGGATCGGGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:170)	Fig. 14C
1344	1172-Seed-6	TTGGCATGGAAGTCACACCTAGCCGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:171)	Fig. 14C
1345	1172-Spacer-16	CATGGAAGTCACACCTGTTGCAGTCTC CGCCTACATGGGAGTTGTGACCCTAT AGTGAGTCGTATTAATTTC (SEQ ID NO:172)	Fig. 14D
1346	1172-Spacer-18	GCATGGAAGTCACACCTTGTGTCAGT CTCCGCCTACATGGGAGTTGTGACCCT ATAGTGAGTCGTATTAATTTC (SEQ ID NO:173)	Fig. 14D
1347	1172-Spacer-20	GGCATGGAAGTCACACCTTCGTTGCA GTCTCCGCCTACATGGGAGTTGTGAC CCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:174)	Fig. 14D
1348	1172-Spacer-22	TGGCATGGAAGTCACACCTTCGGTTG CAGTCTCCGCCTACATGGGAGTTGTG ACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:175)	Fig. 14D
1349	1172-Spacer-26	ATTGGCATGGAAGTCACACCTTCGG GGTTGCAGTCTCCGCCTACATGGGA GTTGTGACCCTATAGTGAGTCGTAT TAATTTC (SEQ ID NO:176)	Fig. 14D

1350	1172- Spacer-28	CATTGGCATGGAAGTCACACCTTCGG GAGTTGCAGTCTCCGCCTACATGGGA GTTGTGACCCTATAGTGAGTCGTATTA ATTTC (SEQ ID NO:177)	Fig. 14D
1351	1172- Spacer-30	GCATTGGCATGGAAGTCACACCTTCG GGAAGTTGCAGTCTCCGCCTACATGG GAGTTGTGACCCTATAGTGAGTCGTAT TAATTTC (SEQ ID NO:178)	Fig. 14D
1215	<i>ORF1</i> LwaCas13a crRNA	GTAAACCAGGTGGAACCTCATCAGG AGATGTTTTAGTCCCCTTCGTTTTTGG GGTAGTCTAAATCCCCTATAGTGAGTC GTATTAATTTC (SEQ ID NO:179)	Fig. 2E
1363	Tcc- crRNA- HCV-1	TAGTGGTCTGCGGAACCGGTGAGTGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:180)	Figs. 22A- 22C
1370	Tcc- crRNA- TYLCV-2	AAGGCCTTATGGAAGCAGCCCAATGT TGCAGTCTCCGCCTACATGGGAGTTGT GACCCTATAGTGAGTCGTATTAATTTC (SEQ ID NO:181)	Figs. 22A- 22C
CV627	AapCas12b- RnaseP- sgRNA-1	AGGGTCACACCCAAGTAATTGTGCCA CTTCTCAGATTTGAG (SEQ ID NO:182)	Figs. 21A- 21B
CV628	AapCas12b- RnaseP- sgRNA-2	CTCGGACCAGAGCCATGTAAGTGCCA CTTCTCAGATTTGAG (SEQ ID NO:183)	Figs. 21A- 21B

CV629	AapCas12b- RnaseP- sgRNA-3	AATTACTTGGGTGTGACCCTGTGCCAC TTCTCAGATTTGAG (SEQ ID NO:184)	Figs. 21A- 21B
CV241	AapCas12b sgRNA scaffold- Top	GAAATTAATACGACTCACTATAGGG TCTAGAGGACAGAATTTTCAACGG GTGTGCCAATGGCCACTTTCCAGGT GGCAAAGCCCGTTGAGCTTCTCAA TCTGAGAAGTGGCAC (SEQ ID NO:185)	

Pre-crRNAs of LwaCas13a, HheCas13a, and TccCas13a

crRNA #	Name	Sequence (5' → 3')
1377	LwaCas13a- pre-crRNA-5' FAM labeled	FAM- AUCAUGCGAGGAUUUAGACUACCCCAAAAA CGAAGGGGACUAAAACUUCAGAUAUAGCCU GGUGGUUCAGGC (SEQ ID NO:186)
1378	HheCas13a- pre-crRNA- 5' FAM labeled	FAM- ACUGAGGUAUGUAACAAUCCCCGUAGACAG GGGAACUGCAACCAGAUAUAGCCUGGUGGU UCAGGC (SEQ ID NO:187)
1379	TccCas13a- pre-crRNA- 5' FAM labeled	GUCGACACGGGUCACAACUCCCAUGUAGGC GGAGACUGCAACUAUUACCACUCAGGGUAA UGUCCG (SEQ ID NO:188)

gBlocks used in this study.

Name	Sequence (5' → 3')
SARS-CoV-2 <i>ORF1</i> gBlock	GAAATTAATACGACTCACTATAGGGCCTCACTTGTTCTTGCTCGCAAACATACAACGTGTTGTAGCTTGTCACACCGTTTCTATAGATTAGCTAATGAGTGTGCTCAAGTATTGAGTGAAATGGTCATGTGTGGCGGTTCACTATATGTTAAACCAGGTGGAACCTCATCAGGAGATGCCACAACTGCTTATGCTAATAGTGTTTTTTAACATTTGTCAAGCTGTCACGGCCAATGTTAATGCACTTTTATCTACTGATGTAAACAAAATTGCCGATAAGTATGTC (SEQ ID NO:189)
HCV genotype 1a	GAAATTAATACGACTCACTATAGGGTTGGGGGCGACACTCCACCATGAATCACTCCCCTGTGAGGAAGTACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTCTGTCAGCCTCCAGGACCCCCCTCCCGGGAAGGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGATAAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGCTAGCCGAGTAGTGTGGGTGCGGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGAGTGCCCCGGAGGTCTCGTAGACCGTGCACCATGAGCACGAATCTAAACCCCAAAGAAAAACCAAACGTAACACCAACCGTCGCCCACAGGACGTCAAGTTCCCGGGTGGCGGTCAATCGTTGGTGGAGTTTACTTGTGCGCGCAGGGGCGTCATAGCTGTTTC CTG (SEQ ID NO:190)

HCV genotype 2b	GAAATTAATACGACTCACTATAGGGTTGGGGGCGACACTC CGCCATGAATCACTCCCCTGTGAGGAACTACTGTCTTCACG CAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTCGTAC AGCCTCCAGGCCCCCCCCCTCCCGGGAGAGCCATAGTGGTCT GCGGAACCGGTGAGTACACCGGAATTACCGGAAAGACTGG GTCCTTTCTTGGATAAACCCTCTATGTCCGGTCATTTGG GCACGCCCCCGCAAGACTGCTAGCCGAGTAGCGTTGGGTT GCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCTTGCGA GTGCCCCGGGAGGTCTCGTAGACCGTGCATCATGAGCACA AATCCTAAACCTCAAAGAAAAACCAAAAGAAACACAAACC GCCGCCCACAGGACGTTAAGTTCCCGGGTGGCGGTCAGAT CGTTGGCGGAGTTTACTTGCTGCCGCGCAGGGGCCGTCATA GCTGTTTCCTG (SEQ ID NO:191)
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crRNA sequences shown as 5'→3' reverse complement to be annealed with T7 oligo for IVT.

Table 6: Clinical samples used in this study.

Sample number	Sample ID	Ct value
391	160935	16
392	160938	14
393	160939	16
394	160941	16
395	160942	27
396	160943	14
397	160944	20
398	160947	14

#	Sample ID in figures	Sample ID	Ct value (SARS- CoV-2 N gene)	SARS- CoV-2 strain
Used in Fig. 16C&16D				
95		A1	20.20318222	Delta
96		A2	23.8019371	Delta
97		A3	20.38153458	Delta
98		A4	23.91605568	Beta
99		A5	21.15932465	Delta
100		A6	20.17287445	Beta
101		A7	20.63743782	Delta
102		A8	26.51057625	Delta
103		A9	24.15272903	Delta
104		A10	26.29830933	Delta
105		A11	22.10408401	Delta
106		A12	26.71198273	Delta
107		B1	32.79143906	Delta
108		B2	30.94537354	Delta
109		B3	19.86674881	Delta
110		B4	26.80452538	Delta
111		B5	23.44918823	Delta
112		B6	23.47177696	Delta
113		B7	25.94287682	Delta
114		B8	26.83697891	Beta
115		B9	26.61860275	Delta
116		B10	29.60500526	Beta
117		B11	28.60080528	Delta
118		B12	21.05815697	Delta
119		C1	23.73565865	Delta
120		C2	20.14953613	Delta
121		C3	24.65905762	Delta

122		C4	20.69680595	Delta
123		C5	24.33411217	Alpha
124		C6	30.21217537	Delta
125		C7	30.52635384	Beta
126		C8	25.62018967	Delta
127		C9	26.6070385	Delta
128		C10	16.92820358	Alpha
129		C11	26.35331726	Alpha
130		C12	16.99542999	Beta
131		D1	15.77931976	Other
132		D2	26.65350533	Delta
133		D3	18.5001297	Alpha
134		D4	31.98776627	Delta
135		D5	16.7693367	Delta
136		D6	34.24099731	Delta
137		D7	21.01351547	Other
138		D8	16.75134087	Delta
139		D9	29.01878738	Delta
140		D10	27.75723839	Delta
141		D11	29.67990685	Other
142		D12	20.9514904	Other
143		E1	24.57509232	Delta
144		E2	20.96550179	Delta
145		E3	26.76332283	Beta
146		E4	25.0199337	Other
147		E5	29.9657135	Beta
149		E7	27.49075508	Alpha
150		E8	22.13245583	Delta
151		E9	23.42307281	Delta
152		E10	24.99167442	Delta
153		E11	23.46128845	Delta
154		E12	19.64938736	Delta

155		F1	28.25497818	Delta
156		F2	20.91403008	Beta
157		F3	23.19413948	Delta
158		F4	33.09214783	Alpha
159		F5	34.40586853	Delta
160		F6	30.73725128	Other
161		F7	23.17684937	Other
162		F8	21.75904465	Alpha
163		F9	34.17617035	Beta
164		F10	27.16244316	Alpha
165		F11	19.42002296	Beta
166		F12	33.7602272	Alpha
167		G1	35.41335297	Alpha
168		G2	17.82286072	Other
169		G3	Undetermined	
170		G4	Undetermined	
171		G5	Undetermined	
172		G6	Undetermined	
173		G7	Undetermined	
174		G8	Undetermined	
175		G9	Undetermined	
176		G10	Undetermined	
177		G11	Undetermined	
178		G12	Undetermined	
179		H1	Undetermined	
180		H2	Undetermined	
181		H3	Undetermined	
182		H4	Undetermined	
183		H5	Undetermined	
184		H6	Undetermined	
185		H7	Undetermined	
186		H8	Undetermined	

187		H9	Undetermined	
188		H10	Undetermined	
189		H1	Undetermined	
190		H2	Undetermined	
192		H4	Undetermined	
193		H5	Undetermined	
194		H6	Undetermined	
195		H7	Undetermined	
197		H9	Undetermined	
Results described in text				
396	A12	160943	14	
397	B1	160944	20	
398	B2	160947	14	
410	C2	160992	23	
421	D1	164191	17	
444	E12	164515	14	
445	F1	164520	27	
446	F2	164525	27	
447	F3	164527	19	
448	F4	164530	19	
449	F5	164532	27	
450	F6	164537	27	
451	F7	164539	18	
452	F8	164553	20	
453	F9	164580	34	
454	F10	164581	20	
455	F11	164583	18	
456	F12	164589	22	
457	G1	164596	31	
458	G2	164597	15	
459	G3	164598	24	

460	G4	164603	18	
461	G5	164604	18	
462	G6	164605	19	
463	G7	164624	25	
465	G9	164649	21	
466	G10	164650	22	
467	G11	164651	17	
468	G12	164654	26	
469	H1	164661	22	
470	H2	164662	18	
472	H4	164665	25	
473	H5	164667	20	
474	H6	166758	24	
475	H7	166759	19	
476	H8	166760	21	
477	H9	166761	14	
478	H10	166762	23	
479	H11	166763	16	
480	H12	166764	24	
K0002	–ve 1	K0002	Undetermined	
K0005	–ve 2	K0005	Undetermined	
K0006	–ve 3	K0006	Undetermined	
K0007	–ve 4	K0007	Undetermined	
K0008	–ve 5	K0008	Undetermined	
Used in Fig. 17C				
K0244	14		14	
K0246	15.6		15.6	
K0250	16.8		16.8	
K0247	17.5		17.5	
K0249	17.8		17.8	
K0254	15.5		15.5	

K0252	17.5		17.5	
K0203	16.7		16.7	
K0209	19.2		19.2	
K0200	16.9		16.9	
K0253	30.7		30.7	
K0213	14.7		14.7	
K0068	17		17	
K0265	16.1		16.1	
Used in Fig. 17D				
1	22.23623848		22.23623848	
2	20.20703506		20.20703506	
4	14.83642673		14.83642673	
6	16.92188454		16.92188454	
7	22.07608795		22.07608795	
8	20.47973633		20.47973633	
9	21.8478508		21.8478508	
10	26.23923874		26.23923874	
11	22.94090843		22.94090843	
12	22.38891029		22.38891029	
13	22.45388031		22.45388031	
14	22.93203545		22.93203545	
15	28.23180008		28.23180008	
16	27.80712509		27.80712509	
5	-Ve		Undetermined	
18	-Ve		Undetermined	
Described in text				
1	1		18.57	
2	2		24.62	
3	3		19.65	
4	4		30.72	
5	5		32.33	

6	6		21.14	
7	7		31.9	
8	8		34.9	
9	9		19.93	
10	10		16.59	
11	11		18.46	
12	12		17.81	

13	13		19.82	
14	14		18.2	
15	15		23.64	
16	16		35.57	
17	17		20.72	
18	18		16.57	
19	19		30	
20	20		18.98	
21	21		31.13	
22	22		27.63	
-Ve	-Ve		Undetermined	
-Ve	-Ve		Undetermined	

OPTIMA-dx reaction

The reaction was performed using RT-LAMP primers at a final concentration of 1.6 μ M FIP/BIP primers (with the T7 promoter sequence added to the FIP primer), 0.2 μ M F3/B3 primers, and 642 0.4 μ M LF/LB primers, in 1X Isothermal Amplification Buffer from Lucigen (30027, Lucigen), 1.4 mM dNTPs, 2.4 U/ μ L Bst DNA Polymerase (30027, Lucigen), 0.3 U/ μ L of WarmStart RTx Reverse Transcriptase (M0380, NEB), 6 mM MgSO₄, 0.8 U/ μ L RNasin plus (N2611, Promega), 0.5 mM NTPs, 4 U/ μ L Hi-T7 RNA polymerase (M0658S, NEB), 0.4 U/ μ L thermostable inorganic pyrophosphatase (M0296, NEB), 1 μ M ssRNA FAM reporter or 750 nM ssRNA HEX reporter, 50 nM Cas13, 50 nM crRNA, and 4.5 μ L of template RNA in 25- μ L reactions. A detailed protocol for the OPTIMA-dx reaction setup is provided in supplementary note 1.

One-pot multiplexed OPTIMA-dx reaction

The multiplexed reaction was performed as described above (OPTIMA-dx reaction) with the following modification. 50 nM of AapCas12b protein, 50 nM of AapCas12b sgRNAs-1 for RNase P detection, 250 nM HEX ssDNA reporter (the FAM reporter used at 250 nM instead of 1 μ M in multiplexed detection), and RT-LAMP primers for RNase P detection (LF and LB were 655 used at 0.2 μ M final

concentration) were added to OPTIMA-dx SARS-CoV-2 or HCV detection components. The final reaction volume is 50 μ L. These reactions were incubated in a 96-well plate (BioRad) at 56°C for 1–2 h in a 96-well Real-Time PCR detection system (CFX96 qPCR machine, BioRad), with fluorescence measurements taken every 2 min using both FAM and HEX channels.

Pre-crRNA processing assays

RNA oligos of 5' FAM-labelled pre-crRNAs were custom-synthesized (IDT). Pre-crRNA processing assays were performed in 1x isothermal buffer (B0537, NEB) supplemented with an additional 6 mM MgSO₄ (final of 8 mM MgSO₄) in a 20- μ L reaction volume. In all assays, 100 nM of each Cas13a orthologue was incubated with 200 nM of their cognate 5'-FAM labeled pre-crRNAs for 1 hour at different temperatures. The reactions were then heated at 70°C for 3 min in 1X RNA Loading Dye (B0363S, NEB) and cooled down on ice for 3 min before loading onto a 15% polyacrylamide-urea denaturing gel. Electrophoresis was conducted for 80 min at 25 W. The gels were visualized using fluorescein channel in Bio-Rad Molecular Imager Gel Doc system.

Two-pot detection reactions

Reverse transcription and LAMP isothermal amplification of target nucleic acids were conducted using the previously reported RT-LAMP primers [Joung *N Engl J Med*, 2020. 383(15): p. 1492-1494]. Reactions were performed using 1.6 μ M FIP/BIP primers (with the T7 promoter sequence added to either the FIP or BIP primer), 0.2 μ M F3/B3 primers, and 0.4 μ M LF/LB primers in 1X Isothermal Amplification Buffer (20 mM Tris- HCl pH 8.8, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Tween 20) (B0537, NEB), 1.4 mM dNTPs, 8 U of Bst 2.0 WarmStart DNA Polymerase (M0538, NEB), 7.5 U of WarmStart RTx Reverse Transcriptase (M0380, NEB) and 6 mM MgSO₄ (B1003, NEB) in 25- μ L reactions containing 100 cp/ μ L of SARS-CoV-2 control standards. The reactions were incubated at 62°C for 40 min in a PCR machine (C1000 touch thermal cycler, BioRad). For subsequent Cas13a-based detection, 50 nM of recombinant HheCas13a or TccCas13a protein was incubated with 50 nM of the respective crRNA,

250 nM of ssRNA reporter (Poly(U) ssRNA reporter for HheCas13a or mix ssRNA reporter for TccCas13a), 0.8 U/ μ L RNaseOUT, 2 U/ μ L Hi- T7 RNA polymerase (M0658S, NEB), 1 mM NTPs, and 2 μ L of the RT-LAMP reaction product. Reactions were run in a 96-well plate (BioRad) at 55°C for 1 h in a 96-well Real-Time PCR detection system (CFX96 qPCR machine, Bio-Rad), with fluorescence measurements taken every 2 min using the FAM channel.

Michaelis-Menten enzyme kinetic parameters calculation

The trans cleavage activity of TccCas13a was investigated by measuring Michaelis-Menten enzyme kinetic parameters following the protocol introduced by Ramachandran et al.[4]. The Michaelis-Menten equation represents the relationship between reaction velocity and substrate concentration, which can be obtained from experimental data:

$$v = \frac{d[P]}{dt} = k_{cat}E_0 \frac{[S]}{K_M + [S]}$$

Where v is reaction velocity, $[P]$ is the concentration of reaction product, E_0 is the initial enzyme concentration, and $[S]$ is the substrate concentration. The reporter cleaved by TccCas13a is the reaction product in this assay. To estimate the kinetic parameters of TccCas13a, 0.5 nM of activated RNP was treated with different concentrations of FAM Mix reporters. In detail, 100 nM RNP was first prepared by incubating 100 nM TccCas13a protein, 125 nM crRNA (# 1172), and 1 U of RNase inhibitor (NEB, M0314L) in 1X isothermal amplification buffer (B0537, NEB) supplemented with 6 mM MgSO₄ at 56°C for 10 minutes. Next, the trans cleavage activity of RNP was activated by mixing 20 nM of N gene target with 2 nM RNP in 1 x isothermal amplification buffer supplemented with 6 mM MgSO₄ and 1 U of RNase inhibitor and incubated at 56°C for 15 min. For the trans cleavage assay, FAM Mix reporter at concentrations of 31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 1 μ M, 2 μ M and 4 μ M was added into 0.5 nM of target-activated RNP together with 6 mM MgSO₄ and 1 U of RNase inhibitor in 1x

isothermal amplification buffer in 20 μL of the final volume. The fluorescence readout was measured every 30 s at 56°C (CFX96 qPCR machine, Bio-Rad). The same reactions described above were also carried out in parallel without the addition of crRNA, which were used as controls to subtract the fluorescence background signal. The data were analyzed by GraphPad Prism software (GraphPad, CA, USA) to calculate K_M and k_{cat} . First, the data obtained from reactions without crRNA were subtracted from those reactions with crRNA to obtain the true fluorescence generated by enzyme-cleaved reporters. The real-time data from the first 600 s were fitted using linear regression to obtain the initial reaction velocity for different reporter concentrations represented by the increase of fluorescence over time, which can be represented as dF/dt . To convert the fluorescence readout into the concentration of the cleaved product, FAM Mix at 31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 1 μM , and 2 μM was incubated with 40 μg of RNase A (Invitrogen, cat: 12091-039) in 20 μL reaction at 37°C for more than 3 hours to ensure complete cleavage. By plotting the end-point data and subtracting it from the background using water-only samples over reporter concentration, the relationship between fluorescence readout and reporter concentration can be obtained: $FP = a[P]$. Where FP is the fluorescence produced by the cleaved reporter, and a is a constant.

The reaction can then be calculated as follows:

$$v = \frac{dP}{dt} = \frac{1}{a} \times \frac{dF}{dt}$$

The curve for reaction velocity dP/dt over reporter concentration was fitted to the Michaelis–Menten equation to calculate the value of K_M and V_{max} . The k_{cat} can be calculated as V_{max} equals the value of $k_{cat}E_0$. To test the validation of calculated kinetic parameters, the back-of-the-envelope test introduced in Ramachandran et al. [Ramachandran, et al., *Anal Chem*, 2021. **93**(20): p. 7456-7464] was conducted. For all the tests, an initial linear time portion t_{lin} of 600 s was used to calculate the α , β , and γ values:

E_0 (nM)	t_{lin} (s)	S_0 (nM)	v (nM/s)	α	β	γ
0.5	600	4000	0.296	0.044	0.371	
		2000	0.195	0.059	0.245	
		1000	0.103	0.062	0.129	
		500	0.039	0.047	0.049	
		250	0.031	0.073	0.038	
		125	0.015	0.074	0.019	
		62.5	0.008	0.073	0.010	
		31.25	0.003	0.060	0.004	

Optimization of one-pot detection reactions

For Bst DNA polymerase screening and other optimization reactions, reverse transcription and LAMP isothermal amplification of the target nucleic acids, coupled with T7-mediated in vitro transcription and Cas13-based detection of the amplified and in vitro-transcribed target RNA, were carried out in the same tube. Reactions were performed using RT-LAMP primers at a final concentration of 1.6 μ M for FIP/BIP primers (with the T7 promoter sequence added to either the FIP or BIP primer), 0.2 μ M F3/B3 primers, and 0.4 μ M LF/LB primers, in 1X Isothermal Amplification Buffer (from a different vendor from the Bst DNA polymerase screening reactions) or from Lucigen (30027, Lucigen) in other optimization experiments, 1.4 mM dNTPs, 0.32 U/ μ L Bst DNA Polymerase (from a different vendor from the Bst DNA polymerase screening reactions) or 2.4 U/ μ L from Lucigen (30027, Lucigen), 0.3 U/ μ L of WarmStart RTx Reverse Transcriptase (M0380, NEB), 6 mM MgSO₄, 0.8 U/ μ L RNasin plus (N2611, Promega), 0.5 mM NTPS, 2 U/ μ L Hi-T7 RNA polymerase (M0658S, NEB), 0.4 U/ μ L thermostable inorganic pyrophosphatase (M0296, NEB), 250 nM RNA reporter, 50 nM Cas13, 50 nM crRNA, and 2 μ L of template RNA in 25- μ L reactions. These reactions were incubated in a 96-well plate (BioRad) at 56°C (or as otherwise indicated) for 1–2 h in a 96-well Real-Time PCR detection system (CFX96 qPCR machine, Bio-Rad), with fluorescence measurements taken every 2 min using the FAM channel.

For the detection of HCV, the one-pot detection reactions were performed as described above with the use of 500 pM in vitro transcribed RNA template. For the detection of TYLCV, the reactions were performed as described above, but without the addition of RTx reverse transcriptase and with use of 2 µL of 1:10 or 1:100 diluted extracted DNA as template. 1 ng of TYLCV plasmid was used as positive control. All RT-LAMP primers are listed above.

Screening of AapCas12b sgRNAs in one-pot reaction

Reactions were performed using RT-LAMP primers at a final concentration of 1.6 µM for FIP/BIP primers, 0.2 µM F3/B3 primers, and 0.2 µM LF/LB primers, in 1X Isothermal Amplification Buffer (30027, Lucigen), 1.4 mM dNTPs, 2.4 U/µL Bst DNA Polymerase (30027, Lucigen), 0.3 U/µL of WarmStart RTx Reverse Transcriptase (M0380, NEB), 6 mM MgSO₄, 0.8 U/µL RNasin plus (N2611, Promega), 0.5 mM NTPS, 4 U/µL Hi-T7 RNA polymerase (M0658S, NEB), 0.4 U/µL thermostable inorganic pyrophosphatase (M0296, NEB), 250 nM ssDNA HEX reporter, 50 nM AapCas12b, 50 nM sgRNAs, and 1 µL of total human RNA template in 25-µL reactions. These reactions were incubated in a 96-well plate (BioRad) at 56°C for 1 h in a 96-well Real-Time PCR detection system (CFX96 qPCR machine, Bio-Rad), with fluorescence measurements taken every 2 min using the HEX channel. T7 RNA polymerase and NTPs were included here to test the compatibility of Cas12b activity with these reagents for the subsequent multiplex detection reactions.

Agroinfiltration inoculation of plants with TYLCV and DNA extraction

Plant infection with TYLCV infectious clones and subsequent DNA extraction was done following the previous protocol (Mahas et al. *Detection of Plant DNA Viruses*. Viruses, 2021. **13**(3)).

Clinical sample collection and RNA extraction.

Oropharyngeal and nasopharyngeal swabs were collected from suspected COVID-19 patients by physicians in Ministry of Health hospitals in Saudi Arabia and placed in 2-mL screw-capped cryotubes containing 1 mL of TRIZOL for inactivation and transport. Each sample tube was sprayed

with 70% ethanol, enveloped with absorbent tissues, and then placed and sealed in individually labeled biohazard bags. The bags were then placed in leak-proof boxes and sprayed with 70% ethanol before placement in a dry ice container for transfer to the lab. Total RNA was extracted from the samples following instructions as described in the CDC EUA-approved protocol and using the Direct-zol kit (Direct-zol RNA Miniprep, Zymo Research; catalog #R2070) following the manufacturer's instructions.

Extraction-free sample processing and concentration

A beads-based extraction mixture was prepared as follows. First, beads were prepared by washing 1 mL of Sera-Mag SpeedBeads Carboxyl Magnetic Beads Hydrophobic (GE Healthcare 65152105050250) with 1 mL of UltraPure DNase/RNase-free distilled water (1097705, Invitrogen) twice and then resuspended in 50 mL of beads binding buffer (10 mM Tris-HCl pH 8.0, 1M KCl, 18 % PEG-8000, and 1mM EDTA). Next, 50 mL of extraction mixture was prepared by mixing 32.5 mL of beads (resuspended in binding buffer), 12.5 mL of 4x Viral RNA Extraction Buffer (VRE100, Sigma-Aldrich), and 5 mL of UltraPure DNase/RNase-free distilled water. The extraction mixture was aliquoted in 1.5 mL tubes, 400 μ L each.

To process clinical samples, 200 μ L of VTM of oropharyngeal swabs were transferred into 400 μ L of extraction mixture, vortexed vigorously and incubated at room temperature for 5 min. The mixture was then placed on a magnetic rack (Invitrogen DYNAL bead Separator) for 2-3 mins until the solution gets clear. Next, the supernatant is removed, and beads are resuspended and washed in 750 μ L of 70% ethanol (v/v). Samples are again placed on the magnetic rack for ~ 2 mins until solution gets clear. The supernatant is removed completely and the tubes are left open for 5-10 min to dry. Beads are then resuspended in 30 μ L of H₂O and vortexed vigorously for 5 seconds and incubated at room temperature for 3 mins. The samples are then placed on the magnetic rack to collect beads, and 15 μ L of H₂O is transferred into 35 μ L of OPTIMA-dx master mix.

For experiment with VTM spiked with non-infectious virus particles, 200 μ L of VTM of oropharyngeal swabs collected from healthy donors were spiked with the indicated concentration of non-infectious virus particles

(NATSARS(CoV2)-ERC, ZeptoMetrix) or with 200 μ L of (NATSARS(CoV2)-NEG, ZeptoMetrix) for negative controls, and the spiked VTM were processed as described above.

Real-time reverse transcription PCR (RT-PCR) for detecting positive SARS-CoV-2 RNA samples.

RT-PCR was conducted on extracted RNA samples using the oligonucleotide primer/probe (Integrated DNA Technologies, 641 catalog #10006606) and Superscript III one-step RT-PCR system with Platinum Taq Polymerase (catalog #12574-026) following the manufacturer's protocol. Freeze-drying of detection reactions

Multiplexed OPTIMA-dx detection reactions were assembled as described above in a final volume of 50 μ L in 1.5 mL tubes. Reactions were snap-frozen in liquid nitrogen and transferred to a LABCONCO Acid-Resistant CentriVap Concentrator (supplemented with LABCONCO CentriVap -105°C Cold Trap and Vacuubrand CVC 3000 Vacuum pump) Freeze Dry System for 2–3 hours of freeze-drying at a minimal temperature under the pressure of 1 to 10 millibar until the water was completely removed. Rehydration of freeze-dried reactions was accomplished with the RNA isolated from clinical samples (20 μ L), 25 H₂O, and 5 μ L of 10X Isothermal Amplification Buffer from Lucigen (30027, Lucigen).

Assembly of OPTIMA-dx reaction.

1- Dilute TccCas13a protein into 1 μ M in 1x isothermal buffer (Lucigen, 30027). 2- Assemble TccCas13a RNP as follows:

Reagents	Concentration	Amount (μ L)	Final concentration
H ₂ O		12	
Isothermal buffer	10X	3	1X
TccCas13a	1 μ M	7.5	250 nM
TccCas13a crRNA#1172	1 μ M	7.5	250 nM
Total		30 (enough for 6 reactions)	

3- Keep the assembly at room temperature while assembling the OPTIMA-dx reaction.

4- Assemble OPTIMA-dx master mix as follows:

Reagents	Concentration	Amount (μL)	Final concentration
H ₂ O		0.65	
Isothermal buffer	10X	2.5	1X
MgSO ₄	100 mM	1.5	6 mM
dNTPs	25 mM	1.4	1.4 mM
SC LAMP primers	10X	2.5	1X
NTPs	10 mM	1.25	0.5 mM
<i>Bst</i> DNA polymerase	50 U/μL	1.2	2.4 U/μL
RTx reverse transcriptase	15 U/μL	0.5	0.3U/μL
RNasin Plus RNase inhibitor	40 U/μL	0.5	0.8 U/μL
Hi-T7 RNA polymerase	50 U/μL	2	4 U/μL
Inorganic pyrophosphatase	20 U/μL	0.5	0.4 U/μL

HEX mix reporter	18.75 μ M	1	750 nM
RNP	250 nM	5	50 nM
template		4.5	
Total		25	

5- Incubate the reaction at 56 °C for 60 mins.

6- Place the reaction tubes in p51 molecular fluorescence viewer and visualize the results.

Assembly of multiplexed OPTIMA-dx reaction.

1- Dilute TccCas13a protein into 2 μ M in 1x isothermal buffer (Lucigen, 30027). 2- Dilute AapCas12b protein into 2 μ M in 1x isothermal buffer (Lucigen, 30027). 3- Assemble of TccCas13a and AapCas12b RNPs as follows in 2 different tubes:

- TccCas13a RNP assembly:

Reagents	Concentration	Amount (μ L)	Final concentration
H ₂ O		4.5	
Isothermal buffer	10X	3	1X
TccCas13a	2 μ M	7.5	500 nM
TccCas13a crRNA (#1172 for SARS-CoV2 or #1363 for HCV)	1 μ M	15	500 nM
Total		30 (enough for 6 reactions)	

- AapCas12b RNP assembly:

Reagents	Concentration	Amount (μL)	Final concentration
H ₂ O		4.5	
Isothermal buffer	10X	3	1X
AapCas12b	2 μM	7.5	500 nM
AapCas12b sgRNA-1 for RNase P detection.	1 μM	15	500 nM
Total		30 (enough for 6 reactions)	

4- Keep the RNPs at room temperature while assembling the multiplexed OPTIMA-dx reaction.

5- Assemble multiplexed OPTIMA-dx master mix as follows:

Reagents	Concentration	Amount (μL)	Final concentration
H ₂ O		0	
Isothermal buffer	10X	5	1X
MgSO ₄	100 mM	3	6 mM
dNTPs	25 mM	2.8	1.4 mM
SC LAMP primers (or HCV primers)	10X	5	1X
RNase P primers	10X	5	1X
NTPs	10 mM	2.5	0.5 mM
<i>Bst</i> DNA polymerase	50 U/μL	2.4	2.4 U/μL

RTx reverse transcriptase	15 U/ μ L	1	0.3U/ μ L
RNasin Plus RNase inhibitor	40 U/ μ L	1	0.8 U/ μ L

Hi-T7 RNA polymerase (High concentration (M0470T, NEB))	1000 U/ μ L	0.2	4 U/ μ L
Inorganic pyrophosphatase	20 U/ μ L	1	0.4 U/ μ L
FAM RNA mix reporter	25 μ M	0.5	250 nM
HEX ssDNA reporter	10 μ M	1.25	250 nM
TccCas13a RNP	500 nM	5	50 nM
AapCas12b RNP	500 nM	5	50 nM
template		9.35	
Total		50.65	

6. Incubate the reaction at 56 °C for 60 mins in a 96-well Real-Time PCR detection system (CFX96 qPCR machine, Bio-Rad), with fluorescence measurements taken every 2 min using both FAM and HEX channels.

5

OPTIMA-dx Software Implementation

The dataset of fluorescent images used for training the software consisted of many random images annotated manually as positive or negative to set the proper fluorescence intensity threshold. The software was then trained and tested multiple times to reach the best mean average precision (mAP) value with this dataset. The application allows the user to easily take a picture of PCR strips or upload an already captured image of a PCR strip illuminated by a transilluminator. The software then determines the location of each tube, calculates a probability score for each target category and classifies each tube as positive (green bounding box) or negative (red bounding box) samples based on the intensity of the fluorescent signal (Fig. 6d). The entire image processing, from capturing the reaction tubes to the final app output results, takes less than 1 min. Once the image is captured, it can be uploaded and processed by the software.

Deep Learning Framework

As of today, there are various deep learning frameworks for engineers and researchers to choose to train machine learning models [Abadi, M., et al., arXiv preprint arXiv:1603.04467, 2016, Chen, T., et al., Mxnet: arXiv preprint arXiv:1512.01274, 2015, Jia, Y., et al. Caffe: Convolutional architecture for fast feature embedding. in Proceedings of the 22nd ACM international conference on Multimedia. 2014, Paszke, A., et al., Pytorch: An imperative style, high-performance deep learning library. arXiv preprint arXiv:1912.01703, 2019]. These frameworks abstract the underlying hardware and software stack to expose a simple API in language such as Python. Among these frameworks, TensorFlow is one of the most popular frameworks in deep learning community [Abadi, M., et al., arXiv preprint arXiv:1603.04467, 2016]. Compared with TensorFlow, TensorFlow-Lite (TensorFlow Lite. tensorflow.org/lite.) is the lightweight version of TensorFlow, which is specifically designed for the mobile

platform and embedded devices. In this project, TensorFlow framework was used to train the model on Linux workstation and use TensorFlow-Lite to deploy the trained model on mobile device with Android operating system.

Object Detection Model

5 Object detection has been witnessing a rapid revolutionary change in the field of computer vision. Basically, it involves two tasks:

 Object localization: determine where objects are located in a given image. Specifically, object detection model will use
10 rectangular bounding boxes to locate all the detected objects in the image.

 Object classification: determine which category each detected object belongs to. Specifically, for each detected object, object detection model will calculate a probability for each target
15 category, indicating how likelihood this detected object belongs to this specific category.

 Currently, there are many popular object detection models (e.g., SSD [10], RetinaNet [Lin, T.-Y., et al. Focal loss for dense object detection. in Proceedings of the IEEE
20 international conference on computer vision. 2017], Faster R-CNN [Ren, et al., arXiv preprint arXiv:1506.01497, 2015], Mask R-CNN [He, K., et al. *Mask r-cnn*. in *Proceedings of the IEEE international conference on computer vision*. 2017]) available in different deep learning frameworks. All these
25 models have one common part called feature extractor, which focuses on calculating high quality features for object detection tasks. In order to deal with the problem of limited resources on the mobile device (e.g., small storage space and limited battery

power), researchers have proposed some efficient feature extraction network architecture specifically tailored for mobile and resource constrained environments, such as MobileNets [Howard, et al., arXiv preprint arXiv:1704.04861, 2017],
5 MobileNetV2 [Sandler, M., et al. *Mobilenetv2: Inverted residuals and linear bottlenecks*. in *Proceedings of the IEEE conference on computer vision and pattern recognition*. 2018]. In this work, the SSD with MobileNetV2 (referred to as SSD-Mobilenet-V2) was the main focus. SSD (Single Shot
10 MultiBox Detector) is a popular algorithm in object detection. Mobilenet-V2 is a convolution neural network used to produce high-level features which can be used as a backbone feature extractor for SSD. It is small, low-latency, low-power and parameterized to meet the resource constraints
15 of a variety of use cases. Specifically, it can be run efficiently on mobile devices with TensorFlow-Lite. SSD-Mobilenet-V2 combines the advantages of the two models, enabling it to efficiently perform target detection tasks on mobile devices.

Transferring well-trained object detection models on one
20 dataset to another new dataset is a common approach called transfer learning. It has several benefits, but the main advantages are saving training time, getting better performance, and not needing a lot of data. Google has trained one SSD-Mobilenet-V2 object detection model using COCO dataset
25 (Microsoft. <https://cocodataset.org>), which has 90 different categories. This pre-trained model can be used as a good starting point for OPTIMA-dx detection model to help us save time and get better performance. Specifically, since this project only has 2 categories (positive and negative), final layers were

modified to make it produce only 2 outputs corresponding to the 2 categories.

Dataset

To train the model, one OPTIMA-dx image dataset was created using P51™ Molecular Fluorescence Viewer (minipcr. <https://www.minipcr.com/product/p51-molecular-glow-lab>). Specifically, cameras were used on different mobile devices to take pictures of the tubes shown on this device. At least 391 pictures have been directly taken from mobile device cameras. When taking these pictures, due to some random lighting, angles, jitter and other issues, some pictures are distorted and blurred, which are not suitable for training and testing the model, and need to be deleted. These images were further augmented by randomly modifying the brightness, contrast, color and sharpness, which generated a new set of images. Each image was made square by cropping to make sure each tube in each image can maintain a normal aspect ratio during model training and testing. Next, the images that meet the requirement were randomly divided into a training set (540 pictures) and a test set (66 pictures). Finally, the ground-truth bounding boxes for each picture in training and test set were created by LabelImg (tzutalin. github.com/tzutalin/labelimg).

Model training

In order to transfer visual knowledge learned from the large-scale generic dataset COCO to the model, it was initialized using Google's pre-trained SSD- Mobilenet-V2. The model was then trained using the training set. Specifically, it was trained on one GeForce GTX 1080 Ti GPU with batch size 10. RMSprop optimizer was used with initial learning rate as 0.004. A total of 35000 steps/batches were trained and one checkpoint was saved every 10 minutes. Then all the testing can be done using these saved checkpoints.

Model testing

The most common metric used to evaluate the performance of object detection model is the mAP (mean average precision) [Howard, A.G., et al.,

arXiv preprint arXiv:1704.04861, 2017; Sandler, M., et al. Mobilenetv2: Inverted residuals and linear bottlenecks. in Proceedings of the IEEE conference on computer vision and pattern recognition. 2018.]. In the testing process, mAP was calculated on the test set for each saved check point, and finally take the checkpoint with the largest mAP. Currently, the best mAP is 97.6%.

Android App development

The selected checkpoint was converted into a TensorFlow Lite model using the model converter tool (tensorflow.org/lite/convert) provided by TensorFlow. Then it can be deployed and run this TensorFlow Lite model on mobile device. For each input image, the TensorFlow Lite model can output 3 different kind of information: 1) the location of the bounding box for each detected tube, containing four coordinate values on the image plane: left, top, right and bottom. 2) Category for each detected tube and its value is either positive or negative. 3) One confidence score indicating the probability that the model thinks one tube belongs to the category. Finally, the bounding boxes, categories and confidence scores are drawn over the input image and display the final image on screen.

The code for the smart phone app and to download the app is available at: [hi-zhengcheng.github.io/optima-dx](https://github.com/hi-zhengcheng/optima-dx)

Results

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a highly sensitive, robust and practical method that holds great promise in developing a viable POC detection platform. However, when used independently as a method to detect nucleic acids, it suffers from a high rate of false positives due to primer-dimer formation and cross-contamination. In order to address these drawbacks, introducing additional level of specificity is highly desirable. Coupling RT-LAMP to specific CRISPR/Cas target recognition and *in trans* reporter cleavage ensures signal appearance only in reactions where a correct amplicon has been generated. Nevertheless, most methods reported to date rely on transferring RT-LAMP

product to the second tube, which complicates handling and, more importantly, leads to aerosol formation that is detrimental to accuracy of subsequent reactions. In order to address these shortcomings, an assay where all components are added in a single step and the reaction is incubated at a single temperature is needed. To achieve this goal, thermostable reagents are needed that can tolerate the relatively high temperatures needed for the RT-LAMP reactions.

Identification, screening and characterization of thermophilic Cas13 enzymes

Recently, Cas13 variants from mesophilic bacteria have been employed for biosensing, including pathogen detection, genotyping, and diagnostics of viruses and disease markers. The SARS-CoV-2 pandemic highlighted the need to develop POC diagnostics. RT-LAMP is a practical approach for POC diagnostics. Still, it needs to be developed in a one-pot closed system to avoid cross-contamination, facilitate user-friendliness and enhance sensitivity and specificity. The current Cas13 homologs can only be used in a two-pot assay which is problematic and poses a risk of cross-contamination. Therefore, it was aimed to identify Cas13 proteins from thermophilic bacteria and test their thermostability in a wider temperature range especially at higher temperatures suitable for developing one-pot RT-LAMP assay involving virus genome amplification and CRISPR-mediated detection in a single tube.

Cas13 variants were interrogated to determine whether some of these originate from thermophilic hosts. HheCas13a originating from *Herbinix hemicellulosilytica* thermophilic bacterium was identified as a potential thermophilic protein. Subsequently, the HheCas13a (1285 amino acids) was used as a query in BLAST-P NCBI searches of non-redundant protein sequences datasets to interrogate databases for potential thermophilic Cas13 homologs. A TccCas13 homolog originating from *Thermoclostridium caenicola*, sharing 87% identity at the amino acid level, was identified as another likely thermophilic protein. The gene sequence of TccCas13a was

synthesized and the available clone of HheCas13a was used for heterologous expression in *E. Coli* followed by purifying the proteins to homogeneity. Subsequently, differential scanning Fluorimetry (DSF) was conducted to test the thermostability of the HheCas13 and TccCas13a proteins.

5 The data showed that both proteins possess a denaturation temperature higher than those of mesophilic bacteria, e.g., LwaCas13a. Next, it was hypothesized that the complexing of sgRNA and the HheCas13 and TccCas13 proteins would further stabilize the proteins at higher temperatures. *In silico* prediction of the TccCas13a crRNA was performed
10 and the recently reported crRNA of the HheCas13a was used. TccCas13a and HheCas13a were incubated with and without their respective *in vitro* transcribed sgRNAs at 37, 60, 70 and 90°C for 30 minutes. The SDS-PAGE analysis demonstrated that the TccCas13a and HheCas13a loaded with sgRNA exhibit higher thermostability. However, TccCas13a exhibited a
15 better thermostability compared to HheCas13a.

 The data showed that both proteins exhibit thermostability and thus are promising candidates for downstream applications requiring *cis* and *trans* catalytic activities at higher temperatures, e.g., one-pot RT-LAMP for diagnostic applications as well as targeted gene knockdown, virus
20 interference, and RNA editing and imaging.

Characterization of the cis and trans catalytic activities of the TccCas13a and HheCas13a thermophilic proteins

 The data showed that both proteins remain folded at higher temperatures and that loading of sgRNA enhances the thermostability of the
25 proteins. Next, it was tested whether the RNP complex of these proteins is active and mediates *cis* and *trans* activities required for the downstream applications of these proteins. sgRNAs were designed to cleave a synthetic target sequence to determine the ability of both proteins to exhibit catalytic *cis* activities at higher temperatures.

The data showed that both proteins exhibit robust *cis* catalytic activities at higher temperatures.

In order to couple RT-LAMP with specific CRISPR/Cas based detection, two thermophilic Cas13 enzymes, namely HheCas13a and TccCas13a, were identified and their activity tested at relevant temperatures. When Cas13/crRNA ribonucleoproteins (RNP) recognize and cleave their target sequence, they exhibit collateral cleavage activity that degrades surrounding ssRNAs [8, 9]. Such collateral activity has been harnessed in nucleic acid detection applications, where a ssRNA probe (reporter) molecule present in the Cas13 reaction is degraded by target-dependent Cas13 collateral activity [7]. The ssRNA reporter contains a fluorophore linked by a short ssRNA sequence to a quencher, which emits fluorescence after the ssRNA sequence is cleaved, indicating the presence, and therefore the detection, of the target of interest. Because different Cas13 proteins exhibit different cleavage preferences depending on ssRNA sequences [6], the ssRNA reporter that can be cleaved by TccCas13a and HheCas13a effectors was investigated.

It was determined whether these proteins retain the non-specific *trans* degradation activities of ssRNA reporter molecules in the presence of ssRNA target at higher temperatures. Different Cas13a variants trigger collateral RNase activity at exposed uridine residues, so the *trans*-cleavage nucleotide preference of TccCas13a and HheCas13 at higher temperatures was tested. Therefore, 3 different ssRNA probes were screened. Each probe was conjugated to a 5' fluorescent molecule (FAM) and a 3' fluorescence quencher (FQ). 6-mers of poly(A) and poly(U) homopolymers and an additional 6-mer probe of mixed U, G, A, C nucleotides were used. Using two targeting crRNAs and one non-specific (NS) crRNA, it was observed that TccCas13a exhibited a *trans*-ssRNA cleavage preference against a mixed RNA sequence (not homopolymer ssRNA sequence) containing different nucleotides: UGACGU (FIG. 1A). HheCas13 exhibited a cleavage preference for a homo-uridine ssRNA substrate, consistent with previous

observations (Fig. 1B) [10]. Moreover, the rate at which the trans-cleavage activity of ssRNA reporters reached saturation was determined using different target, ssRNA activator, concentrations. The data show that TccCas13 exhibited a higher sensitivity with only fM of the ssRNA activator
5 resulting in detectable cleavage of the reporter.

Based on the observation that both HheCas13a and TccCas13a are derived from thermophilic host bacteria, it was determined whether both effectors exhibit cleavage activity at high temperatures. It was observed that both proteins were highly active at high temperature (60 °C), with
10 TccCas13a showing stronger and faster activity at high temperature compared with its activity at 37 °C (Figs. 2A-2B).

These results prompted the investigation of the activity of both Cas13 effectors at a broad range of elevated temperatures. Although LwaCas13a exhibited very robust and fast signal when incubated at 37°C, no activity was
15 observed at high temperatures. In contrast, both HheCas13a and TccCas13a maintained robust trans cleavage activity at 56°C and 60°C. HheCas13a maintained strong activity at temperatures up to 60 °C. On the other hand, TccCas13a maintained a robust activity at temperatures as high as ~ 70 °C (Fig. 2C). In addition, crRNAs with increased spacer lengths was tested
20 using 24 and 28 nt long spacers. Subsequently, trans cleavage activity assays using a broad temperature range (37-72C) was conducted. The data showed that TccCas13a loaded with crRNA2 is active over a wide range of temperature (37-70C). HheCas13, however, showed, robust activity between 37-60C. Upon exploring the crRNA spacer length requirements of the
25 previously uncharacterized TccCas13a effector, it was observed that TccCas13a showed comparable activity using 24 or 28 nt long spacer sequences (Fig. 2D). These data provide compelling evidence on the thermostability and catalytic activities of TccCas13a and HheCas13a proteins and their usefulness in applications requiring cis and trans catalytic
30 activities. See also Figs. 1C-1E, 1F, and 2E.

Biochemical characterization of thermostable TccCas13a and HheCas13a

To further characterize the activity of the identified thermostable Cas13 proteins, the crRNA requirements were tested by introducing various modifications to the crRNA spacer sequences. The effect of single mismatches between crRNA and target RNA on HheCas13a and TccCas13a RNA detection activities was tested. Single bases across the crRNA spacer sequence were mutated to the respective complementary bases. Results show that both HheCas13a and TccCas13a were tolerant to single mismatches across the spacer, as such mismatched spacers enabled RNA detection with similar efficiency as fully matched spacers (Fig. 14A and Fig. 14F).

However, double mismatches resulted in different tolerances for different regions of the spacer sequence with both HheCas13a and TccCas13a proteins (Fig. 14 and Fig. 14G). In addition, when stretches of 4 mismatches were introduced in the spacer, a significant reduction was found in the activity of both Cas13 enzymes for mismatch stretches in the center of the spacer, while 4 consecutive mismatches at the extreme 5' or 3' ends of the crRNA spacer had less of an effect, indicating the presence of a seed region in the center of the spacer sequence, similar to previous observations with other Cas13 effectors (Fig. 14C and Fig. 14H) [8].

The minimal spacer length required for efficient Cas13-mediated RNA detection was investigated. A series of spacer truncations ranging from 30 nt to 16 nt long spacers. While HheCas13a required 24 nt or longer spacers to maintain efficient RNA detection activity (Fig. 14I), TccCas13a exhibited robust activity with spacer sequences as short as 20 nt (Fig. 14D).

Notably, previous studies have shown the ability of CRISPR/Cas13 systems to process pre-crRNAs and generate mature crRNAs capable of guiding Cas13 enzymes to the target RNA [9]. Interestingly, HheCas13a is the only known Cas13 orthologue that is incapable of processing pre-crRNAs, at least in vitro [10]. Considering that both HheCas13a and TccCas13a are thermostable proteins and evolutionarily closely related (Fig.

1C), the ability of TccCas13a to process pre-crRNAs in vitro was investigated. Using 5'-FAM labeled pre-crRNA sequences, the pre-crRNA processing activity of TccCas13a was tested in comparison to HheCas13a and LwaCas13a proteins. LwaCas13a exhibited robust pre-crRNA processing activity, but HheCas13a did not process the cognate pre-crRNA when it was incubated at 37 or 60 °C, [10]. pre-crRNA processing activity with TccCas13a at the tested temperatures was also not detected. These results, together with previous findings [10], indicate that the thermostable HheCas13a and TccCas13a enzymes are the only pre-crRNA processing defective Cas13 homologs known to date, pointing to a possible relationship between the thermostability of these Cas13 variants and the lack of pre-crRNA processing activities.

The robust activity of TccCas13a observed in the previous experiments led to further study the enzyme kinetics. Michaelis-Menten kinetic measurements of TccCas13a ssRNA trans cleavage activity were performed at elevated temperature and it was found that when TccCas13a is activated with ssRNA targets, it catalyzes trans ssRNA cleavage with catalytic efficiency (kcat/Km) of $0.24 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 14E). The observed high value of the Km indicates the low affinity of Cas13 enzyme with the non-specific, in trans, RNA target. Similar observations of high Km values were recently reported with other Cas13 variants [77].

Use of thermophilic Cas13s for SARS-CoV-2 detection in one-pot and two-pot assays

To ensure sensitive detection, pre-amplifying the RNA target of interest is a necessary step [7]. RT-LAMP isothermal amplification was chosen because it possesses several advantages over other amplification methods, including high sensitivity, rapid turnaround time, simple operation, and low cost [11]. Primer sets well-established in previous reports were used to target and amplify conserved regions in the SARS-CoV-2 N gene, named here as STOPCovid (SC) primer sets [4, 12]. However, because Cas13s target RNA, these primers were modified by appending a T7 promoter

sequence to the 5' end of the first half of either the forward inner primer (FIP) or the backward inner primer (BIP). Therefore, during LAMP amplification, the T7 promoter sequence should get integrated into the amplified DNA products, providing a suitable template for the T7 RNA polymerase to transcribe the amplified LAMP product *in vitro* and generate RNA targets for Cas13 detection (Table 1).

Various reports have shown the utilization of T7 RNA polymerase for subsequent *in vitro* transcription of the amplified product and Cas13 based detection. However, coupling of the RT-LAMP amplification, T7-mediated transcription, and Cas13 detection in one pot at relatively high temperature suitable for RT-LAMP amplification is an unmet need. Therefore, the thermostable Hi-T7 RNA polymerase, which exhibits optimal performance at temperatures close to where both RT-LAMP and thermophilic Cas13 variants are active, was utilized to accomplish this goal.

crRNAs targeting the highly conserved region in the SARS-CoV-2 *N*-gene were designed and tested with primer sets previously reported to be highly efficient and specific. Initial screening of these crRNAs and primer sets in two-pots settings, where RT-LAMP was performed first, and the amplified product was used in the second step for T7 RNA polymerase-mediated *in-vitro* transcription and Cas13-based detection, identified few crRNAs that were highly active (Figures 3A-3D). Most of the tested crRNAs targeting the RNA transcript produced from LAMP products harboring T7 promoter sequence from FIP-T7 or BIP-T7 primers showed robust performance and high detection signal. However, crRNAs targeting regions of LAMP amplicons that are not transcribed showed no activity. These results supported the strong detection of RT-LAMP products when using crRNAs, confirming that 1) the amplification of the synthetic SARS-CoV-2 genome was successful with the modified primers; 2) the T7 promoter was successfully integrated into the amplified products; 3) T7 RNA polymerase could use the amplified amplicons to generate Cas13a substrates

that activate Cas13a enzymes to degrade ssRNA reporters and generate signal output (Fig. 15B, Figs. 18A-18D).

It was then investigated whether target detection in one-pot assay is feasible at a single temperature using all three enzymes. Therefore, all crRNAs and primer sets were rescreened in one pot settings. The screening assays identified a combination of crRNA and primer set that showed the most specific and efficient detection of the SARS-CoV-2 RNA in comparison to the other tested crRNAs and primers, namely crRNA#1172 when used with SC T7-FIP modified primer sets (Figs. 4A-4D).

Consistently, this combination of primers and crRNA specifically and efficiently detected SARS-CoV-2 target in one-pot (Figs. 5A-5E). Interestingly, although HheCas13a exhibited strong detection signal in two-pot settings, no significant detection signal was identified in one-pot settings with any crRNA. By contrast, TccCas13a was consistent in specifically and efficiently detecting SARS-CoV-2 target in one-pot with the optimized combination of primers and crRNA.

Optimization of the one-pot assay

In order to maximize the efficiency of this system, the reaction chemistry was optimized in terms of the type of *Bst* DNA polymerase used, and the concentrations of *Bst* DNA polymerase, Hi-T7 RNA polymerase, Mg^{2+} and Cas13 RNP in the reaction (Fig. 6 and Figs. 7A-7D). It was observed that the optimal sensitivity and efficiency of the one-pot detection assay can be achieved if the system is highly tuned with regards to the type and concentration of enzymes used at every step (Fig. 6 and Figs. 7A-7D).

With the optimized reaction, the analytical limit of detection (LoD) of the Cas13-based one pot assay was evaluated using synthetic SARS-CoV-2 RNA as an input. The LoD of the one pot assay was estimated to be 20 cp/uL, an improved sensitivity relative to reported Cas13-based one pot assays for SARS-CoV-2 detection (Fig. 8) [13].

Because different biochemical reactions perform optimally at different temperatures in the one-pot assay, the performance of the one-pot

assay was tested at different temperatures. The optimal temperature as determined by this experiment for the one-pot detection assay was 56°C, with diminished performance at higher or lower temperatures, probably due to the reduced performance of LAMP at lower temperatures, and of the Hi-
5 T7 RNA polymerase at higher temperatures (Figs. 15C, 15D).

Validation of the thermophilic Cas13-based one-pot assay on clinical samples

Next, the assay was validated with total RNA extracted from SARS-CoV-2 patient swab samples. Oropharyngeal or nasopharyngeal swab
10 samples were collected from suspected COVID-19 patients. After RNA extraction following the CDC EUA-approved protocol, the samples were confirmed positive for SARS-CoV-2 using RT-qPCR. The Cas13-based one-pot assay was first tested on 8 samples (provided in the chart above) with Ct values of 14-27. Using the one-pot Cas13 detection assay, all samples were
15 correctly identified (Fig. 9). These results indicate that this newly developed detection system can reliably detect SARS-CoV-2 in clinical samples.

Evaluation and clinical validation of OPTIMA-dx assay for SARS-CoV-2 visual detection

To provide for large-scale screening during a pandemic, performing
20 diagnostic assays at POC or outside of laboratory settings is important. Since the use of sophisticated fluorescence detection instruments such as qPCR machines or plate readers complicates the achievement of such goal, CRISPR diagnostic approaches have adapted lateral flow detection in an effort to develop a simple visual readout that can expedite accurate
25 diagnostics in POC settings [43]. However, despite the efficiency and simplicity of this approach, the reaction tubes need to be opened for lateral flow detection readouts, thus increasing the chance of aerosols and cross-contamination.

As an alternative, a strategy was sought to couple the assay with a
30 portable device. Using RNA reporter molecules conjugated to a 5' HEX or FAM fluorophores at the appropriate concentration, TccCas13a collateral

cleavage produced a bright signal visible with a hand-held, inexpensive fluorescence visualizer (P51 Molecular Fluorescence Viewer), allowing simple visualization and interpretation of the results (Fig. 16A). This one-pot assay with visual detection was termed OPTIMA-dx (One-pot thermophilic Cas13 and isothermal amplification module for nucleic acid detection).

With these optimized reaction conditions, the analytical limit of detection (LoD) of OPTIMA-dx was examined using synthetic SARS-CoV-2 RNA as input. The LoD of OPTIMA-dx assay was estimated to be 10 copies (cp)/ μ L, which can be achieved within 45–60 min of reaction time (Fig. 16B). To test specificity and absence of cross-reactivity, OPTIMA-dx was challenged with other common human viruses, including SARS-CoV-1, MERS-CoV, H1N1, HCoV-OC43, HCoV-229E, and HCoV-NL63. OPTIMA-dx showed high specificity to SARS-CoV-2, with no cross-reactivity against any of the other tested viruses.

Next, how storage at common storage temperatures influenced the performance of a pre-assembled OPTIMA-dx master mix was assessed. Although the OPTIMA-dx reaction did lose activity after storage for 48 h at 4°C, the detection reaction remained active when stored at –20°C for at least 10 days and after multiple freeze-thaw cycles.

To ensure reliability of SARS-CoV-2 detection kits, the Federal Drug Administration (FDA) guidelines (Catalog # 2019-nCoV-EUA-01, CDC, 2019) emphasize the importance of including a positive sample, or internal control, as an indicator of proper sample handling, RNA extraction, template quality and integrity, and validity of reagents. In particular, a negative SARS-CoV-2 readout should be considered invalid if the internal control is negative as well. Thus human ribonuclease P (RNase P) transcripts were tested as an internal control for OPTIMA-dx SARS-CoV-2 detection assay, whereby each sample can be evaluated by two OPTIMA-dx reactions for the detection of SARS-CoV-2 and the RNase P internal control. Accordingly, two crRNAs targeting a region of RNase P were designed amplified with RT-LAMP primers developed in previous reports that were modified with

the T7 promoter sequence appended to the FIP primer [78]. Both crRNAs showed efficient and specific detection, with crRNA 1 showing a faster detection signal compared to crRNA 2, prompting selection of crRNA 1 for the OPTIMA-dx RNase P assay.

5 The one-pot detection assay was also conducted using RNA isolated from patient samples. SARS-CoV-2 detection was performed at King Faisal Specialist Hospital & Research Centre using 73 RT-qPCR–positive samples with a broad range of Ct values and different strains of SARS-CoV-2 and 27 RT-qPCR–negative samples extracted following the protocol approved by
10 the Center for Disease Control Emergency Use Authorization (CDC EUA). The one-pot SARS-CoV-2 detection assay demonstrated 94.5% sensitivity and 100% specificity, showing high concordance with the RT-qPCR data (Fig. 16C). Interestingly, although all 4 samples showing false negative results had Ct values above 30, the detection assay was able to detect other
15 samples with Ct values as high as 34, indicating the high sensitivity of the assay (Fig. 16C). Also tested were all clinical samples for the RNase P gene. OPTIMA-dx detected the RNase P internal control in all tested samples, except one of the negative samples (Fig. 16D).

 In addition, OPTIMA-dx was utilized for visual detection of SARS-
20 CoV-2 with total RNA extracted from another set of swab samples collected from suspected SARS-CoV-2 patients. The validation assays of OPTIMA-dx were conducted using RNA extracted from 45 randomized samples (different from samples used in Fig. 16C), 40 positive samples with Ct values ranging between 14–34, and 5 negative samples. A positive OPTIMA-dx signal was
25 detected with all tested samples, with the exception of the negative samples and no template control (NTC) within 1 h. However, samples with Ct values above 30 show a weaker signal compared to samples with Ct values below 30. These results indicated that OPTIMA-dx can reliably detect SARS-CoV-2 in patient samples within 1 h, with a simple visual readout, for Ct values up
30 to 34.

Development and assessment of simple extraction method for POC sample processing

An important consideration for rapid and simple POC diagnostics is to avoid complicated nucleic acid extraction procedures and kits that are labor-intensive, costly, and require specialized laboratory equipment and personnel training. Therefore, the development of an extraction-free sample processing protocol is critical to simplify CRISPR-dx and increase their user-friendliness for POC applications. Several extraction-free sample processing protocols have been previously developed and applied with CRISPR-dx, including HUDSON and proteinase K-based treatments [13, 44, 79]. These sample processing methods usually require heating steps for efficient lysis of viral particles and inactivation of nucleases. In addition, unlike conventional extraction methods that usually concentrate the extracted RNA, direct application of the treated sample to detection reactions introduces an upper bound and limited amount of inactivated sample input, resulting in decreased detection sensitivity [12].

Therefore, a simple extraction protocol was developed that can be performed at ambient temperature, thus avoiding the need for heating steps, and allowing the processing of large sample volumes, and increasing input via sample concentration. To this end, a viral RNA extraction buffer (Sigma Aldrich) was employed that provides rapid sample lysis and coupled it with sample concentration using magnetic beads. This RNA extraction buffer has several advantages, including short time of processing (5 minutes), room temperature incubation, stabilizing the released RNA, and compatibility with different sample types such as viral transport medium (VTM) and saliva. To streamline the protocol and allow the processing and RNA concentration from a large sample volume, the sample lysis step was combined with binding and concentration of released RNA using magnetic beads in a single step. This rapid protocol allows both steps (sample lysis and RNA binding to beads) to occur at room temperature in a short period of time (5 minutes).

First investigated was the ability of this method to lyse cells and capture the released RNA by detecting the RNase P template using oropharyngeal swabs collected from healthy donors and stored in VTM. RNA was released and concentrated from 200 μ L of VTM into 30 μ L final volume. To maximize the amount of sample input, the OPTIMA-dx reaction volume (50 μ L instead of 25 μ L) was doubled, which allowed use of up to 15 μ L of the concentrated RNA sample. It was found that the extraction protocol could efficiently lyse cells and capture target templates, which enabled efficient detection of RNase P internal control with OPTIMA-dx.

The performance of the extraction protocol and OPTIMA-dx was tested with oropharyngeal swabs collected from healthy donors and stored in VTM that was spiked with different concentrations of non-infectious SARS-CoV-2 virus particles. OPTIMA-dx was able to detect viral load as low as 5000 cp/sample ($\sim$ 50 cp/ μ L of reaction), and RNase P in all tested reactions.

Given the encouraging performance of the developed extraction protocol with OPTIMA-dx detection, this assay was conducted on clinical samples. 22 individual oropharyngeal swabs collected from COVID-19 patients and 2 oropharyngeal swabs collected from healthy donors stored in VTM were obtained. Part of these samples had previously undergone RNA extraction and tested with RT-qPCR in a diagnostic laboratory, allowing comparison of test results with the obtained RT-qPCR Ct values. These samples were processed with the developed extraction procedure and OPTIMA-dx was performed. The assay correctly detected all positive clinical samples with Ct values <34 , indicating that the performance of OPTIMA-dx with the developed extraction protocol is equivalent to the performance observed with extracted RNA.

One-pot multiplexed nucleic acid detection with thermostable AapCas12b and TccCas13a enzymes

An ideal diagnostic platform should allow multiplexed detection of more than one target in a single reaction [80]. Most of the developed CRISPR-based detection platforms detect only one pathogen or target in a

given reaction. This can be attributed mainly to the non-specific collateral cleavage activity of CRISPR Cas systems that complicates the integration of more than one reporter in a single reaction. Therefore, in many cases, several separate and independent reactions are developed to detect different targets or internal controls [37, 12, 81]. The development of a one-pot multiplexed detection reaction is of great importance to improve the detection efficiency, accuracy, and clinical applicability of CRISPR-based diagnostics. Recently, a Cas12b enzyme from *Alicyclobacillus acidiphilus* (AapCas12b) was shown to be thermostable and can be coupled with RT-LAMP for the detection of SARS-CoV-2 [12]. Different from Cas13, Cas12 collateral activity cleaves ssDNA molecules [82]. Therefore, it was reasoned that since both TccCas13a and AapCas12b are thermostable enzymes and have distinct cis and trans cleavage activities, the specific cleavage preference of these two enzymes could be used to develop a one-pot, RT-LAMP coupled multiplexed detection assay. Therefore, a one-pot multiplexed reaction was developed to detect SARS-CoV-2 and the internal control RNase P in the same reaction.

The already established and optimized TccCas13a reaction was used for the detection of SARS-CoV-2 using FAM-labelled RNA reporters, and develop AapCas12b-based detection of RNase P with the use of HEX-labelled ssDNA reporters, which would allow the differentiation between the two fluorescent signals using different detection channels (Fig. 17A). To this end, 3 different AapCas12b single guide RNAs (sgRNAs) were designed targeting the LAMP amplicon amplified by the primers used for RNase P detection, but with FIP primer lacking the T7 promoter. The 3 different sgRNAs were used in one-pot, RT-LAMP-coupled AapCas12b RNase P detection reaction using the same OPTIMA-dx reaction components and conditions, and found that all tested sgRNAs showed comparable performance and mediated robust detection as measured from the HEX fluorescence signal (Fig. 21A).

However, sgRNAs 1 and 3 showed faster and more specific signals compared to sgRNA 2. Therefore, sgRNA 1 were selected to develop the one-pot multiplexed OPTIMA-dx reaction. Next, it was tested if multiplexed RT-LAMP reaction is possible and if the Cas12 and Cas13-mediated fluorescent signals can be produced and distinguished from each other. The reaction mix contained both RT-LAMP primer sets for the detection of SARS-CoV-2 and RNase P targets and both FAM and HEX reporters. SARS-CoV-2 and RNase P were detected in the same reaction using HEX and FAM channels without any fluorescence signal interference from each Cas enzyme's collateral activity (Fig. 17B and Fig. 21B).

Next, the performance of the multiplexed OPTIMA-dx reaction was evaluated for the detection of SARS-CoV-2 and the internal control RNase P on RNA extracted from 14 clinical COVID-19 samples. The OPTIMA-dx multiplexed reaction showed an unambiguous positive result for both SARS-CoV-2 and RNase P in all tested clinical samples (Fig. 17C). The multiplexed detection assay was evaluated on clinical swabs using the simple and quick crude sample extraction method developed above. To this end, 14 oropharyngeal swabs from patients with COVID-19 infection and 2 COVID-19 negative swabs were obtained. These samples were processed with the extraction procedure and OPTIMA-dx multiplexed detection was performed of both SARS-CoV-2 and RNase P. The multiplexed OPTIMA-dx reaction reliably detected both SARS-CoV-2 and RNase P in all SARS-CoV-2 positive samples in 60 min (Fig. 17D).

Although the OPTIMA-dx master mix showed strong stability when stored at -20°C , it was sought to test whether the OPTIMA-dx reaction components can be freeze-dried, which would further simplify storage and distribution for POC applications. Therefore, OPTIMA-dx reaction was freeze-dried for multiplexed detection and the reaction was tested with the same samples processed with the quick extraction protocol in Fig 17D. The OPTIMA-dx reaction remained functional and was able to detect SARS-

CoV-2 and RNase P in all tested samples. However, a reduction in the reaction speed and performance was observed.

Development of a mobile phone application to collect and share SARS-CoV-2 test results

5 Fluorescence detection devices like any plate reader or real-time PCR machines are choice devices to measure any end-point or real-time signal in a sample. However, at a POC settings with fewer resources and no specialized training, smartphone-based imaging is becoming popular in biomedical applications for easy data accessibility and sharing. To facilitate
10 data collection and sharing of SARS-CoV-2 test results as well as interpretation of the OPTIMA-dx readout, a deep learning-based approach was developed to design and develop a mobile phone application capable of collecting and reading OPTIMA-dx results from the low-cost P51 Molecular Fluorescence Viewer at POC settings.

15 The ability of the OPTIMA-dx smartphone application to identify and call positive and negative readouts from OPTIMA-dx results was validated: the app correctly determined the fluorescence status of each sample with good accuracy. The app was next evaluated on the 45 clinical samples tested with OPTIMA-dx in (discussed above): the app identified 38
20 out of the 45 samples as positive. Notably, the two SARS-CoV-2 positive samples deemed negative by the app had the highest Ct values (31 and 34) and thus lowest intense fluorescent signal of all samples. OPTIMA-dx was also run for RNase P in the same samples, resulting in 43 samples testing positive for
25 RNase P out of the 45 samples tested, using the OPTIMA-dx one-pot assay and app. It was concluded that OPTIMA-dx can reach a performance of 95% sensitivity and 100% specificity in patient samples when combined with the mobile app, exhibiting high concordance with RT-qPCR data.

Versatility of OPTIMA-dx for pathogen diagnostics

30 The one-pot detection assay of OPTIMA-dx can be adapted for the detection of other pathogens. To demonstrate the versatility of OPTIMA-dx,

the system was also employed for the detection of the human RNA virus hepatitis C virus (HCV) and the plant ssDNA virus Tomato yellow leaf curl virus (TYLCV). Using in vitro transcribed RNAs of two common HCV genotypes, it was shown that OPTIMA-dx can efficiently detect the two
5 viruses within 1 hour (Fig. 22A). In addition, OPTIMA-dx was used to detect TYLCV ssDNA virus isolated from plants infected with the virus. After DNA isolation from infected, as well as non-infected (healthy), plants, the extracted DNA was diluted 1:10 and 1:100, and the diluted DNA was used as template for OPTIMA-dx reactions. OPTIMA-dx detected the virus
10 only in the DNA extracted from infected plants in both DNA dilutions within 1 hour, indicating the high sensitivity and specificity of the OPTIMA-dx platform for efficient detection of plant DNA viruses (Fig. 22B). Having established multiplexed detection of SARS-CoV-2 and RNase P with OPTIMA-dx, also shown was the versatility of OPTIMA-dx for multiplexed
15 detection of HCV and RNase P internal control, further demonstrating the capability of OPTIMA-dx for multiplexed detection (Fig. 22C).

Discussion

Initially, CRISPR/Cas diagnostic systems were designed as two-pot assays [4, 5, 14], where reverse transcription and isothermal reactions were
20 performed separately. Following the amplification step, a fraction of the reaction mixture was transferred to another tube containing the Cas enzyme and reporter. This approach, however, dramatically increases the chances of carry-over contamination and complicates handling, rendering such modules unfeasible as real-life diagnostic kits. Numerous attempts were reported to
25 address these shortcomings [15] including one-pot reaction systems using Cas12b [16] and amplification-free detection protocols [17, 18]. Amplification-free strategies based on LbuCas13a have the advantage of facile protocol and absence of cross-contamination. However, sensitivity of such approaches remains low, with samples having Ct values above 29
30 evading detection. Furthermore, all present amplification-free techniques rely on a sophisticated device that must distinguish between genuine signal

and the background. An advantage of the thermophilic Cas13-based one-pot detection assay described herein is the straightforward assay workflow that can be performed without sophisticated equipment or trained personnel. The whole assay can be performed in less than 80 minutes with only minimal equipment or risk of cross-contamination.

Frequent, rapid and cost-effective testing without relying on centralized facilities is a key advancement in early screening during pandemic situations [19].

This study shows 1) the first identification and characterization of thermophilic Cas13a enzymes, thereby expanding the molecular engineering toolbox of CRISPR systems for RNA substrates; 2) the first report of a one-pot assay using RT-LAMP coupled to Cas13 for specific and sensitive detection of SARS-CoV-2 to facilitate POC applications and limit cross-contamination; 3) the utilization of the identified thermostable Cas13 with a thermostable Cas12b enzyme to develop a one-pot multiplexed detection reaction; 4) the development and use of mobile phone application coupled with the portable, affordable P51 detection to collect and share testing data with central facilities.

Since their discovery, Type VI CRISPR Cas13 systems have provided efficient and versatile tools for RNA manipulation [100, 101]. However, all Cas13 work to date has been restricted to temperatures around or below 42°C. Mining datasets from diverse natural contexts, including thermophiles, helped uncover novel thermostable variants that evolved naturally to provide immunity to their hosts. These thermophilic Cas13a enzymes will open diverse biotechnological applications for RNA-guided Cas13a ribonucleases at a broad temperature range and under harsh experimental or environmental conditions, especially if complexed with their corresponding crRNAs. For example, Cas13 has recently been gaining interest for therapeutics and disease research, including cancer gene therapy and antiviral therapeutics [83-86]. However, *in vivo* protein stability is important for successful applications [87] and

some proteins, such as LwaCas13a, mediate efficient knockdown only when fused to a stabilizing domain [88].

Notably, thermostabilization of proteins results in better stability *in vivo* [89, 90]. Therefore, the robust activity and thermostability of TccCas13a make this protein a promising candidate for further structural studies and potential *in vivo* RNA targeting applications. Moreover, thermostable enzymes (including thermostable Cas9) provide important genome editing applications in thermophiles [91]; specific RNA targeting at elevated temperatures beyond the range of previously reported Cas13 proteins is key to creating new tools for use in industrially important thermophiles, for which no CRISPR-Cas13 system has been reported. Phylogenetic analysis showed that HheCas13a and TccCas13a are evolutionarily closely related (Fig. 1C). Despite the strong conservation of pre-crRNA processing activity among Cas13 orthologues, HheCas13a has been shown to be the only known pre-crRNA processing defective Cas13 [10]. Interestingly, in addition to both proteins being thermostable Cas13s, the data show that both HheCas13a and TccCas13a are incapable of processing pre-crRNA. These observations could indicate a possible relationship between thermostability and the lack of pre-crRNA processing activity in these Cas13 variants. Besides the wide use of CRISPR-Cas13 enzymes for *in vivo* applications [92], Cas13 proteins have been increasingly used to develop various diagnostic platforms [80], and their usefulness for the development of diagnostics has become apparent during the COVID-19 pandemic [102-104, 93-95]. LAMP isothermal amplification was adapted to develop sensitive CRISPR diagnostics for SARS-CoV-2 detection, including DETECTR [37], iSCAN [96], DISCOVER (Cas13-based module) [102] and the first and only FDA-authorized CRISPR-Cas13 based diagnostics test (SHERLOCK CRISPR SARS-CoV-2 Kit, IDT). However, all the above diagnostic methods are performed in two-pot settings, as their Cas enzymes function at around 37°C and cannot tolerate the high temperatures needed for LAMP

(~55–65°C). Such two-pot settings are not ideal for POC settings due to the increased chance of cross-contamination, which can be overcome with the implementation of thermostable DNA-targeting Cas12 proteins [12, 97]. Therefore, the thermophilic Cas13a protein offers a great
5 advance in diagnostics and other applications. Coupling the activity of both thermostable Cas12 and Cas13 enzymes and the specific recognition of correct amplicons by each CRISPR/Cas system, OPTIMA-dx can be used for multiplexed detection of more than one target in a single reaction. The multiplexed detection capability of OPTIMA-dx could enable
10 additional nucleic acid detection applications, including detecting different virus variants or different pathogens, such as other common respiratory viruses or bacteria in the same reaction. Future developments will include a visual readout of multiplex detection reactions for simple applications at POC.

15 During this work, two different thermostable Cas13 enzymes were characterized and a thermostable Cas13 was identified that could be adapted for a one-pot RT-LAMP coupled Cas13 detection reaction. Although HheCas13a showed good thermostability and activity in two pot system, good performance was not observed in a one- pot reaction.
20 However, this does not exclude the compatibility of HheCas13a for one-pot detection reactions.

OPTIMA-dx detection module has several other advantages that make it suited for POC applications. OPTIMA-dx demonstrated excellent sensitivity with an LoD of 10 cp/μL of synthetic SARS-
25 CoV-2 RNA and can detect samples with Ct values up to 34. Therefore, OPTIMA-dx exhibited robust sensitivity that provides its use for reliable SARS-CoV-2 detection in clinical samples. In addition, besides the strong stability of OPTIMA-dx reaction master mix at –20°C and tolerance to multiple freeze-thaw cycles, it was also demonstrated that
30 OPTIMA-dx reagents can be lyophilized, which would facilitate pre-assembly of OPTIMA-dx reactions for transportation or long-term storage

for large-scale screening in POC settings or remote areas. Moreover, OPTIMA-dx detection module does not require RNA extraction and is compatible with simple lysis and extraction methods. The ambient-temperature sample lysis and concentration method further increases the simplicity of the assay for POC applications.

The detection module was also integrated with the portable P51 Molecular Fluorescence Viewer to facilitate sample readout and developed a machine learning module for efficient data collection and sharing of the test results. Overall, the software provides an additional diagnosis validation and enables fast data sharing, making the entire diagnostic process affordable and accessible to a larger section of society. In conclusion, this work provides a thermophilic Cas13a variant and development of a one-pot RT-LAMP-coupled CRISPR-Cas13a assay for sensitive and specific SARS-CoV-2 detection. The work provides a viable platform for COVID-19 detection in limited-resource settings. Moreover, the thermophilic Cas13a variants reported in this work have other applications beyond diagnostics, including in RNA knockdown, editing, imaging, and virus interference. This work thus expands the applications of CRISPR- Cas13 systems and offers new possibilities for transcriptome engineering and diagnostics at higher temperatures.

Example 2: Development of a miniature CRISPR-Cas13 system that facilitates SARS-CoV-2 detection.

CRISPR/Cas systems possess great potential for various applications, so there are ongoing efforts to search for, identify, and characterize new Cas effectors to increase utility and develop new tools for *in vivo* and *in vitro* applications [48, 49]. Recently, computational approaches for metagenomic mining resulted in the discovery of previously unknown CRISPR/Cas systems, including class II/type VI Cas proteins that exclusively target ssRNA substrates [50-53]. Besides repurposing these RNA targeting

CRISPR/Cas13s for *in vivo* applications [54-58], Cas13s can be used in diagnostics that exhibit unprecedented sensitivity, specificity, and speed [40, 42-44, 47]. Different Cas13 variants have been used for nucleic acid detection. For example, Cas13a [41, 59], Cas13b [60], and Cas13d [61] exhibit collateral cleavage activities and work for nucleic acid detection. This Example expands the existing Cas13-based toolbox for diagnostic applications by identifying and characterizing CRISPR/Cas13 effectors. In this Example, an mCas13 variant was identified, characterized, and its utility for SARS-CoV-2 detection demonstrated. This work illustrates the untapped potential of mCas13 enzymes in diagnostics and other *in vivo* RNA applications.

Materials and Methods

Computational identification of CRISPR/mCas13

Protein sequences of miniature Cas13s in a recent report [62] were kindly provided by Dr. Hui Yang (Chinese Academy of Sciences, Beijing, China). These protein sequences were used as queries in the Basic Local Alignment Search Tool (BLAST) against the NCBI non-redundant (nr) protein database (before January 2021) using default settings. Only subject sequences with query coverage (Query cover) above 90% were considered. Protein sequences of the miniature Cas13f variants did not identify any subject sequences with query coverage above 90%. However, when using protein sequences of the miniature Cas13e variants, especially Cas13e.1 (accession# RKY08123), mCas13 (accession# HFH51004) showed up as the only subject sequence with query coverage above 90% (96%). Protein sequence alignment of mCas13 and the miniature Cas13e and Cas13f variants was performed using ClustalW [73] in MEGAX with default settings, and the alignment was visualized using ESPrpt [74]. The RxxxxH (SEQ ID NO:192) HEPN motif was subsequently identified in the mCas13 protein sequence on the basis of this alignment and was further confirmed by manually searching for this motif using SnapGene. CRISPRCasFinder [75] was performed on

the genomic DNA sequence (GenBank# DSVK01000191.1) to identify the associated CRISPR array. CRISPRDetect [76] was then used to predict the orientation of the direct repeat in the mCas13 CRISPR array.

Cas13 protein expression and purification

- 5 To produce the expression plasmid for Cas13 expression and purification, the *E. coli* codon-optimized Cas13 coding sequence was synthesized (GenScript) *de novo* and subcloned in frame with His and SUMO tags on the N-terminus into the His6-TwinStrep-SUMO bacterial expression vector (Addgene #115267) using BamHI and NotI (Table 8).
- 10 Purification of mCas13 protein was performed following the protocol of Kellner *et al.* (2019) with a few modifications. Briefly, the mCas13 expression vector was transformed into BL21 *E. coli* cells. Starter cultures were prepared by growing single colonies in LB broth supplemented with 100 µg/mL ampicillin for 12 h at 37 °C. Next, 20 mL of starter culture was
- 15 used to inoculate 2 L of Terrific Broth medium (TB) (IBI scientific) supplemented with 100 µg/mL ampicillin for growth at 37 °C until an OD₆₀₀ of 0.5. Cells were incubated on ice for 30 mins, expression was induced with 0.5 mM IPTG, and cultures were then transferred to 16 °C for overnight expression. Cells were harvested by centrifugation for 20 min at 4 °C at 4000
- 20 rpm. Cell pellets were resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM DTT, EDTA-free protease inhibitor (Roche)) and supplemented with 1 mg/mL lysozyme (L6876, Sigma). Cells were lysed by sonication and clarified by centrifugation at 11,000 rpm for 50 min. The soluble 6xHis-SUMO-mCas13 in cleared lysate was then purified
- 25 with an affinity chromatography column (HiTrap Q HP, 5 mL GE Healthcare) (AKTA PURE, GE Healthcare) followed by concurrent removal of the 6xHis-SUMO tag by SUMO protease and overnight dialysis in dialysis buffer. Cleaved protein was concentrated to 1.5 mL by Amicon Ultra-15 Centrifugal Filter Units (50 kDa NMWL, UFC905024, Millipore)
- 30 and further purified via size-exclusion chromatography on a S200 column (GE Healthcare) in gel filtration buffer (50 mM Tris-HCl, 600 mM NaCl,

10% glycerol, 1 mM DTT, pH 7.5). The protein-containing fractions resulting from the gel filtration were pooled, snap frozen, and stored at -80 °C.

Nucleic acid preparation

5 A short region of the SARS-CoV-2 *N* gene sequence was used as a synthetic target in the preliminary mCas13 characterization and optimization experiments to screen crRNAs and collateral reporters and establish mCas13-based detection (Figure 10). The *N* gene target RNA sequences were prepared by *in vitro* transcription of PCR amplicons containing the T7 promoter sequence using the 2019-nCoV_N_Positive Control plasmid as a
10 PCR template (10006625, IDT). Purified PCR amplicons (QIAquick PCR Purification Kit, QIAGEN) were transcribed *in vitro* using HiScribe T7 Quick High Yield RNA Synthesis Kit (E2050, NEB). The transcripts were purified using Direct-zol RNA Miniprep Kits (R2050, Zymo Research)
15 following the manufacturer's instructions, and the purified RNA was stored at -80 °C.

 mCas13 crRNAs were designed to target the *N* gene sequence of the SARS-CoV-2 genome. For crRNA preparation, templates for *in vitro* transcription were generated using single-stranded DNA oligos
20 containing a T7 promoter, scaffold, and spacer in reverse complement orientation (IDT), and were then annealed to T7 forward primer in Taq DNA polymerase buffer (Invitrogen). The annealed oligos were then used as templates for *in vitro* transcription as described above.

 To establish RT-LAMP coupled with T7-mCas13-based detection
25 and LoD range, control synthetic SARS-CoV-2 viral genomic sequences used in Figure 11 were ordered as synthetic RNA from Twist Bioscience, and were diluted to 10,000 RNA copies/μL and used at indicated concentrations to create simulated clinical samples.

 For RT-LAMP amplification (described below), previously published
30 LAMP primers designed to amplify the SARS-CoV-2 *N* gene (Joung *et al.*, 2020 [36], Broughton *et al.*, 2020 [37]) were used, with modifications. The

FIP or BIP primers were each designed with the appended T7 promoter sequence at the 5' end of the first half of the primer. Such modification allows the modified primer to integrate the T7 promoter sequence in the LAMP-amplified product for subsequent T7-mediated *in vitro* transcription.

5 All oligo sequences and substrates are listed in Tables 1, 2 and 7.

Screening of crRNAs and reporters and establishing mCas13 collateral detection

Activity and collateral assays of Cas13 were performed in 1X cleavage buffer (20 mM HEPES-Na pH 6.8, 50 mM NaCl, 5 mM MgCl₂,
 10 1mM DTT) in a 20-μL final reaction volume. Cas13 and crRNAs RNP were first assembled by mixing 500 nM purified Cas13 with 500 nM crRNA (unless otherwise indicated) in 1X cleavage buffer and 20 units RNaseOUT (Invitrogen), followed by incubation at 37 °C for 15 minutes. Next, the assembled RNP was combined on ice with 2 μL of 500 ng/μL *in vitro*-
 15 transcribed target RNA and 250 nM RNA reporter, and reactions incubated for 1 hr at 37 °C (unless otherwise indicated). Real time or end-point fluorescence measurements were collected on a microplate reader M1000 PRO (TECAN) at 2-min intervals (for real time measurements) using 384-well, black/optically clear flat-bottomed plate (Thermofisher).

20 *RT-LAMP reactions*

Reverse transcription and isothermal amplification of target nucleic acids were performed using final concentrations of 1.6 μM FIP/BIP primers (with the T7 promoter sequence fused to either the FIP or BIP primer), 0.2 μM F3/B3 primers, and 0.4 μM LF/LB primers, 1X Isothermal
 25 Amplification Buffer (20 mM Tris-HCl, 50 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Tween 20, pH 8.8) (B0537, NEB), 1.4 mM dNTPs, 8 units of Bst2.0 WarmStart DNA Polymerase (M0538, NEB), 7.5 units of WarmStart RTx Reverse Transcriptase (M0380, NEB) and 6 mM MgSO₄ (B1003, NEB) in 25-μL reactions containing variable concentrations of
 30 SARS-CoV-2 control standards, or 4 μL of isolated RNA from clinical

samples. LAMP reactions were performed at 62 °C for 35 minutes in a PCR (C1000 touch thermal cycler, BioRad) machine.

One-step T7 transcription and mCas13 detection

The two reactions, T7-mediated *in vitro* transcription and mCas13-based detection of the amplified and *in vitro*-transcribed target RNA, were carried out in the same tube. Briefly, 2 µL of the RT-LAMP reaction product was combined with 1X cleavage buffer (described above), 500 nM mCas13/crRNA assembled RNPs, 25 units T7 RNA polymerase (M0251, NEB), 1 mM NTPs, and 250 nM RNA reporter in 20-µL reactions. The reactions were incubated at 37 °C for 20-30 minutes.

Visual Cas13-based detection

For simple visualization of mCas13-based detection, RNA reporters labeled with the HEX fluorophore were used instead of the FAM fluorophore (Table 3). Collateral cleavage of HEX reporters results in a bright signal that can be easily visualized upon excitation with LED light (Ali et al., 2020). Cas13-based reactions were carried out as described above, with modifications. For each reaction, 1 µM of HEX reporter (unless otherwise indicated) was used in 20-µL T7-mCas13 detection reactions. Reactions were incubated at 37 °C for 30 minutes. Reaction tubes were then transferred into the P51 Molecular Fluorescence Viewer (miniPCR) and photos were taken using a smartphone with default settings.

Clinical sample collection and RNA extraction

Oropharyngeal and nasopharyngeal swabs were collected from suspected COVID-19 patients by physicians in Ministry of Health hospitals in Saudi Arabia and placed in 2-mL screw-capped cryotubes containing 1 mL of TRIZOL for inactivation and transport. Each sample tube was sprayed with 70% ethanol, enveloped with absorbent tissues, then placed and sealed in an individual labeled biohazard bag. The bags were then placed in leak-proof boxes and sprayed with 70% ethanol before placement in a dry ice container for transfer to King Abdullah University of Science and

Technology (KAUST). Total RNA was extracted from the samples following instructions as described in the CDC EUA-approved protocol and using the Direct-zol kit (Direct-zol RNA Miniprep, Zymo Research; catalog #R2070) following the manufacturer's instructions.

- 5 *Real-time reverse transcription PCR (RT-PCR) for detecting positive SARS-CoV2 RNA samples.*

RT-PCR was conducted on extracted RNA samples using the oligonucleotide primer/probe (Integrated DNA Technologies, catalog #10,006,606) and Superscript III one-step RT-PCR system with Platinum

- 10 Taq Polymerase (catalog #12574–026) following the manufacturer's protocol.

*Nucleotide and Amino acid sequences used***Table 7: crRNA sequences used in this study.**

Name	Sequence	Note
Sense-mCas13-T1 (crRNA 1)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAC AAGGAACTGATTACAAACATTGGCCGCACCCTATAGTG AGTCGTATTAATTTT (SEQ ID NO:41)	crRNAs for SARS-CoV-2 N gene Target 1
Sense-mCas13-T2 (crRNA 2)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAC TGATTACAAACATTGGCCGCAAATTGCACCCTATAGTG AGTCGTATTAATTTT (SEQ ID NO:42)	
Sense-mCas13-T3 (crRNA 3)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCG TTCTTCGGAATGTCGCGCATTGGCATGGCCCTATAGTGA GTCGTATTAATTTT (SEQ ID NO:43)	
Sense-mCas13-T4 (crRNA 4) was used for all SARS- CoV-2 detection experiments	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCAGCGCTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTT (SEQ ID NO:44)	
Sense-mCas13-N target 2-1 (crRNA 5)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAA GATCACATTGGCACCCGCAATCCTGCTACCCTATAGTG AGTCGTATTAATTTT (SEQ ID NO:45)	crRNAs for SARS-CoV-2 N gene Target 2
Sense-mCas13-N target 2-2 (crRNA 6)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAC AATGCTGCAATCGTGCTACAACCTCCCTATAGTGAG TCGTATTAATTTT (SEQ ID NO:46)	
Sense-mCas13-N target 2-3 (crRNA 7)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCG TTCCTCATCACGTAGTCGCAACAGTTCACCCTATAGTGA GTCGTATTAATTTT (SEQ ID NO:47)	
Sense-mCas13-N target 2-4 (crRNA 8)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAC AGTTCAAGAAATTCAACTCCAGGCAGCACCCCTATAGTG AGTCGTATTAATTTT (SEQ ID NO:48)	
Sense-mCas13-N target 2-5 (crRNA 9)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCGA TGCTGCTCTTGCTTTGCTGCTGCTTGACCCTATAGTGAG TCGTATTAATTTT (SEQ ID NO:49)	

Sense-mCas13-N target 2-6 (crRNA 10)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAG GCCAAACTGTCTACTAAGAAATCTGCTGCCCTATAGTGA GTCGTATTAATTTTC (SEQ ID NO:50)	
Sense-mCas13-NS (NS)	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCTT ATGCCTCTTCCGACCATCAAGCATTTTACCCTATAGTGA GTCGTATTAATTTTC (SEQ ID NO:51)	Non-specific targeting
Sense-mCas13-4- Mis-1	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCAA ATTTGCCCCCAGCGCTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:52)	
Sense-mCas13-4- Mis-2	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ACTTGCCCCCAGCGCTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:53)	
Sense-mCas13-4- Mis-3	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTACCCCCAGCGCTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:54)	
Sense-mCas13-4- Mis-4	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCTCCAGCGCTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:55)	
Sense-mCas13-4- Mis-5	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCCGGCGCTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:56)	
Sense-mCas13-4- Mis-6	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCCAGCACTTCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:57)	
Sense-mCas13-4- Mis-7	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCCAGCGCTCCAGCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:58)	
Sense-mCas13-4- Mis-8	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCCAGCGCTTCATCGTTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:59)	
Sense-mCas13-4- Mis-9	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCCAGCGCTTCAGCGGTCTTCCCTATAGTGAG TCGTATTAATTTTC (SEQ ID NO:60)	

Sense-mCas13-4-Mis-10	GCTGGAGCAGCCCTCGATTTGCAGGGTAATCACAGCCA ATTTGCCCCCAGCGCTTCAGCGTTCCTCCCTATAGTGAG TCGTATTAATTTC (SEQ ID NO:61)	
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Table 8: mCas13 protein sequence and tag sequences for protein purification.

Name	Protein sequence
mCas13	MGSSHHHHHSSGLVPRGSHMASWSHPQFEKGGSGGGSGGSAWSHPQFEKMSD SEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFKRQ GKEM DSL RFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGSGMGIDYSLTSDCYRGINKSC FAVALNIAYDNC DHKGCRTLLSEVLR SKGGISDEQIKSQVVDGIQKRLKDIRNYFSH YYHAEDCLRFGDQDAVKVFLEEIYKNAESKT V GATKESDYKGVVPLFELHNGTY MITAAGVIFLASFFCHRSNVYRMLGAVKGFKHTGKEQLSDGQKRDYGFTRRLAY YALRDSYSVGAEDKTRCFREILSYLSRVPQLAVDWLNEQQLLTPEEKEAFLNQPAE DEGGDISDSSSSDKNKKSKEKRRSLRRDEKFI LFAIQFIEGWAAEQGLDVT FARYQK TVEKAENKNQDGKQARAVQLKYRNQGLNPDFNNEWMYI QNEHAI IQIKLNNKK AVAARISENELKYL VLLIFEKGNDAVQKLNCYIYSMSQKIEGEWKHRPDERWMP SFTKRADRTVTPEAVQSRLSYIRKQLQETIEKIGQEEPRNNKWL IYKGGKISMILKFIS DSIRDIQRRPNVKQYHILRDALQRLDFDGFYKELQNYVNDGRIAVSLYDQIKGVNDI SGLCKKVCELT LERLAGLEAKNGSELRRYIGLEAQEHPKYGEWNTLQEKA KRFLE SQFSIGKNFLRKM FYGDCCQKRCFDEEKGYNTQAKERKSLYSIVKEKLKDIKPIHDD RWYLIDRNPKNYDNKHSRIIRQMCNTYIQDVLCMKMAMWHYEKLISATEFRNKLE WNCIGQGNMGYERYSLWYKTGCGVVIQFTPADFLRLDIEKPAMIENICQCFVLGN KKLNSGAEEKITWDKFNKDGI AKYRKRQAEAVRAIFAFEEGLKIQEDKWSHERYFP FCNILDEAVKQGKIKDTGKDKEALNRGRNDFH EEFKSTEDQQAIFQKYFPIVERKD DTKKRRDKKQK* (SEQ ID NO:62)

5 6x His affinity tag: residues 5-10; Thrombin site: residues 14-19;
Strep-tag II: residues 24-31 and 44-51; SUMO: residues 52-148;
mCas13 protein: residues 151-1023.

Results

Identification, design, construction, and expression of a miniature Cas13 system

Xu *et al.* [62] describes compact Cas13 effectors that are classified as CRISPR/Cas type VI-E and VI-F. These compact Cas13s are used for *in vivo* applications, including endogenous RNA interference, RNA editing, and as an anti-coronavirus approach that targets and combats SARS-CoV-2 and other influenza viruses *in vivo* [62].

Some of the compact Cas13 protein sequences from Xu *et al.* were used as queries in the Basic Local Alignment Search Tool (BLAST) to find potentially uncharacterized mCas13 proteins. The alignment identified various proteins, including a few uncharacterized putative Cas13 sequences with two predicted RxxxxH (SEQ ID NO:192) motifs of the conserved Cas13 Higher Eukaryotes and Prokaryotes Nucleotide-binding (HEPN) ribonuclease domains [62, 63]. One small (837 amino acids) candidate showed high similarity (>95% query coverage) to the most efficient Cas13 identified in Xu *et al.*, namely Cas13e.1 [62]. Further analysis of the metagenomic contigs showed that the putative mCas13 protein has an associated CRISPR array in its immediate vicinity. In silico analysis of the associated CRISPR array predicted that the mCas13-associated crRNAs share high similarity with the length and architecture of the compact Cas13e.1 crRNA (30-nt long spacer sequence at the 5' end of the crRNA followed by a 36-nt long direct repeat (DR) sequence at the 3' end), and to previously reported crRNAs of the Cas13b family [55]. A schematic illustration of the computational strategy is shown in Fig. 10H. Furthermore, multiple sequence alignment analysis showed high similarity and the two predicted HEPN domains conserved among the putative mCas13 protein, Cas13e.1, and the other compact proteins, Cas13e and Cas13f (Fig. 10K). Based on these predictions, it was hypothesized that the putative mCas13 is functionally active. Next, the corresponding gene sequence was codon-optimized and synthesized and

a bacterial expression plasmid for heterologous expression in BL21 *Escherichia coli* containing the mCas13 sequence was designed and constructed. Subsequently, the protein was produced, and its activity tested *in vitro* for diagnostic applications.

5 *Characterization of CRISPR-mCas13 cis and trans catalytic activities*

When Cas13/crRNA ribonucleoproteins (RNP) recognize and cleave their target sequence, they also exhibit non-specific, collateral cleavage activity that degrades ssRNAs nearby [63, 64]. Such collateral activity can
10 be used in nucleic acid detection applications, where a ssRNA probe (reporter) molecule provided in the Cas13 reaction is cleaved by target-dependent Cas13 collateral activity [61]. The ssRNA reporter can contain a fluorophore linked by a short ssRNA sequence to a quencher, which emits fluorescence after the ssRNA sequence is cleaved, indicating the presence,
15 and therefore the detection, of the target sequence (Fig. 10A).

To test the mCas13's *cis* and *in-trans* activities *in vitro* and to determine the most effective crRNAs to use in the mCas13 SARS-CoV-2-based detection assays, 10 different crRNAs targeting two different regions in the SARS-CoV-2 nucleocapsid gene (*N*) were designed and screened. The
20 *in vitro* cleavage activity of mCas13 was first evaluated with 4 different crRNAs targeting single-stranded RNA substrates harboring target sequences complementary to the crRNA spacers. mCas13 exhibited different cleavage efficiencies with different crRNAs, with crRNA 4 mediating the highest efficiency relative to other crRNAs and controls (Figure 10L).

25 Because different Cas13 proteins can exhibit different cleavage preferences depending on ssRNA sequences [63], efforts were directed to identifying the best ssRNA reporter for the mCas13 SARS-CoV-2 based detection module. Therefore, 5 different ssRNA probes, each conjugated to a 5' fluorescent molecule (FAM) and a 3' fluorescence quencher (FQ) were
30 screened. mCas13 was incubated with each of the four targeting crRNAs and non-specific (NS) crRNA and reporters in the presence of the synthetic (*N*

gene) ssRNA target. The screening consistently identified crRNA 4 with a significantly higher fluorescence signal relative to the NS crRNA control, indicating the cleavage preference of mCas13 for poly(U) reporter sequences (Figs. 10B-10F). Using the poly (U) reporter molecule to screen six more
5 crRNAs targeting different *N* gene regions indicated that different crRNAs exhibited overall different signal levels.

To determine the optimal concentration of mCas13 and crRNA for maximal detection signal, the reaction was performed with titrated mCas13 and crRNA concentrations. It was observed that the optimal concentration of
10 Cas13/crRNA RNP for a true positive signal with no significant signal in NS crRNA control was 500 nM (Fig. 10I). In addition, to determine the optimal temperature for mCas13 catalytic activity, different cleavage temperature conditions were tested ranging from 37 °C to 55 °C. The highest activity was observed at 37 °C, which is similar to the optimal temperature of other
15 known Cas13 enzymes used for nucleic acid detection (Fig. 10J).

Using optimized conditions and the most effective crRNA 4, the effect of single mismatches between crRNA and target RNA on mCas13 RNA detection activity was investigated. 10 crRNAs (based on crRNA 4) were designed to contain a single nucleotide mismatch at different sites, with
20 one mismatch for every 3 nucleotides on the spacer sequence. A low mismatch tolerance should cause a much lower detection signal than the positive control (which is a perfect match). This analysis revealed different mismatch tolerances for different regions of the spacer sequence, where mismatches at the extreme 5' or 3' ends of the crRNA were not well
25 tolerated. In contrast, mismatches at other regions were tolerated better (Fig. 10G, Fig. 10M, Fig. 10N). The observed different tolerances for mismatches may be advantageous to designing crRNAs useful for detecting variants that harbor different SNPs. Altogether, these data indicated that the identified mCas13 is catalytically active with robust *trans*-cleavage activity, and thus is
30 suitable for developing nucleic acid detection platforms.

RT-LAMP coupled with CRISPR-mCas13 for SARS-CoV-2 detection

To ensure sensitive detection, pre-amplifying the RNA target of interest is necessary [61]. RT-LAMP isothermal amplification was chosen because it possesses several advantages over other amplification methods, including high sensitivity, rapid turnaround time, simple operation, and low cost [65]. To initiate the RT-LAMP reaction, primer sets well-established in previous reports to target and amplify conserved regions in the SARS-CoV-2 N gene were used (named here as STOPCovid [36] and DETECTR [37] primer sets). Because mCas13 targets RNA, these primers were modified by appending a T7 promoter sequence to the 5' end of the first half of either the forward inner primer (FIP) or the backward inner primer (BIP). During LAMP amplification, the T7 promoter sequence integrates into the amplified DNA products, providing a suitable template for the T7 RNA polymerase to transcribe the amplified LAMP product *in vitro* and generate RNA targets for mCas13 detection (Fig. 11A). The performance of these modified primers was tested using a synthetic SARS-CoV-2 viral genome at 500 copies/ μ L. Gel electrophoresis indicated that these modified primers successfully amplified the target RNA with no observed amplification in the no-template control (NTC) (not shown). In addition, no substantial differences were found between the amplifications using primer sets with T7-containing FIP primer (T7-FIP) or T7-containing BIP primer (T7-BIP). Therefore, T7-FIP primers were chosen to establish the RT-LAMP mCas13 detection platform.

Since mCas13 performs optimally at 37 °C, a temperature also optimal for T7 RNA polymerase, the T7-mediated transcription of the RT-LAMP product was coupled with the Cas13-based detection of the transcribed RNA in a single tube. Therefore, after RT-LAMP pre-amplification of SARS-CoV-2 synthetic RNA using STOPCovid or DETECTR T7-FIP modified primers, the RT-LAMP products were added to the T7 transcription and mCas13 detection reaction. Real-time measurement of the T7-coupled mCas13-based detection indicated robust detection of RT-LAMP product only when using targeting crRNA (crRNA 4) and T7 RNA

polymerase, confirming that the amplification of the synthetic SARS-CoV-2 genome was specific and that T7 promoters were successfully integrated into the amplified products (Fig. 11B). These results show the successful development of an mCas13-based two-pot SARS-CoV-2 detection platform.

5 Next, the limit of detection (LoD) of the two-pot SARS-CoV-2 detection assay was determined. When establishing the two-pot assay, it was verified that both STOPCovid and DETECTR primer sets could be used to effectively detect SARS-CoV-2 RNA (Figs. 11C-11D). Therefore, the performance and the LoD of both STOPCovid and DETECTR primer sets
10 were assayed to determine the primer set that is most suitable for the mCas13 SARS-CoV-2 detection platform. Serial dilutions of the synthetic SARS-CoV-2 viral genome were used as an input for the pre-amplification RT-LAMP reaction. It was observed that both primer sets allowed sensitive detection of the synthetic RNA, but the STOPCovid primers reproducibly
15 detected as few as 4 copies/ μ L viral RNA, compared with 8 copies/ μ L for DETECTR primers (Figs. 11C-11D). Although extending the RT-LAMP pre-amplification time could enhance its sensitivity, the RT-LAMP pre-amplification step was limited to 35 mins and the mCas13 detection reaction was limited to 20–30 mins, resulting in a total detection time of $\approx$ 1 hour or
20 less. Due to its outstanding LoD, the STOPCovid primer set was chosen for the mCas13 detection platform.

The RT-LAMP coupled CRISPR-mCas13 detection assay is specific for SARS-CoV-2

25 To test the assay's specificity and ensure no cross-reactivity with other common viruses, this system was challenged with other SARS-CoV-2-related or non-related viruses, including SARS-CoV-1, MERS, TMV, and TuMV, together with SARS-CoV-2. All tested viruses (other than SARS-CoV-2) showed only near-background signals, indicating that the developed assay was highly specific (Fig. 11E). Collectively, these results indicated that
30 the developed mCas13-based detection platform exhibits reliable, highly

sensitive, and highly specific detection of SARS-CoV-2 with a turnaround time of 1 hour from extracted RNA to results.

Validation of RT-LAMP coupled with CRISPR-mCas13 SARS-CoV-2 detection platform in a clinical setting

5 Next, the assay was validated with total RNA extracted from SARS-CoV-2 patient swab samples. Oropharyngeal or nasopharyngeal swab samples were collected from suspected COVID-19 patients. After RNA extraction following the CDC EUA-approved protocol, the samples were confirmed positive for SARS-CoV-2 using RT-qPCR. The assay was first
10 tested with 17 samples with Ct values of 15–39. Using the mCas13-based detection assay, all samples with Ct values of less than 34 were correctly identified within one hour (Fig. 12A). These results indicate that the system can reliably detected SARS-CoV-2 in samples with Ct values up to 34.

 To facilitate large-scale screening during the SARS-CoV-2
15 pandemic, performing diagnostic assays at POC or outside of laboratory settings is critical. Therefore, it was determined whether the assay could be coupled with a portable device to permit a simple readout suitable for POC and routine diagnostics. A hand-held, inexpensive fluorescence visualizer (P51 Molecular Fluorescence Viewer) was adapted to easily visualize and
20 interpret the results. This portable device illuminates reaction tubes with blue light, and fluorescent reactions are visible through a film used as an optical filter. Using this device, fluorescence is readily visible to the human eye without the need for sophisticated instruments like qPCR machines or plate readers (Fig. 12B). Using a modified RNA reporter molecule conjugated to a
25 5' HEX fluorescent molecule instead of FAM, it was observed that mCas13 collateral cleavage of this reporter produced a bright signal visible with the P51 fluorescence visualizer. Investigation of the HEX reporter concentration needed for definitive visual detection of true positive samples indicated that 1 μ M of HEX RNA reporters produced a clear signal with no substantial
30 background in negative controls (Fig. 12G).

To ensure that the sensitivity of the assay was preserved with the change in fluorescent molecule, the LoD assay was repeated using the STOPCovid primer set. The same results as obtained previously with the machine-based readout in Figure 11C were obtained, demonstrating that the 5' HEX modification did not affect assay performance (Fig. 12C). Next, this assay's performance was evaluated with the same 17 SARS-CoV-2 RNA samples used in Figure 12A and the results from the visual-based detection assay were compared to the results in Figure 12A. There was 100% concordance between the two assays, indicating that the developed mCas13 visual detection assay is reliable (Fig. 12D, Fig. 12H). Finally, to clinically validate and further test the reliability of the visual mCas13 detection assay, the assay's performance was tested with an additional 24 qPCR-confirmed positive clinical samples of total RNA extracted from patient swabs, along with no-template control reactions. The visual-readout mCas13-based detection of SARS-CoV-2 from these 24 samples showed 100% concordance with RT-qPCR results. A clear fluorescence signal relative to the negative controls was detected in each positive sample (Figs. 12E-12F, Figs. 12I-12J). Altogether, this system's effectiveness was validated in 41 clinical samples, facilitating viral detection within 1 hour with a simple visual readout.

Discussion

To control the pandemic of COVID-19, rapid, accurate, reliable, and portable diagnostics for SARS-CoV-2 are needed [21]. However, increasing sensitivity and specificity has remained a common challenge for recently developed diagnostics. Amplification can increase the sensitivity of assays and isothermal amplification techniques like RT-LAMP and RT-RPA are good alternatives for RT-PCR. Both are highly sensitive, provide rapid and more accessible platforms for viral nucleic acid detection, and are suitable for POC uses [66-68]. Despite many advantages of isothermal amplification in relation to other traditional amplification methods, their application is limited by the high rate of non-specific amplification and cross-contamination [69, 70]. Therefore, isothermal amplification methods are

coupled with CRISPR systems to enhance specificity and LoD [36, 41, 71, 72].

Here, a previously unreported/uncharacterized Cas13 variant was identified and characterized and an RT-LAMP coupled with mCas13 assay
5 for SARS-CoV-2 detection was developed. Sequence similarity searching was used to identify the mCas13 variant. The corresponding mCas13 sequence was synthesized and subcloned into a protein expression vector in order to purify the protein and characterize its cleavage activity. The crRNA architecture was identified and synthesized for *in vitro* expression, and the
10 crRNA and mCas13 were used to form an RNP complex for cleavage activity assays. multiple crRNAs targeting different regions in SARS-CoV-2 were designed. One crRNA showed robust collateral activity against poly(U) reporters. Different regions of the crRNA exhibited different tolerances for mismatches, which can be advantageous to designing crRNAs useful for
15 detecting variants that harbor different SNPs.

Because mCas13 targets RNA for cleavage and degradation, the LAMP primers were modified to add the T7 polymerase promoter sequence to permit the T7 RNA polymerase to generate transcripts for mCas13. This modified RT-LAMP enabled the coupling with mCas13 for virus detection,
20 obviating the need for extra steps. This modification in the RT-LAMP primers did not interfere with the RT-LAMP reaction and resulted in robust amplification of the target sequences. The RT-LAMP coupled with mCas13 exhibited high specificity since sequences of other viruses, including SARS-Co-V1, MERS, TMV, and TuMV, did not trigger the collateral reaction;
25 however, the SARS-CoV2 sequence triggered a strong collateral cleavage reaction and fluorescence.

The usefulness of this modality (RT-LAMP coupled with mCas13) for SARS-CoV-2 detection was tested and validated in 41 clinical samples. The data showed that mCas13 successfully detected SARS-CoV-2 in clinical
30 samples, exhibiting strong concordance with RT-qPCR. A simple LED-based visualizer was employed for straightforward and inexpensive detection

of fluorescence in test results. With the affordable and portable P51 LED-based visualizer, a positive signal is observable as a bright green light. The simple fluorescence visualizer showed consistent detection results from fluorescence readers, verifying the effectiveness of the method. It is worth noting that several crRNAs were screened to identify functional ones that activated mCas13, thereby limiting the off-target activities of the mCas13 enzyme. Other CRISPR systems with robust activities can degrade the target and compromise the detection sensitivity. Because this mCas13 system is not overly robust, it may be quite useful for sensitive and specific detection. These features, however, could be quite beneficial in building a highly specific and powerful modality of virus and nucleic acid detection. Briefly, this mCas13-based detection platform enables rapid, accurate, simple, cost-effective, and efficient detection of SARS-CoV-2, and shows potential for POC applications.

Due to its miniature size, the mCas13 variant can be used for a variety of *in vivo* RNA manipulations, including RNA knockdown, editing, splicing regulation, RNA imaging and localization. The recently identified compact Cas13, Cas13e.1, shows efficacy against SAR-CoV-2 and influenza A virus, indicating that Cas13 can be useful as an antiviral therapeutic [56, 62]. These studies demonstrate that bacterial defense systems have untapped potential for diverse synthetic biology applications, diagnostics, and therapeutics as antiviral agents.

In summary, the data demonstrate successful identification and characterization of the catalytic activities of a previously unknown/uncharacterized miniature variant of Cas13, and harnessing of its collateral catalytic activities to develop a system for SARS-CoV-2 detection. The modality coupling RT-LAMP and mCas13 demonstrates key features, including simplicity, specificity, sensitivity, and portability. The readout signal was measured using a low-cost P51 device. The P51 device can be paired with a cell phone camera that processes and shares data, facilitating the integration of this modality to large-scale testing. This work illustrates

the usefulness of mCas13 systems for diagnostics as well as other potential *in vivo* RNA manipulations for diverse applications.

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- 5 Supplemental/Supplementary information associated therewith, is specifically incorporated therein.

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10 It is understood that the disclosed method and compositions are not
limited to the particular methodology, protocols, and reagents described as
these may vary. It is also to be understood that the terminology used herein is
for the purpose of describing particular embodiments only, and is not
intended to limit the scope of the present invention which will be limited
15 only by the appended claims.

Although the description of materials, compositions, components,
steps, techniques, etc. may include numerous options and alternatives, this
should not be construed as, and is not an admission that, such options and
alternatives are equivalent to each other or, in particular, are obvious
20 alternatives.

Every composition disclosed herein is intended to be and should be
considered to be specifically disclosed herein. Further, every subgroup that
can be identified within this disclosure is intended to be and should be
considered to be specifically disclosed herein. As a result, it is specifically
25 contemplated that any composition, or subgroup of compositions can be
either specifically included for or excluded from use or included in or
excluded from a list of compositions.

Those skilled in the art will recognize, or be able to ascertain using
no more than routine experimentation, many equivalents to the specific
30 embodiments of the method and compositions described herein. Such
equivalents are intended to be encompassed by the following claims.

Unless defined otherwise, all technical and scientific terms used
herein have the same meanings as commonly understood by one of skill in

the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific
5 embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A polynucleotide comprising a nucleotide sequence encoding a class II, type VI CRISPR/Cas effector protein (Cas13) and optionally a heterologous sequence, wherein the Cas effector protein comprises an amino acid sequence encoded by SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67, or a sequence with at least 70% sequence identity thereto;
optionally wherein the sequence encoding the Cas effector protein comprises SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67, or a sequence with at least 70% sequence identity to SEQ ID NO:63, SEQ ID NO:65 or SEQ ID NO:67.
2. The polynucleotide of claim 1, wherein the sequence encoding the Cas effector protein is codon optimized for expression in a prokaryotic or eukaryotic cell.
3. The polynucleotide of claim 1 or 2, comprising a heterologous sequence, wherein the heterologous sequence comprises a promoter, transcription terminator, multiple cloning site, drug resistance marker, one or more protease recognition sites, one or more epitope tags, or a combination thereof.
4. The polynucleotide of claim 3, wherein the heterologous sequence is operably linked to the sequence encoding the Cas effector protein.
5. The polynucleotide of any one of claims 1-4 further comprising a sequence encoding an RNA comprising a crRNA sequence, wherein the RNA is capable of complexing with the Cas effector protein and hybridizing to a target RNA sequence.
6. The polynucleotide of any one of claims 1-5, wherein the Cas effector protein is derived from *Thermoclostridium caenicola* or a Proteobacteria bacterium.
7. A vector comprising the polynucleotide of any one of claims 1-6, optionally wherein the vector comprises the nucleotide sequence of SEQ ID

NO:69 or SEQ ID NO:70, or a sequence having at least 60% sequence identity to SEQ ID NO:69 or SEQ ID NO:70.

8. The vector of claim 7, wherein the vector is a viral vector or plasmid.
9. A prokaryotic or eukaryotic cell comprising the vector of claim 7 or 8.
10. A method of producing a class II, type VI CRISPR/Cas effector protein comprising contacting the vector of claim 7 or 8 with a prokaryotic or eukaryotic cell under conditions suitable for expression of the sequence encoding the Cas effector protein.
11. The method of claim 10 further comprising isolating and/or purifying the Cas effector protein.
12. An isolated class II, type VI CRISPR/Cas effector protein, wherein the Cas effector protein is produced by the method of claim 10 or 11.
13. An isolated class II, type VI CRISPR/Cas effector protein comprising the amino acid sequence of SEQ ID NO:64 or SEQ ID NO:66, or a sequence with at least 70% sequence identity to SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68.
14. A ribonucleoprotein complex comprising the Cas effector protein of claim 12 or 13 complexed with an RNA comprising crRNA sequence, optionally wherein the RNA is capable of hybridizing to a target RNA sequence.
15. A composition comprising a Cas13 protein, wherein the Cas13 protein comprises the amino acid sequence of SEQ ID NO:64, SEQ ID NO:66 or SEQ ID NO:68, or a sequence with at least 70% sequence identity to SEQ ID NO:64, SEQ ID NO:66, or SEQ ID NO:68.
16. The composition of claim 15, wherein the Cas13 protein comprises one or more HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains, preferably two HEPN domains.
17. The composition of claim 16, wherein the HEPN domain comprises a RxxxxH (SEQ ID NO:192) motif sequence, wherein X represents any amino acid.

18. The composition of any one of claims 15-17, wherein the Cas13 protein is complexed with and RNA comprising a crRNA sequence, optionally wherein the RNA is capable of hybridizing to a target RNA sequence.
19. The composition of claim 18, wherein the crRNA sequence comprises a spacer sequence that is capable of hybridizing to the target RNA sequence, and a direct repeat sequence.
20. The composition of claim 18 or 19, wherein the crRNA sequence comprises a spacer of about 20-30 nucleotides, preferably 24-28 nucleotides.
21. The composition of any one of claims 18-20, wherein the Cas13 protein can cleave the target RNA sequence at a temperature of about 37-70 °C, about 50-70 °C, about 47-60 °C, or about 60 °C.
22. The composition of any one of claims 18-20, wherein the Cas13 protein can cleave the target RNA sequence at a temperature of about 37 °C-42 °C, preferably about 37 °C.
23. The composition of any one of claims 18-22 comprised in a prokaryotic or eukaryotic cell.
24. A method of performing targeted knockdown of an RNA transcript comprising introducing the composition of any one of claims 18-22 to a cell, wherein the crRNA sequence hybridizes to the RNA transcript, thereby inducing cleavage of the RNA transcript by the Cas13 protein.
25. The method of claim 24, wherein RNA transcript is a mRNA or lincRNA.
26. The method of claim 24, wherein RNA transcript is derived from a viral gene, preferably a bacteriophage.
27. A method of detecting the presence of an RNA transcript in a nucleic acid sample comprising contacting the sample with the composition of any one of claims 18-22 in the presence of an activatable single stranded RNA (ssRNA) oligonucleotide comprising a reporter moiety,

wherein the crRNA is designed to hybridize to the RNA transcript, wherein the Cas13 cleaves the ssRNA oligonucleotide upon binding of the

Cas13 crRNA complex to the RNA transcript, wherein detection of the cleavage of the ssRNA oligonucleotide indicates the presence of the RNA transcript.

28. The method of claim 27, wherein the RNA transcript is generated by transcription from a dsDNA molecule, optionally wherein the dsDNA molecule is generated by reverse transcription coupled isothermal amplification of a target RNA.

29. The method of claim 27 or 28, wherein the reporter moiety comprises a fluorophore linked to a quencher via the ssRNA, wherein fluorescence is emitted upon cleavage of the ssRNA oligonucleotide.

30. A method of determining the localization of an RNA transcript in a cell comprising introducing the composition of any one of claims 18-22 to the cell,

wherein the Cas13 is catalytically inactive and further comprises a detectable marker,

wherein the crRNA is designed to hybridize to the RNA transcript, and wherein the Cas13 crRNA complex binds to the RNA transcript, thereby indicating the location of the RNA transcript.

31. The method of claim 30, wherein the detectable marker is selected from GFP, eGFP, RFP, YFP, BFP, CFP, mNeonGreen, mCherry, mOrange, mRaspberry, mPlum, mKO, mRFP, mRFPPruby, mRuby, tagRFP, mKate2, and DsRed.

32. A method for performing targeted editing of an RNA transcript comprising introducing the composition of any one of claims 18-22 to a cell,

wherein the Cas13 is catalytically inactive and further comprises a deaminase domain of an RNA-dependent Adenosine Deaminase (ADAR),

wherein the crRNA is capable of hybridizing with a region in the RNA transcript comprising a target A nucleotide to form an RNA duplex, wherein the duplex comprises an A-C mismatch at the target A nucleotide,

wherein the target A nucleotide is deaminated by the deaminase domain.

33. The method of claim 32, wherein the ADAR is selected from ADAR1, ADAR2, or ADAR3.

Reporter screening with HheCas13a and TccCas13a

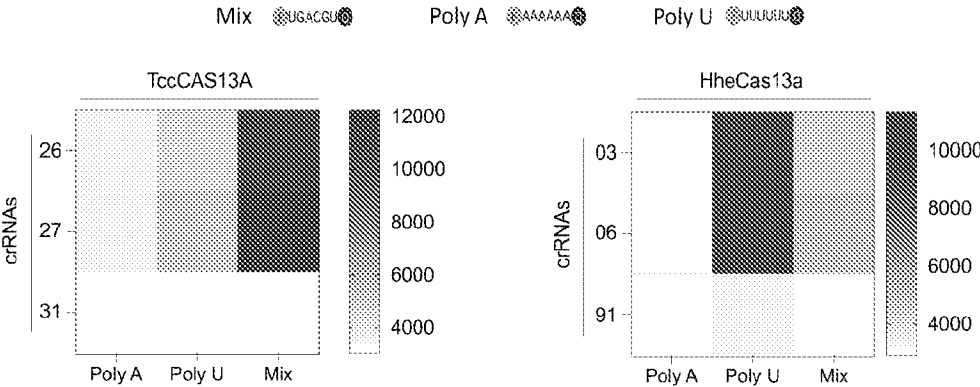


FIG. 1A

FIG. 1B

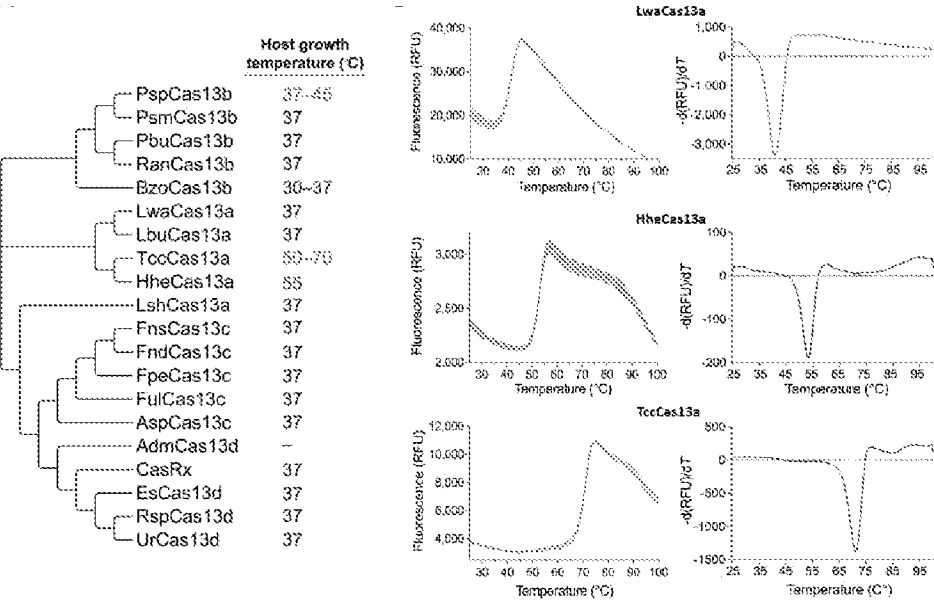


FIG. 1C

FIG. 1D

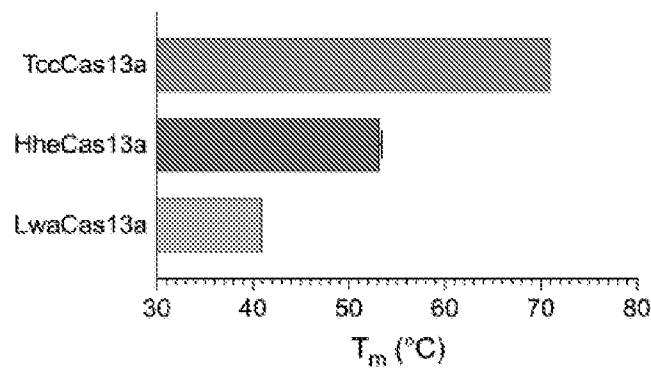


FIG. 1E

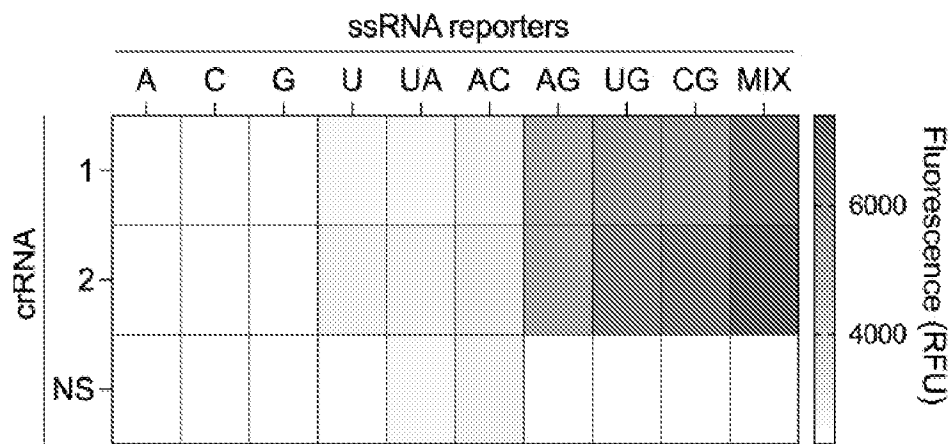


FIG. 1F

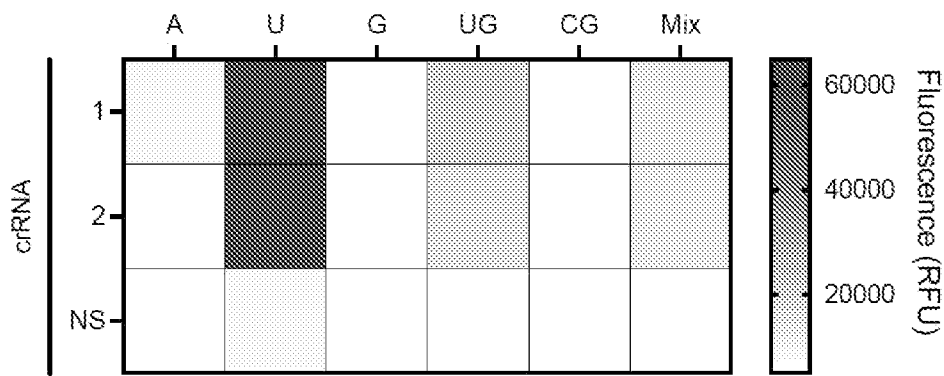


FIG. 1G

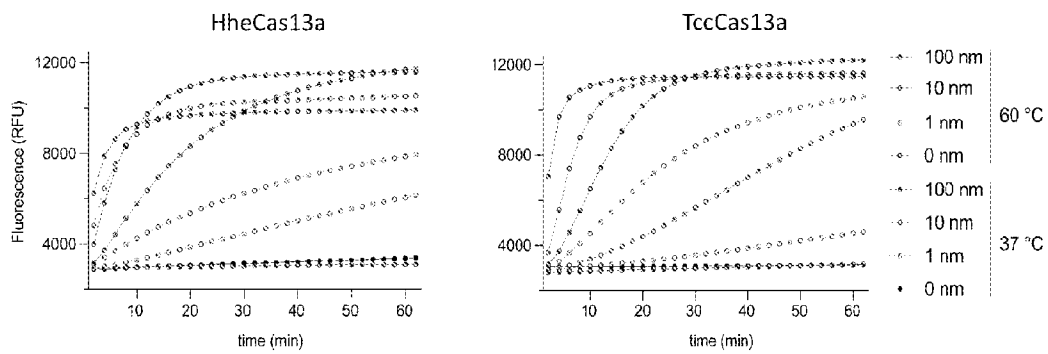


FIG. 2A

FIG. 2B

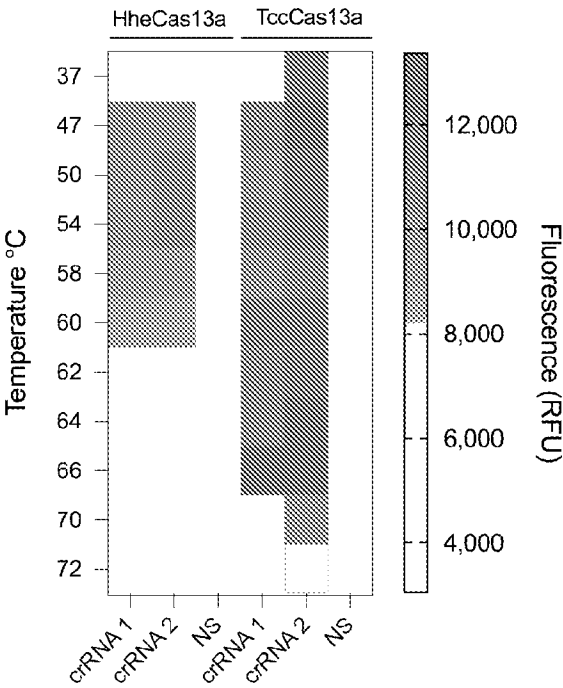


FIG. 2C

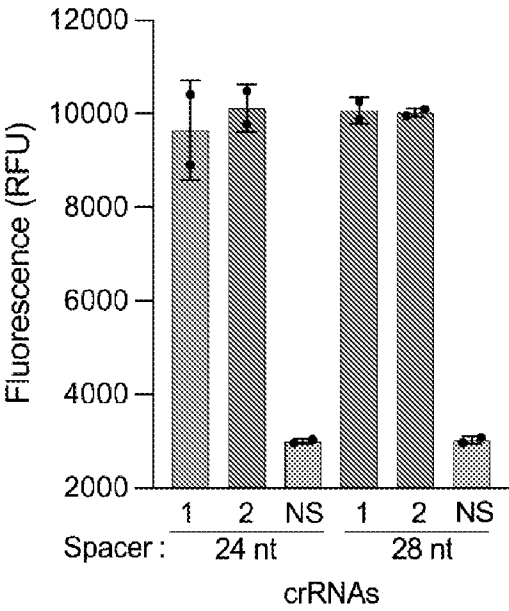


FIG. 2D

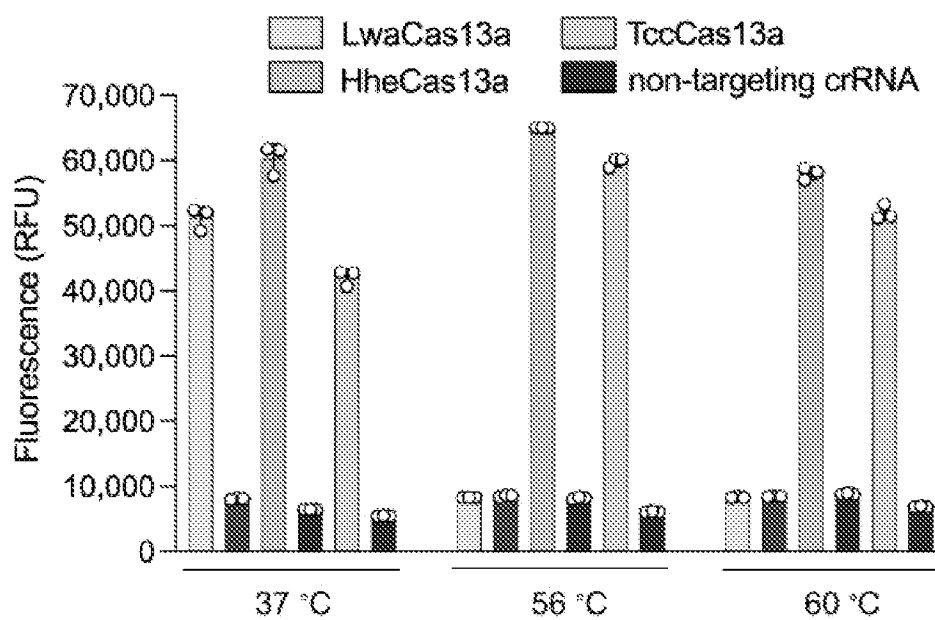


FIG. 2E

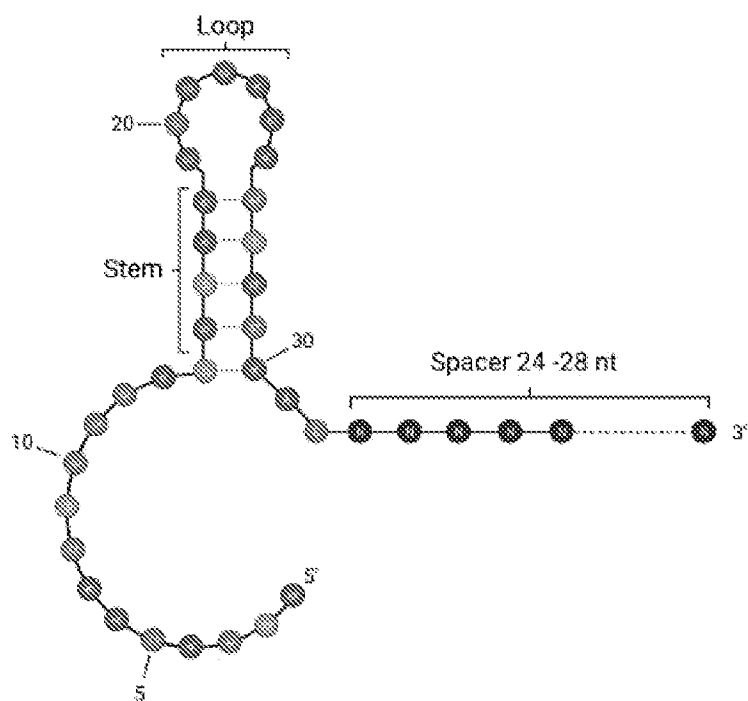


FIG. 2F

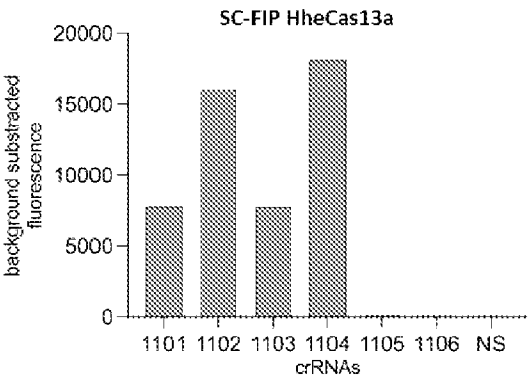


FIG. 3A

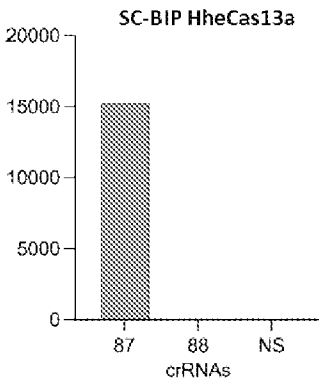


FIG. 3B

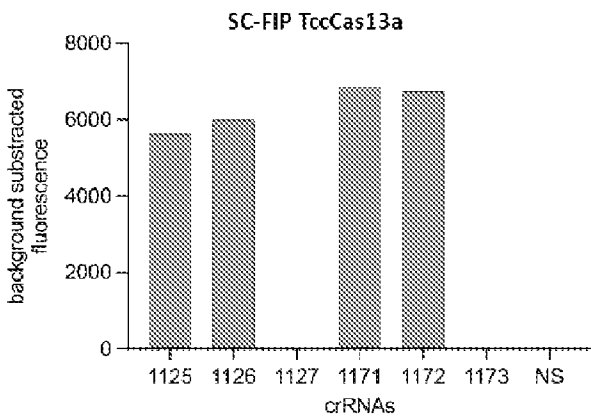


FIG. 3C

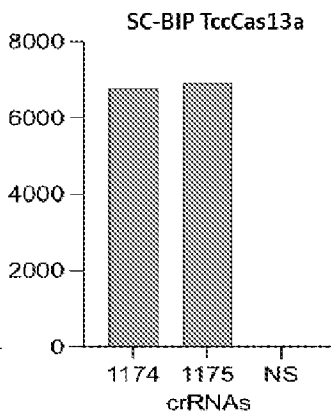


FIG. 3D

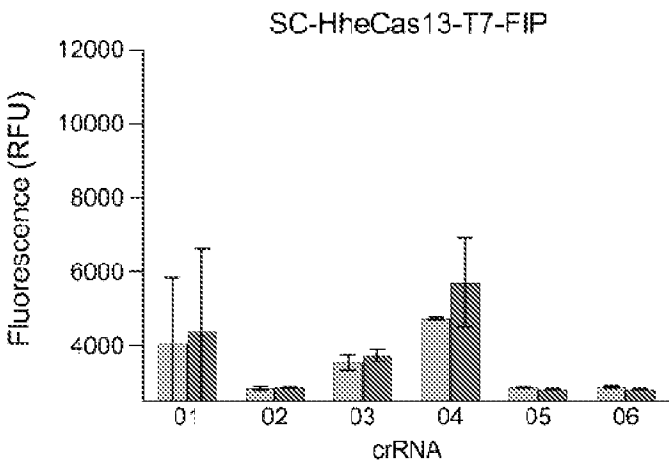


FIG. 4A

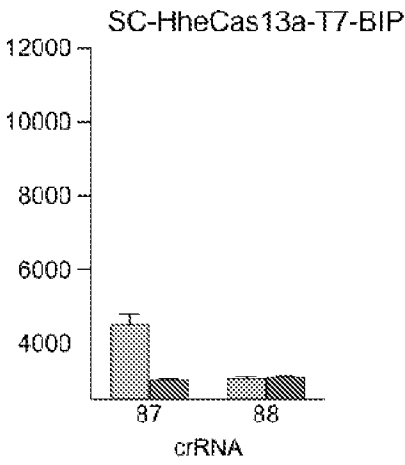


FIG. 4B

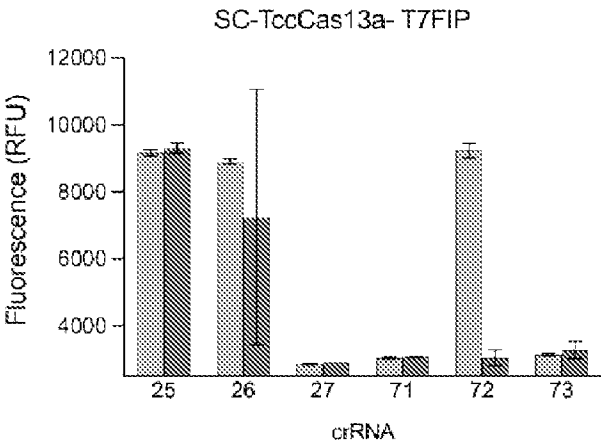


FIG. 4C

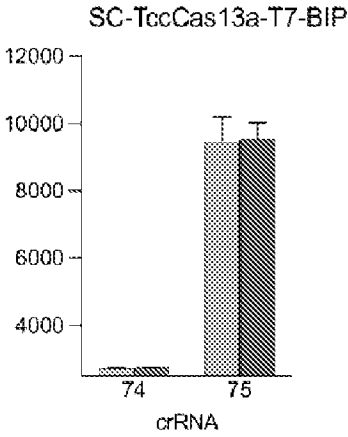


FIG. 4D

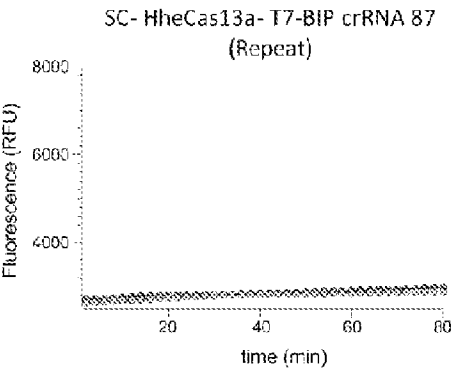


FIG. 5A

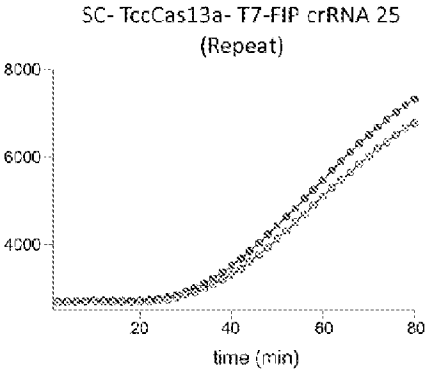


FIG. 5B

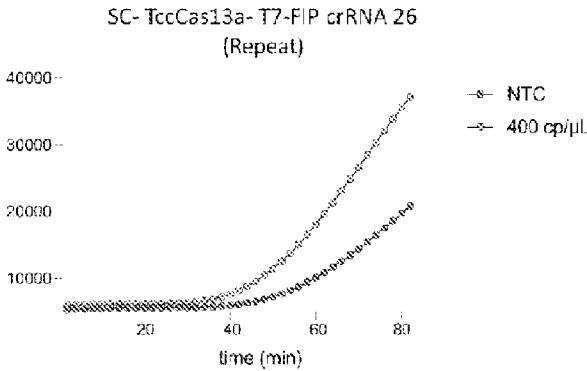


FIG. 5C

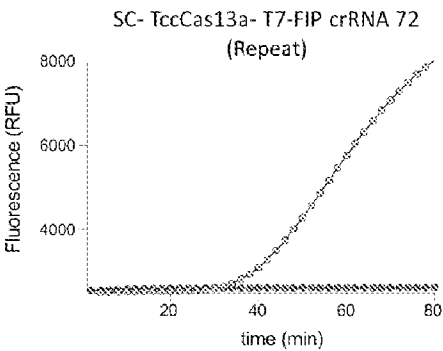


FIG. 5D

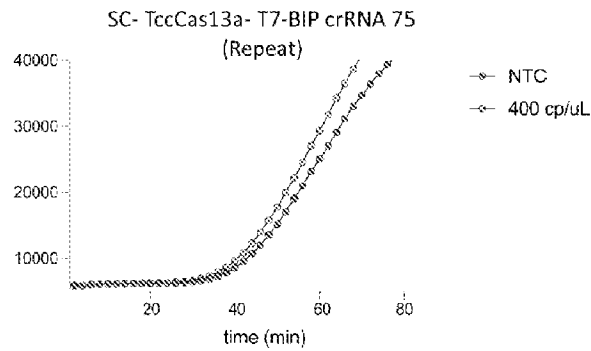


FIG. 5E

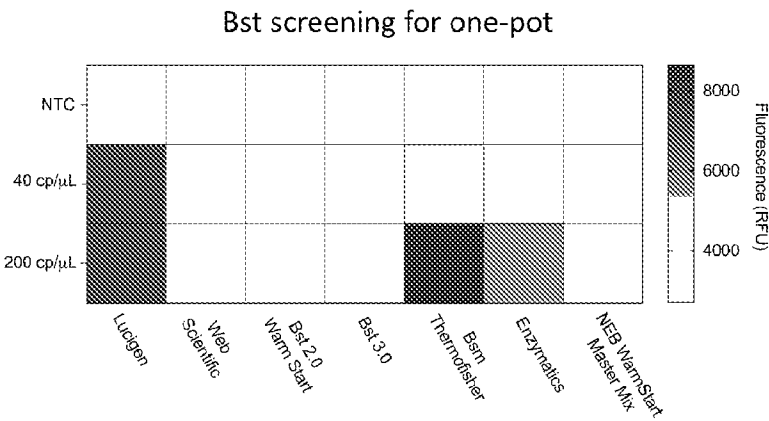


FIG. 6

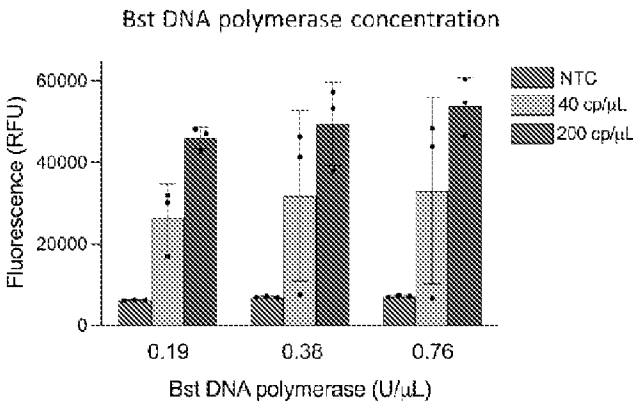


FIG. 7A

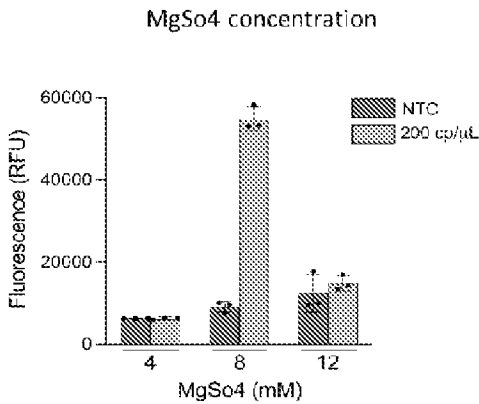


FIG. 7B

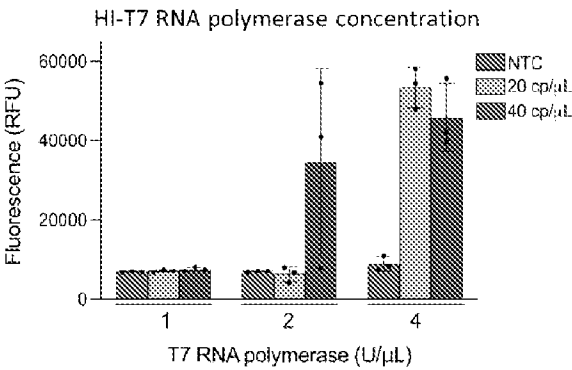


FIG. 7C

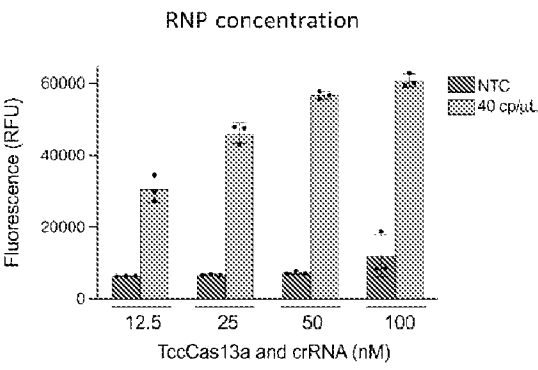


FIG. 7D

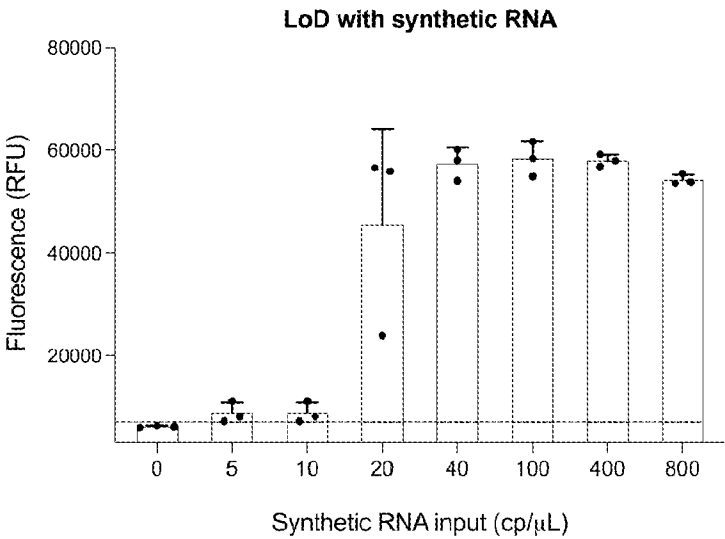


FIG. 8

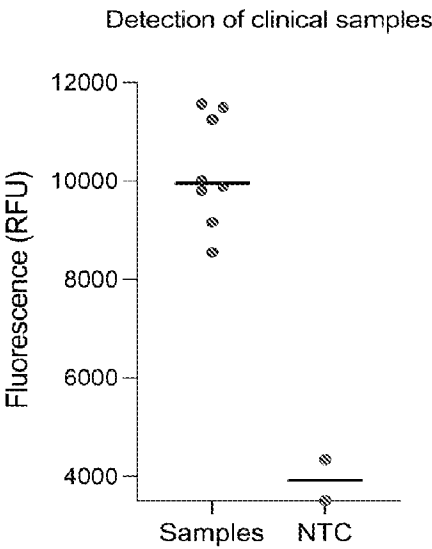


FIG. 9

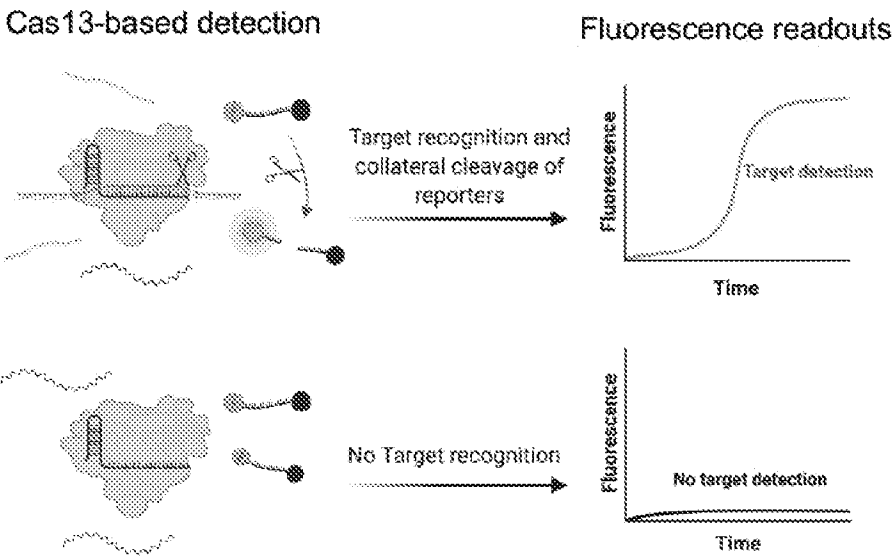


FIG. 10A

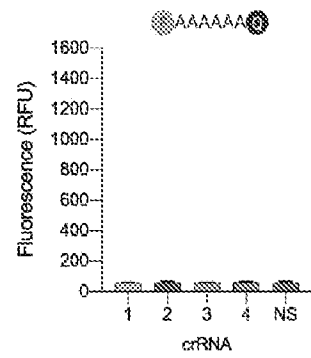


FIG. 10B

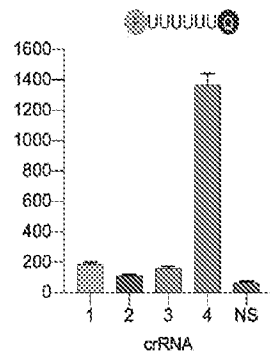


FIG. 10C

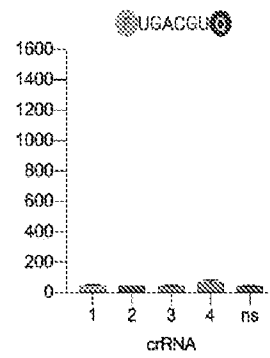


FIG. 10D

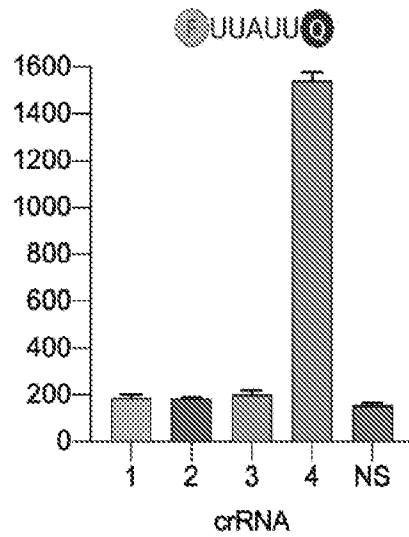


FIG. 10E

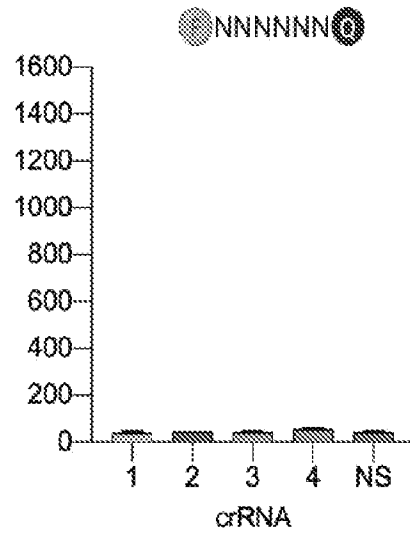


FIG. 10F

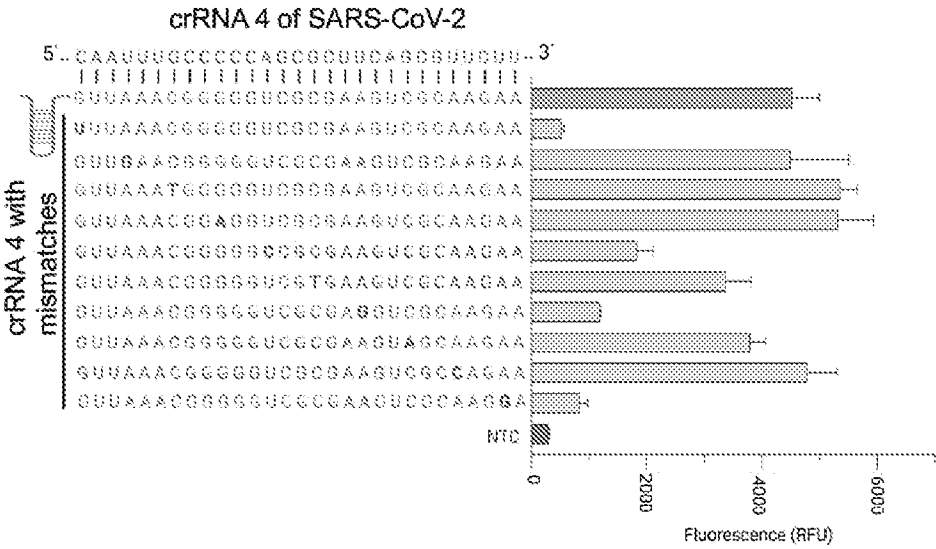


FIG. 10G

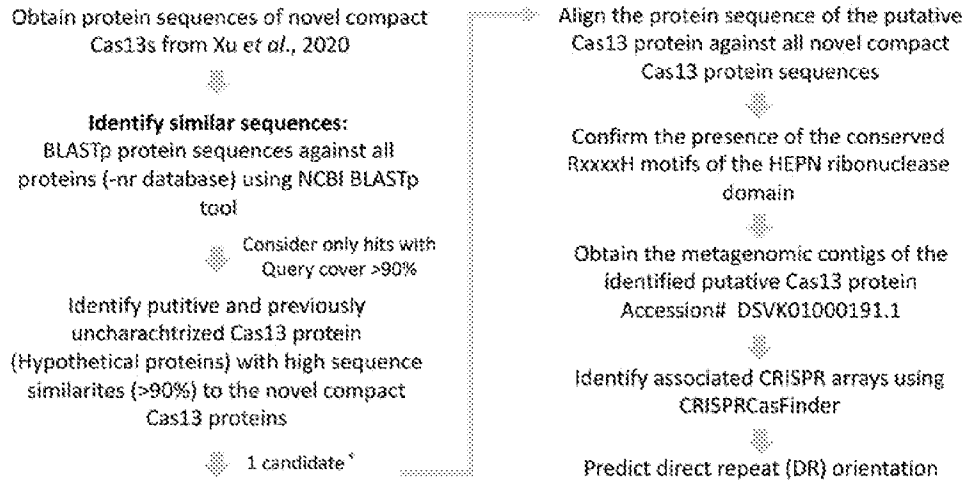


FIG. 10H

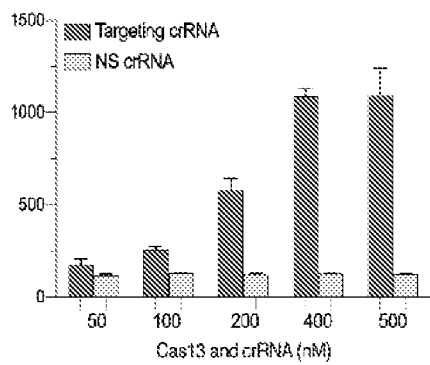


FIG. 10I

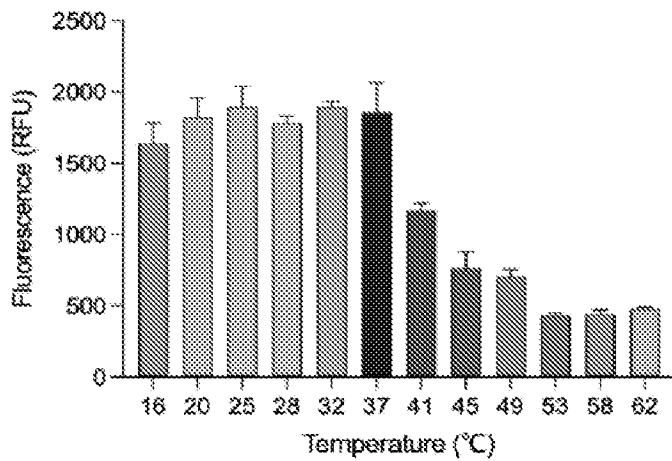


FIG. 10J

FIG. 10K

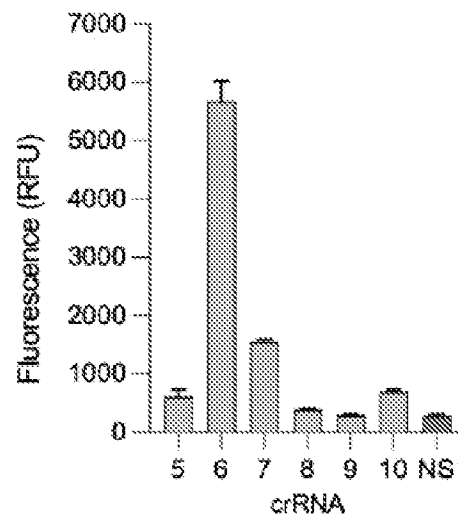


FIG. 10L



FIG. 10M

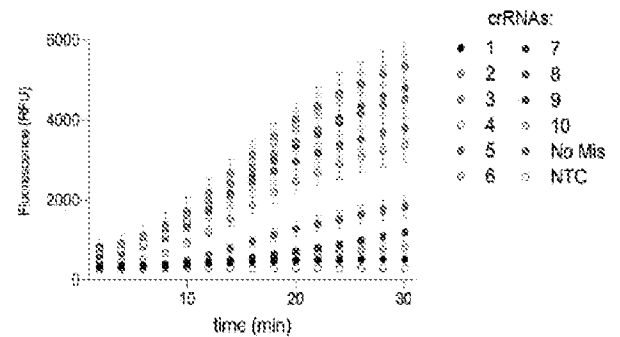


FIG. 10N

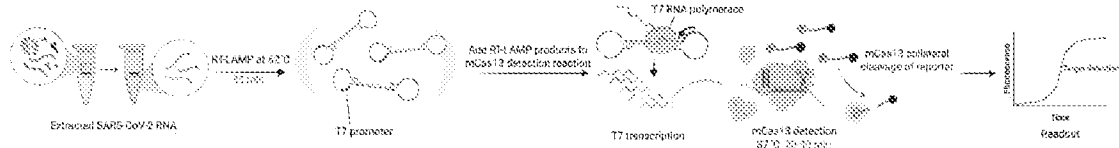


FIG. 11A

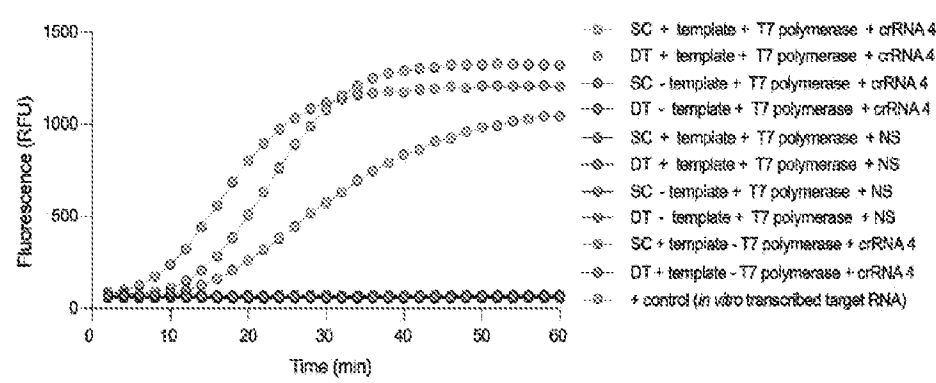


FIG. 11B

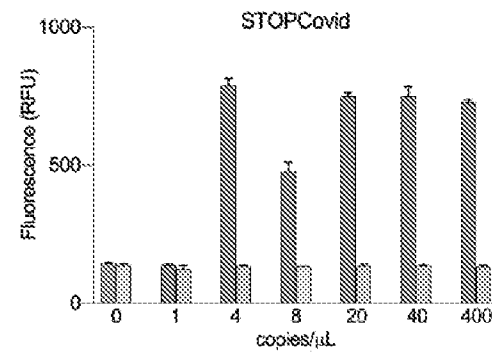


FIG. 11C

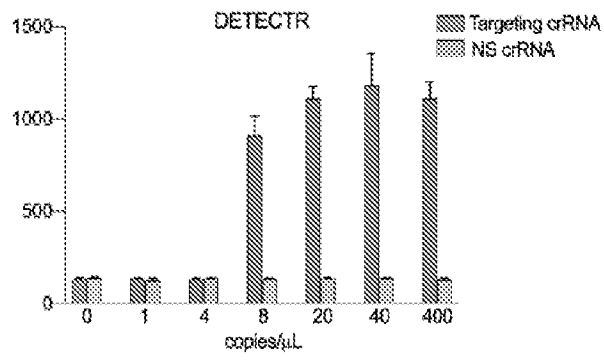


FIG. 11D

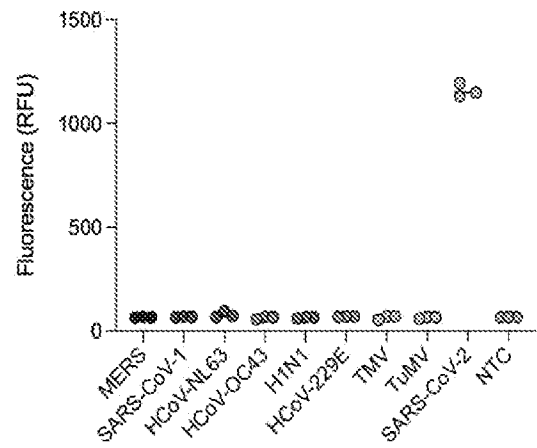


FIG. 11E

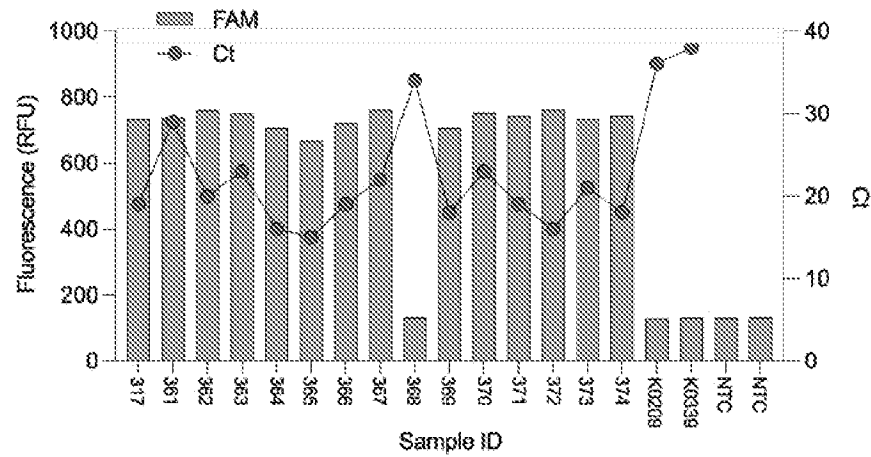


FIG. 12A

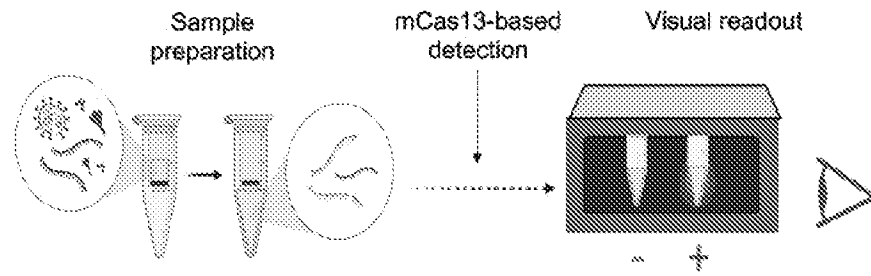


FIG. 12B

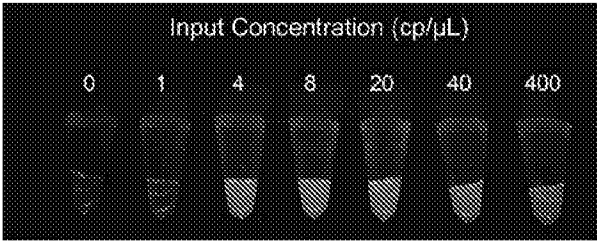


FIG. 12C

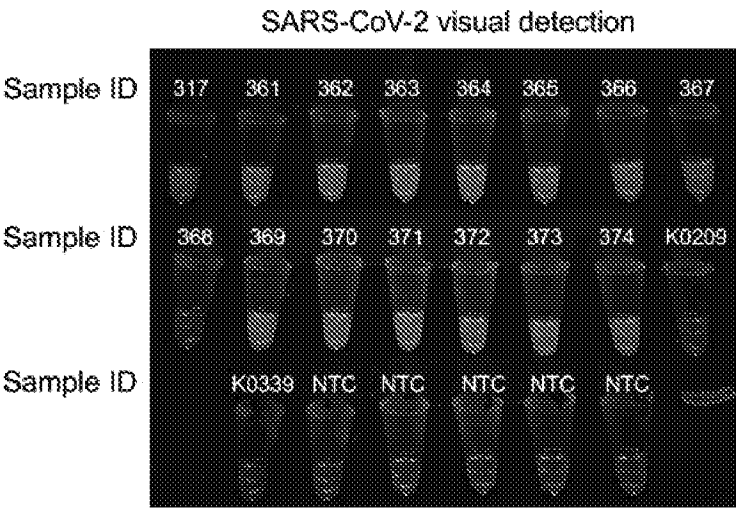


FIG. 12D

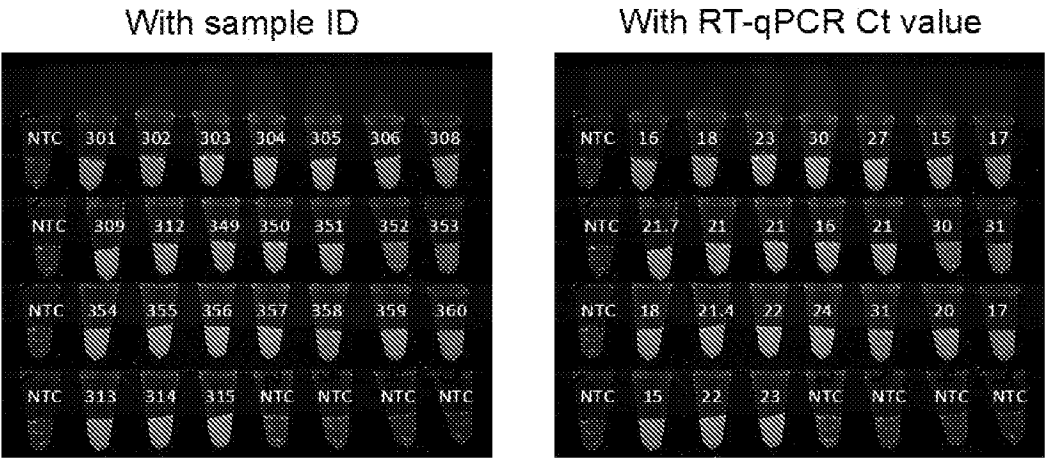


FIG. 12E

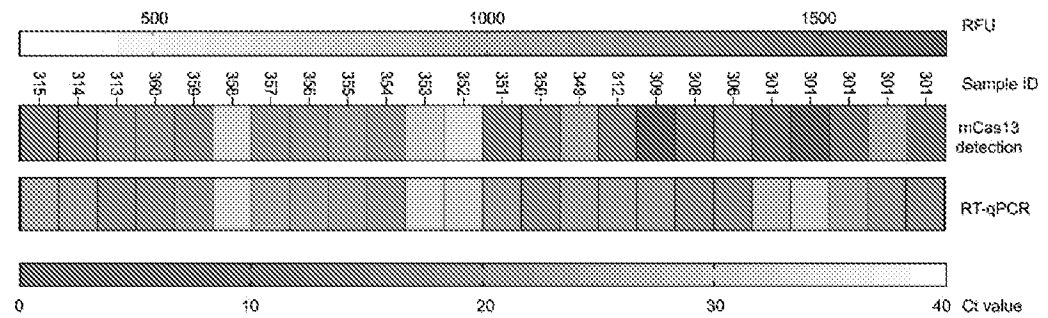


FIG. 12F

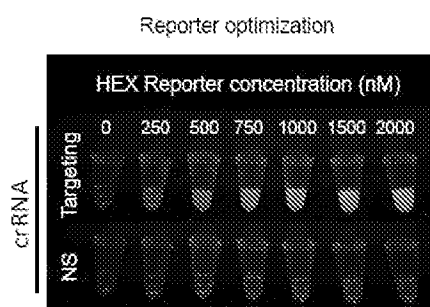


FIG. 12G

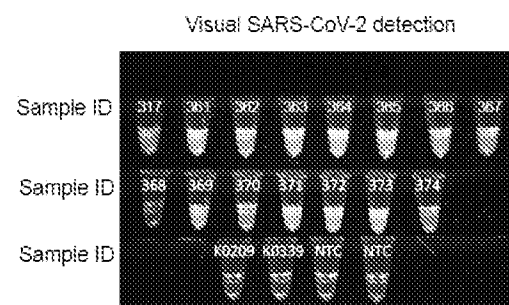


FIG. 12H

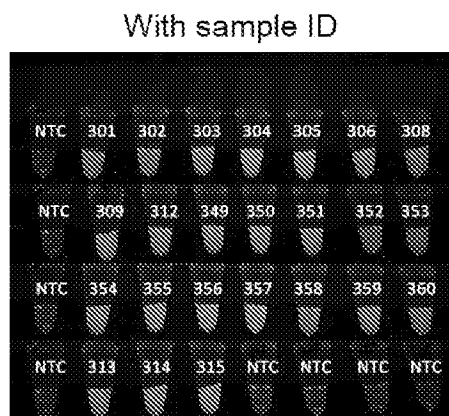


FIG. 12I

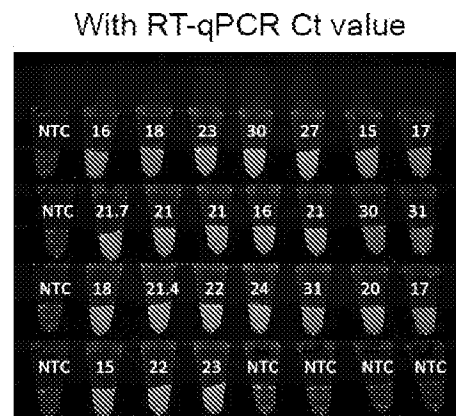


FIG. 12J

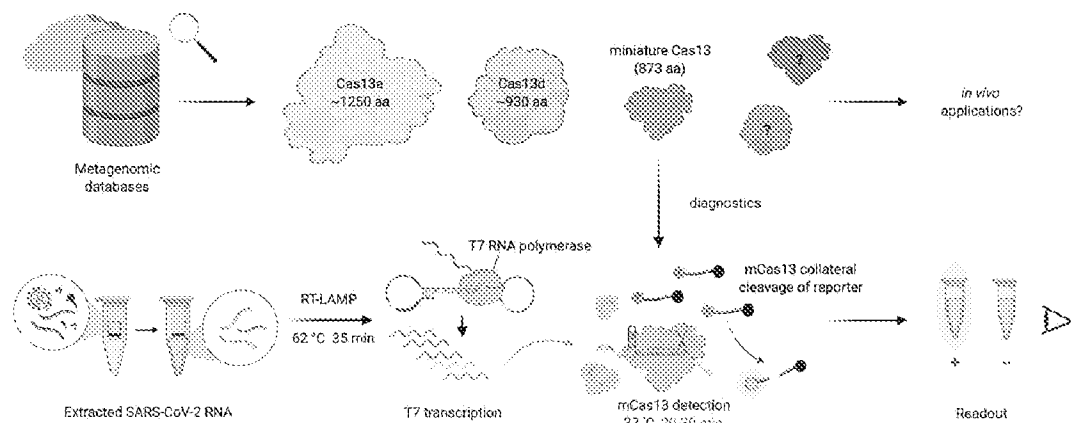


FIG. 13

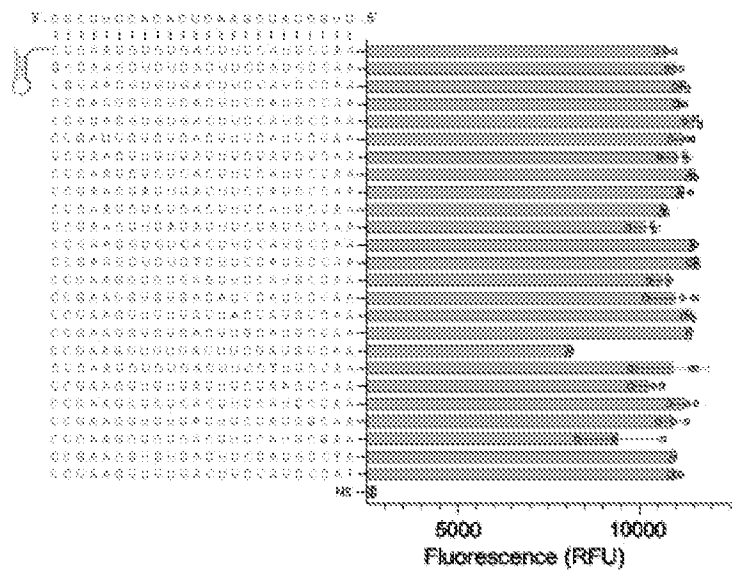


FIG. 14A

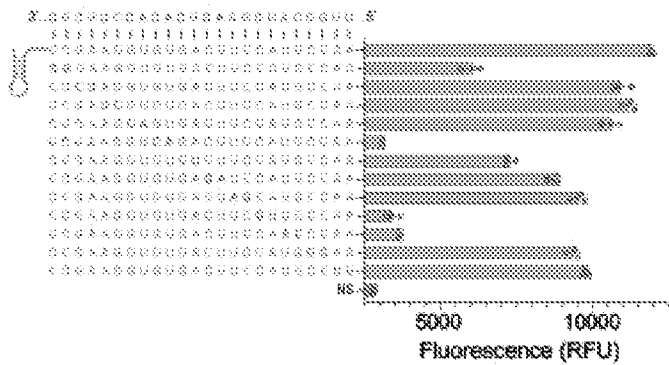


FIG. 14B

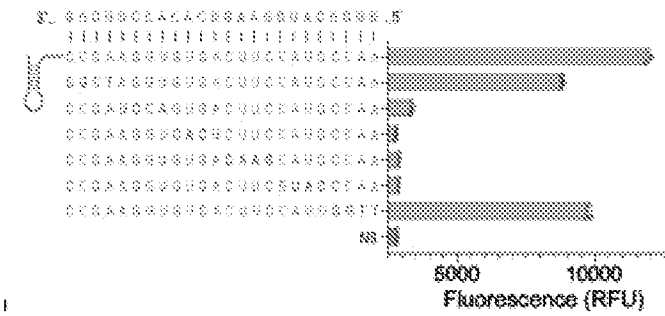


FIG. 14C

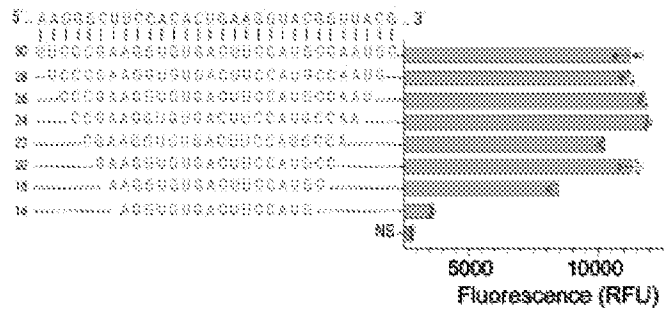


FIG. 14D

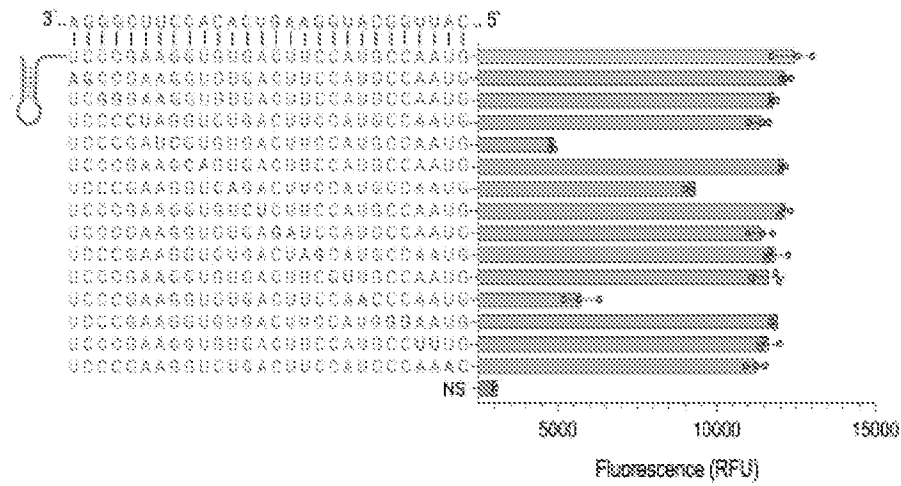


FIG. 14G

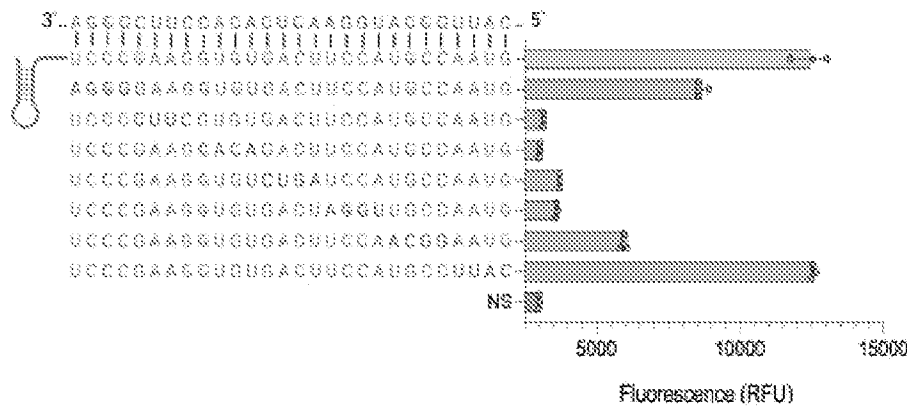


FIG. 14H

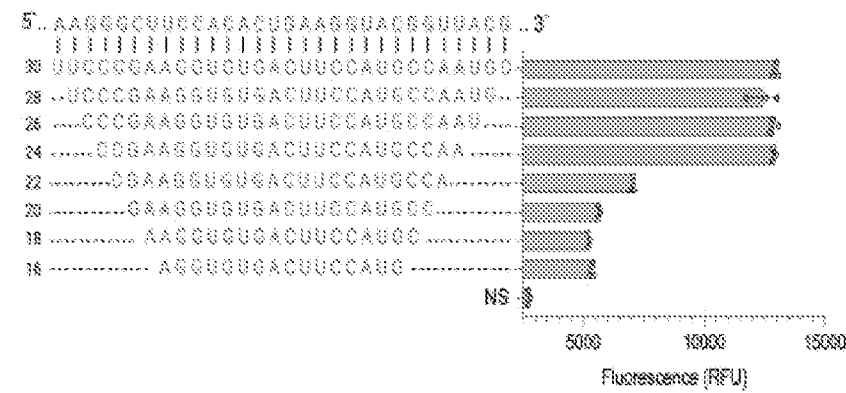


FIG. 14I

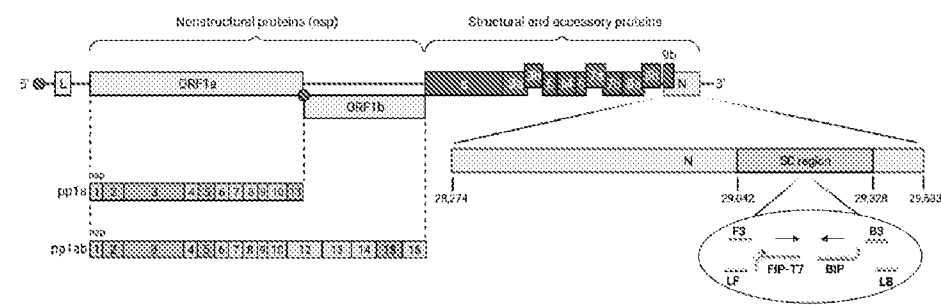


FIG. 15A

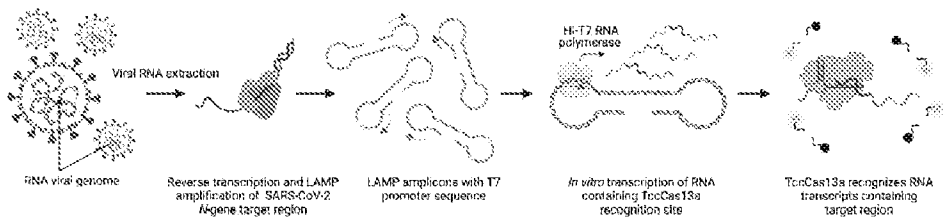


FIG. 15B

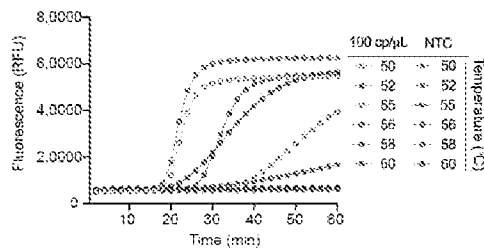


FIG. 15C

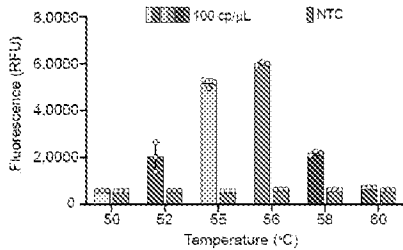


FIG. 15D

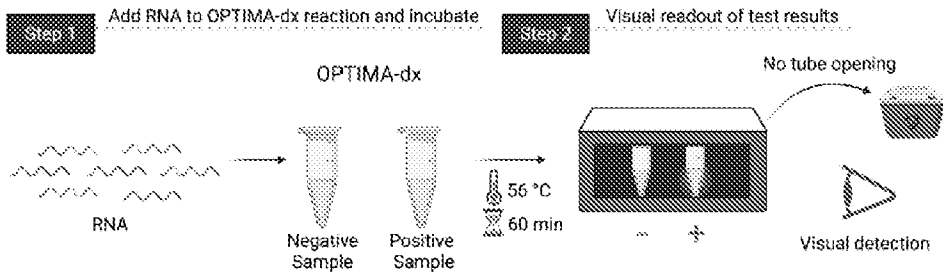


FIG. 16A

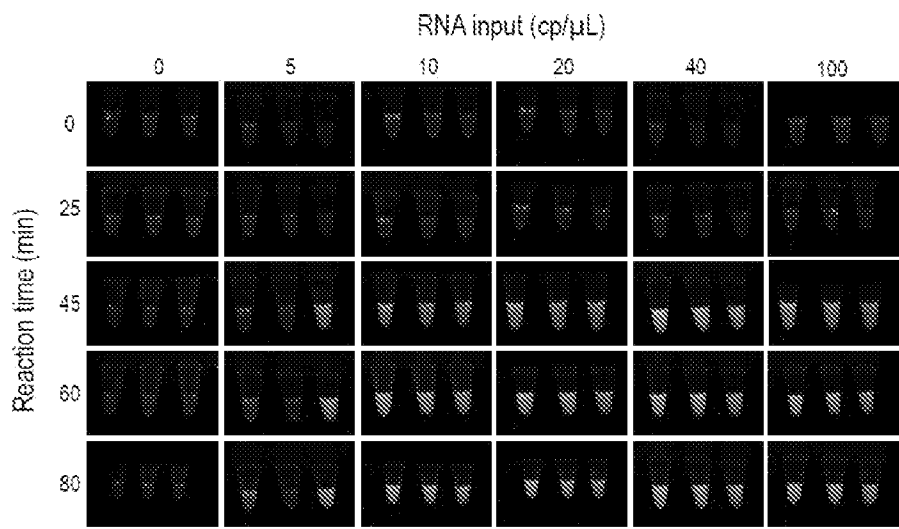


FIG. 16B

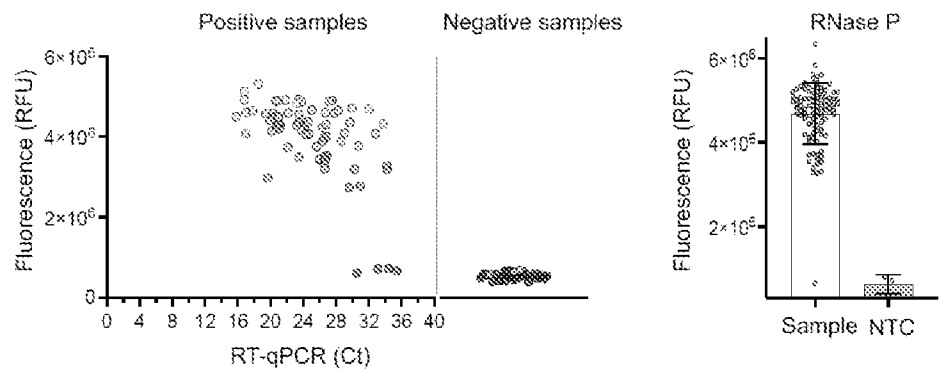


FIG. 16C

FIG. 16D

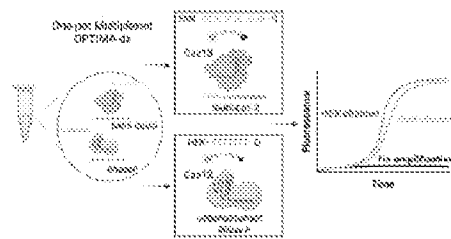


FIG. 17A

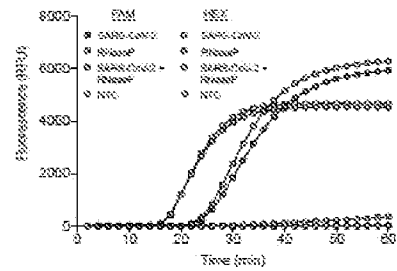


FIG. 17B

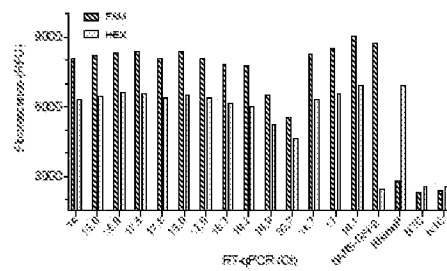


FIG. 17C

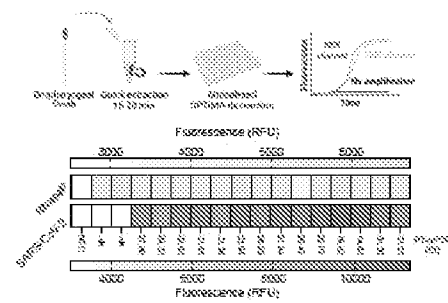


FIG. 17D

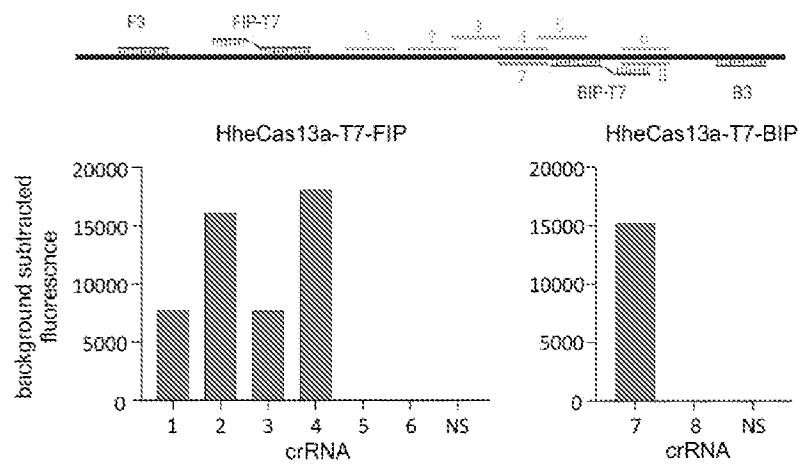


FIG. 18A

FIG. 18B

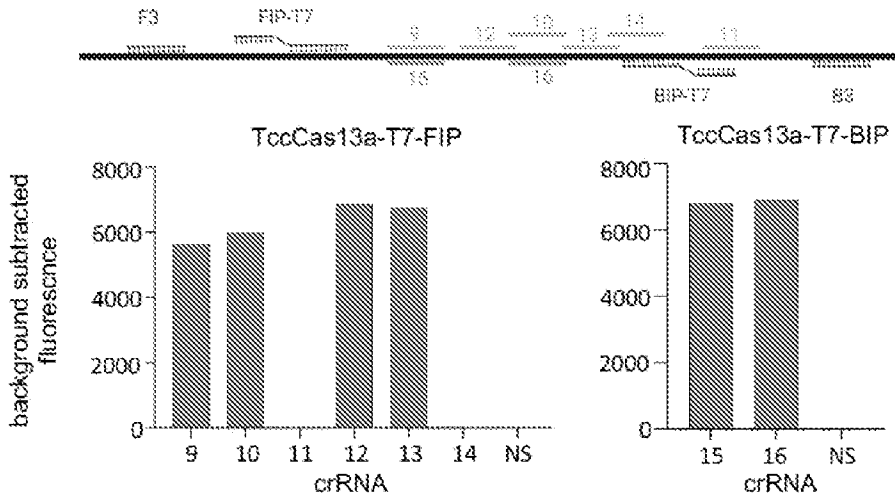


FIG. 18C

FIG. 18D

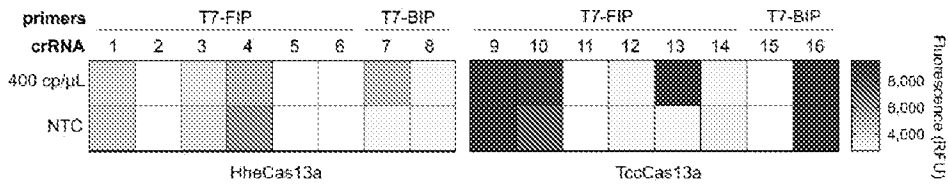


FIG. 19

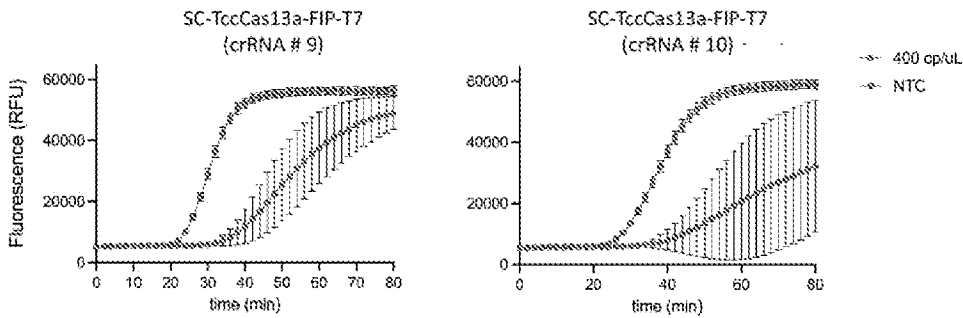


FIG. 20A

FIG. 20B

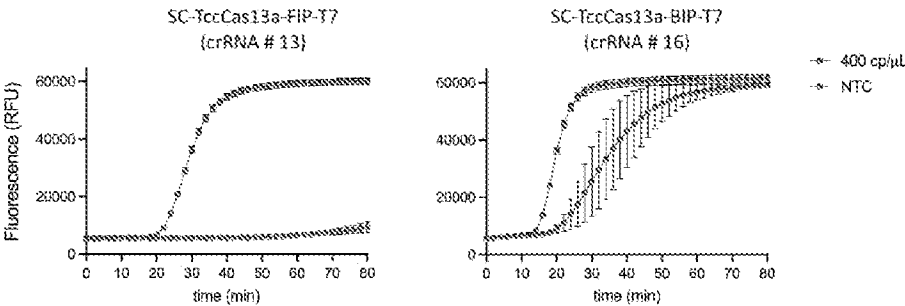


FIG. 20C

FIG. 20D

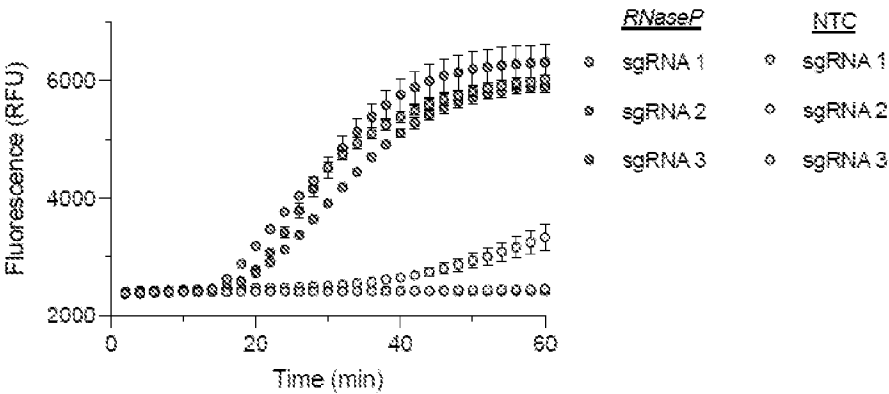


FIG. 21A

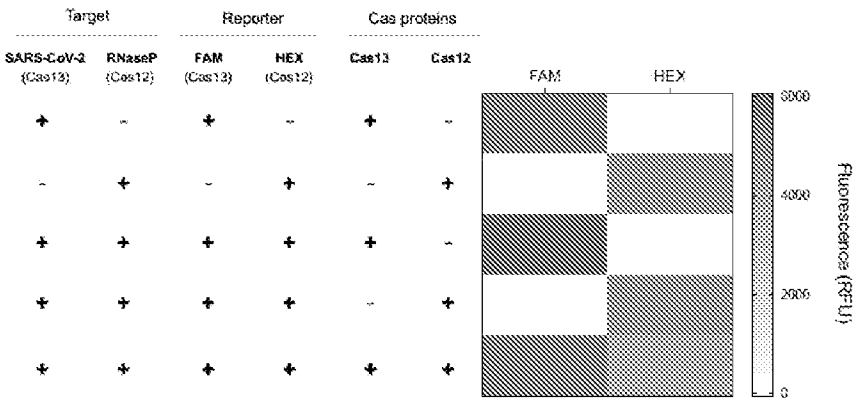


FIG. 21B

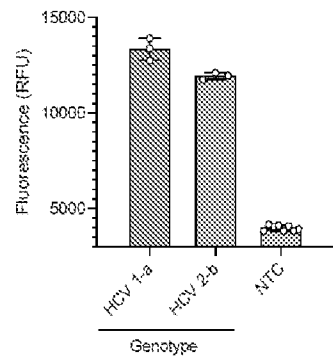


FIG. 22A

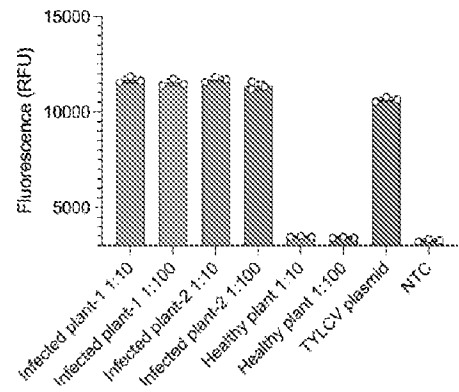


FIG. 22B

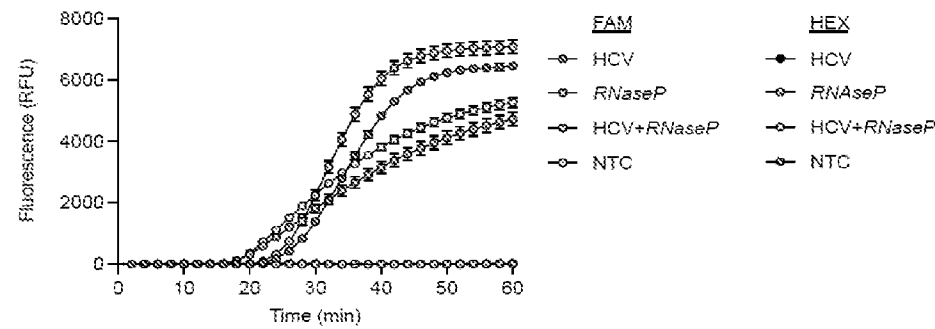


FIG. 22C

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

Applicant's or agent's file reference KAUST2021-164-02PCT	IMPORTANT DECLARATION	Date of mailing(<i>day/month/year</i>) 27 September 2022 (27-09-2022)
International application No. PCT/IB2022/055036	International filing date(<i>day/month/year</i>) 27 May 2022 (27-05-2022)	(Earliest) Priority date(<i>day/month/year</i>) 27 May 2021 (27-05-2021)
International Patent Classification (IPC) or both national classification and IPC C12Q1/6844		
Applicant KING ABDULLAH UNIVERSITY OF SCIENCE AND		

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below

1. ☐ The subject matter of the international application relates to:
 - a. ☐ scientific theories
 - b. ☐ mathematical theories
 - c. ☐ plant varieties
 - d. ☐ animal varieties
 - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes
 - f. ☐ schemes, rules or methods of doing business
 - g. ☐ schemes, rules or methods of performing purely mental acts
 - h. ☐ schemes, rules or methods of playing games
 - i. ☐ methods for treatment of the human body by surgery or therapy
 - j. ☐ methods for treatment of the animal body by surgery or therapy
 - k. ☐ diagnostic methods practised on the human or animal body
 - l. ☐ mere presentations of information
 - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art

2. ☒ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

☐ the description
☒ the claims
☐ the drawings

3. ☒ The failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions prevents a meaningful search from being carried out:

☐ the written form has not been furnished or does not comply with the standard.
☒ the computer readable form has not been furnished or does not comply with the standard.

4. Further comments:

Name and mailing address of the International Searching Authority  European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040 Fax: (+31-70) 340-3016	Authorized officer WEZYK, Magdalena Tel: +49 (0)89 2399-8315
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 203

Non-establishment according to Article 17(2) (a) PCT

The claims of the present application have, according to Rule 13ter.1(d) PCT, not been searched since the sequences disclosed in the application do not comply with WIPO Standard as prescribed under Rule 5.2 PCT.

A Standard-compliant sequence listing has not been furnished in text (TXT) format and the applicant has not remedied the deficiencies within the time limit fixed in the invitation pursuant to Rule 13ter.1(a) PCT (see Guidelines for Search and Examination at the EPO as PCT Authority, B-VIII, 3.2).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) PCT declaration be overcome.