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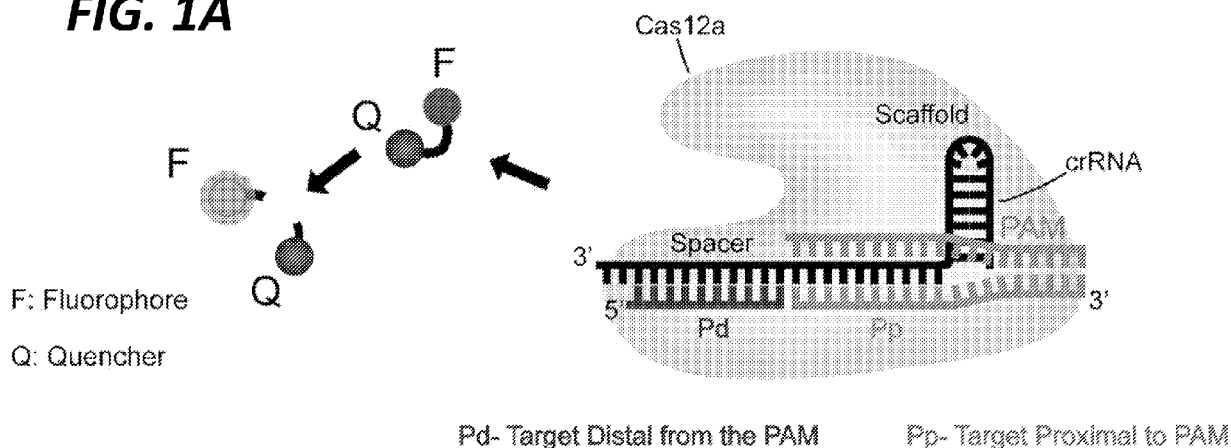
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Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

(54) Title: SYSTEMS AND METHODS FOR TARGET POLYNUCLEOTIDE DETECTION WITH CRISPR/CAS12A USING ACTIVATORS

FIG. 1A

(57) Abstract: The present disclosure provides novel CRISPR associated (Cas) systems, methods, and kits for detection of a target, including RNA targets, without reverse transcription. The systems of the present disclosure can be adapted for use with other CRISPR-based target detection systems and methods. Aspects of methods, systems and kits of the present disclosure enable detection of target polynucleotides in a sample with the use of CRISPR/Cas12a systems and DNA activators.

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SYSTEMS AND METHODS FOR TARGET POLYNUCLEOTIDE DETECTION WITH CRISPR/CAS12A USING ACTIVATORS

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Ser. No. 63/276,074, titled "SYSTEMS AND METHODS FOR REVERSE TRANSCRIPTION FREE RNA DETECTION WITH CRISPR/CAS12A USING SPLIT ACTIVATORS," filed November 5, 2021. This application is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant Nos. R21 AI156321, R21 AI168795 and R35 GM147788 awarded by the National Institutes of Health. The government has certain rights in the invention.

This invention was made in part or whole with funds received from Grant No. AGR00018466 awarded by the Florida Breast Cancer Foundation.

SEQUENCE LISTING

The instant application contains a Sequence Listing filed in ST.26 format entitled 222111_2500_Sequence_Listing.xml created on November 7, 2022. The content of the sequence listing is incorporated herein in its entirety.

FIELD

The present disclosure relates to CRISPR/Cas complex-based systems and methods.

BACKGROUND

The discovery of CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) systems has provided new platforms and approaches to the field of genome engineering and advanced applications in biology, agriculture, biotechnology, diagnostics, and treatment of genetic disorders. Originally derived from different species of bacterial adaptive immune systems, the CRISPR/Cas technology works by introducing a Cas nuclease and a short guide RNA sequence with a region complementary to a target sequence/site that acts as a guide by binding with Cas and directing the crRNA/Cas complex to a target site.

The development of CRISPR/Cas systems for rapid, point-of-care detection of nucleic acid targets for diagnosing diseases, such as cancer and viruses, has increased recently. The ongoing SARS-CoV-2 pandemic has vastly underscored the need for developing rapid, accurate and sensitive techniques for pathogen detection. Contemporary diagnostic methods that are based on reverse transcriptase polymerase chain reaction (RT-qPCR) are widely used, but are handicapped by their dependency on expensive reagents, sophisticated equipment, and trained personnel. CRISPR-Cas systems have emerged as a widely adopted diagnostic tool for the detection of SARS-CoV-2 and other viruses and conditions within the past year.

Class 2 type V and VI single effector Cas proteins, such as Cas12a and Cas13a, have been employed for the development of rapid, sensitive, and cost-effective detection platforms including DETECTR and SHERLOCK (Gootenberg et al., *Science*, 2017; Gootenberg et al., *Science*, 2018; Chen et al., *Science*, 2018; Broughton et al, *Nat. Biotechnol.*, 2020; Young et al, *NEJM.*, 2020) due to their robust trans-cleavage activity. The Cas12a-based DETECTR technology from Mammoth Biosciences and Cas13a-based SHERLOCK technology from Sherlock Biosciences are two CRISPR-based detection systems that are now approved by the FDA under EUA as lab-based diagnostics for detecting SARS-CoV-2 RNA. These platforms combine nucleic acid pre-amplification methods, such as RT-LAMP, RT-RPA, RT-HDA and other isothermal amplification steps, with the trans-cleavage ability of Type V and Type VI Cas effectors, for specific recognition of nucleic acid targets. Also, since many CRISPR systems, such as Cas12a CRISPR systems, detect DNA, for detection of RNA reverse transcription of the target is required before detection with CRISPR.

For the above-mentioned CRISPR-based detection methods, the pre-amplification step (such as RT-LAMP) takes additional time and/or must be done in a separate reaction (e.g., a separate “pot”) from the CRISPR detection, since the pre-amplification has to be conducted at elevated temperatures above the melting temperature of most Cas enzymes used in the CRISPR/Cas detection step. The need for separate amplification and detection steps increases the time, equipment, reagents, and costs needed for the assay and can reduce sensitivity. There is a need for detection of RNA without reverse transcription.

SUMMARY

According to various aspects, the present disclosure provides novel CRISPR associated (Cas) systems, methods, and kits for detection of a target, including RNA targets, without reverse transcription. The systems of the present disclosure can be adapted for use with other CRISPR-based target detection system and gene therapy application.

Methods of the present disclosure for detecting a target polynucleotide in a sample involve the following steps: combining the sample in a reaction vessel with the following: a Cas12a CRISPR-associated (Cas) enzyme; a CRISPR RNA (crRNA) having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide; a plurality of probes, each probe comprising an oligonucleotide element labeled with a detectable label, wherein the probe is configured to be cleaved by the Cas12a enzyme upon formation of an activated CRISPR/Cas complex thereby generating a CRISPR-generated detectable signal or detectable molecule; and a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA, wherein the activator is a single stranded or double stranded DNA sequence, wherein an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide. The method further includes incubating the reaction vessel and detecting the CRISPR-generated detectable signal or detectable molecule if the target polynucleotide is present in the sample. In embodiments, the target polynucleotide is an RNA, and the method does not require reverse transcription of the RNA before detection. Thus, methods of the present disclosure include reverse-transcription free detection of RNA. Methods of the present disclosure also include multiplexed detection of two or more different target polynucleotides in the sample. In embodiments, the methods can detect an RNA target and a DNA target, 2 or more RNA targets, or two or more DNA targets.

Systems of the present disclosure for detecting a target polynucleotide in a sample include the following components: a Cas12a CRISPR-associated (Cas) enzyme; a CRISPR RNA (crRNA) having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide; a plurality of probes, each probe comprising an oligonucleotide element labeled with a detectable label, wherein the probe is configured to be cleaved by the Cas12a enzyme upon formation of an activated CRISPR/Cas complex thereby generating a CRISPR-generated detectable signal or detectable molecule; and a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA, wherein the activator is a single stranded or double stranded DNA sequence, wherein an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide. In the systems of the present disclosure, the DNA activator can be a ssDNA or dsDNA and the target polynucleotide can be RNA or DNA.

Systems of the present disclosure for detecting multiple different target polynucleotides can include target-specific crRNAs corresponding to one or more different target polynucleotides.

Kits of the present disclosure for detecting a target polynucleotide in a sample include the system of the present disclosure and instructions for use to detect a target polynucleotide in a sample.

Other systems, methods, features, and advantages of the present disclosure will be or will become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional systems, methods, features, and advantages be included within this description, and be within the scope of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Further aspects of the present disclosure will be more readily appreciated upon review of the description of its various embodiments, described below, when taken in conjunction with the accompanying drawings. The components in the drawings are not necessarily to scale, emphasis instead being placed upon clearly illustrating the principles of the present disclosure. Moreover, in the drawings, like reference numerals designate corresponding parts throughout the several views.

FIGS. 1A-1G illustrate that Cas12a orthologs tolerate short ssDNA activators added in combination. FIG. 1A is a schematic representation of Cas12a complexed with crRNA, and *trans-cleavage* activated by combinations of short ssDNA target activators distal and proximal to the scaffold/PAM. FIGS. 1B-1D illustrate fold change at t=60 minutes of *in vitro trans-cleavage* assay with Cas12a orthologs activated by truncated ssDNA activators from 6-20 nt in length. The red graphs represent experiments carried out with LbCas12a, green represents AsCas12a, and orange represents ErCas12a. FIGS. 1E-1G illustrate heat maps representing fold change at t=60 minutes of *in vitro trans-cleavage* assay activated by combinations of truncated ssDNA activators of different lengths ranging from 6-14 nt in the Pp and Pd regions. The reactions contained 25 nM truncated ssDNA GFP-activators, 60 nM Cas12a, and 120 nM crGFP and were incubated for 60 min at 37°C. Error bars represent SD (n=3. Statistical analysis was performed using a two-tailed t-test where ns = not significant with $p > 0.05$, and the asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) denote significant differences.

FIGS. 2A-2H illustrate that RNA activators/targets are tolerated at the Pd end of the crRNA. FIG. 2A is a schematic representation of Cas12a activated by combinations of ssDNA (red), dsDNA (orange), and RNA (blue) in the PAM proximal and distal regions. FIGS. 2B-1D

are heat maps representing the fold changes of *in vitro trans-cleavage* assay (n=3) with Cas12a orthologs for the combinatorial schemes seen in FIG. 2A. FIGS. 2E-2H illustrate WT crRNA and ENHANCE crRNA used in the *in vitro trans-cleavage* assay with Cas12a orthologs. ssDNA and ssRNA were used as targets in the PAM distal region while dsDNA was supplied in the PAM proximal region. Reactions were incubated for 60 min at 37°C. Error bars represent SD (n=3). The reactions contained 25 nM truncated GFP-activators, 60 nM Cas12a, and 120 nM crRNA.

FIGS. 3A-3G illustrate development of activator detection methods and systems of the present disclosure (also referred to as SAHARA in the present disclosure) for the detection of a wide range of RNA targets. FIG. 3A is a schematic representation of Cas12a complexed with WT vs. SAHARA crRNA and short (20-nt) vs. long (730-nt) GFP RNA activators. FIGS. 3B-3D illustrate a comparison of *trans-cleavage* activity among Cas12a orthologs for the short v. long combinatorial schemes seen in FIG. 3A. An S12 activator was supplied at the Pp region of the crRNA for each SAHARA condition. FIGS. 3E-3G illustrate a comparison of *trans-cleavage* activity using a short (20-nt) GFP RNA activator among Cas12a orthologs with varying Pp dsDNA indicated as SR-Scr, a 12-nt scrambled dsDNA, or SR-S12, a 12-nt complementary Pp dsDNA. Both SR-Scr and SR-S12 were complexed with a 24-nt crRNA. WT included a 20-nt crRNA and detected a fully complementary 20-nt RNA activator. All reactions in FIGS. 3B-3G were incubated for 60 min at 37°C and RFU readings were taken at t=60 min using a fluorescence plate reader. Split activator system or SAHARA reactions contain 25 nM S12 dsDNA GFP-activators, 25 nM RNA GFP-activators, 60 nM Cas12a, and 120 nM crGFP. WT CRISPR/Cas reactions contain 25 nM RNA GFP-activators, 60 nM Cas12a, and 120 nM crGFP. Error bars represent SD (n=3). Statistical analysis was performed using a two-tailed t-test where ns = not significant with $p > 0.05$, and the asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) denote significant differences.

FIGS. 4A-4F illustrate that embodiments of the present disclosure can detect picomolar levels of HCV and miRNA-155 RNA targets. FIG. 4A illustrates an S12 dsDNA GFP-activator targeting in the Pp region (green), and the head (orange), tail (purple), and middle (blue) sections of an HCV polypeptide precursor RNA target were detected in the Pd region of each corresponding crRNA. FIG. 4B illustrates a comparison among the head, tail, mid, and pooled HCV targeting crRNA (n=3). The condition contains equimolar amounts of each crRNA represented in FIG. 4A. FIG. 4C illustrates the HCV limit of detection using the pooled crRNA with a split activator system is in the picomolar range. FIG. 4D illustrates head vs Tail detection for a mature miRNA-155 target mediated by a split activator system. crRNAs were designed to target an S12 dsDNA GFP-activator in the Pp region and target either the head or tail region of

a miRNA-155 target in the Pd region. FIG. 4E illustrates a comparison of delta RFU values among cr155-Tail, cr155-Head, and a combination of both Head and Tail targeting crRNAs. FIG. 4F shows a miRNA-155 limit of detection using a pooled crRNA with a split activator system. SAHARA shows significant detection up to 100 picomolar when compared to NTC with a miRNA-155 target. All reactions in figure 5 contain 25 nM S12 dsDNA GFP-activators, 25 nM RNA-activators, 60 nM ErCas12a, and 120 nM crRNA. Reactions were incubated for 60 min at 37°C. Error bars represent SD (n=3). Statistical analysis was performed using a two-tailed t-test where ns = not significant with $p > 0.05$, and the asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) denote significant differences.

FIGS. 5A-I illustrate that embodiments of methods of the present disclosure improve specificity of target detection at certain positions along the crRNA. FIGS. 5A-C illustrate a comparison of fold changes for the *in vitro trans-cleavage* assay between WT CRISPR/Cas reaction and SAHARA. Activator mutants normalized to each system's respective WT activator. Comparison of RFU values at t=60 min for the *in vitro trans-cleavage* assay between WT CRISPR/Cas reaction and SAHARA. FIGS. 5D-5I illustrate comparisons of *trans-cleavage* activity among WT, M09, and M10 SAHARA activator mutants using RFU at t=60 min. Each graph represents a different Cas12a ortholog used for the reaction: LbCas12a (FIGS. 5D and 5G), AsCas12a (FIGS. 5E and 5H), and ErCas12a (FIGS. 5F and 5I). Split activator system or SAHARA reactions contain 25 nM S12 dsDNA GFP-activators, 25 nM ssDNA GFP-activators, 60 nM Cas12a, and 120 nM crGFP. WT CRISPR/Cas reactions contain 25 nM ssDNA GFP-activators, 60 nM Cas12a, and 120 nM crGFP. Reactions were incubated for 60 min at 37°C. Error bars represent SD (n=3). Statistical analysis was performed using a two-tailed t-test where ns = not significant with $p > 0.05$, and the asterisks (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) denote significant differences.

FIGS. 6A-6J illustrate the role of the PAM sequence, GC content, and the concentration of S12 DNA for methods and systems of the present disclosure. FIGS. 6A-6D illustrate PAM sequence tolerance of Cas12a orthologs coupled with SAHARA. Comparison of *trans-cleavage* activity among S12 dsDNA activators containing different PAM sequences (n=3). The PAM sequences TTTA, AAAT, and VVVN were assessed (V representing a non-thymine nucleotide and N representing any nucleotide). FIGS. 6E-6G are graphs illustrating that Cas12a orthologs tolerate a wide range of GC contents in the crRNA and S12 dsDNA for RNA detection (n=3). FIGS. 6H-6J illustrate the *trans-cleavage* activity of Cas12a with varying concentrations of S12 after incubation for 60 min at 37°C. Error bars represent SD (n=3). The *trans-cleavage* is

initiated by S12 DNA concentrations as low as 50 Pm, but SAHARA *trans-cleavage* activity is absent when S12 DNA is not present in the reaction.

FIGS. 7A-7G illustrate simultaneous detection of multiple DNA and RNA targets using embodiments of the present disclosure. FIG. 7A is a schematic illustration of multiplexed detection with SAHARA. Briefly, a mixture of different crRNAs is pooled together. The pooled crRNAs can then be differentiated for *trans-cleavage* activity by the use of sequence-specific S12 activators. FIGS. 7B-7D are heat maps depicting the *trans-cleavage* activity of 3 different pooled crRNAs (crRNA-a, crRNA-b, and crRNA-c) in the presence of 3 different S12 activators (S12a, S12b, S12c) or a no S12 control for Lb, As, and Er cas12a orthologs. Fold change compared to the NTC at t=60 min from the start of the reaction is plotted. 30 nM Cas12a, 60 nM crRNA, 25 nM S12 activators, and 25 nM of DNA or RNA targets were used in the assay (n=3). FIG. 7E is a schematic illustration of multiplexed RNA detection with a combination of SAHARA and Cas13b. DNA or RNA reporters consisting of different colored dyes are used to distinguish the signal produced by Cas12a and Cas13b. FIGS. 7F-7G illustrate multiplexed RNA detection using Lb, As, and Er orthologs of Cas12a and PsmCas13b. Cas12a targets activator T1 and produces a signal in the FAM channel, while Cas13b targets activator T2 and produces a signal in the HEX channel (n=3).

FIGS. 8A-8D illustrate the use of dsDNA activators with and without PAM. FIG. 8A is a schematic illustrating the design of PAM-containing and PAM-less ds activators corresponding to the Pp or Pd end of the crRNA. FIGS. 8B-8D illustrate *trans-cleavage* activity of the combination of PAM-containing or no-PAM containing double-stranded DNA activators binding at either the PAM-proximal (Pp) end or the PAM-distal (Pd) end of the crRNA in a combinatorial fashion. The heat map indicates the fold change in RFU compared to NTC at time t=60 min for Lb, As, and Er Cas12a orthologs (n=3).

FIGS. 9A-9G illustrate chimeric DNA-RNA guides complexed with Cas12a. FIG. 9A is a schematic representation of chimeric DNA-RNA hybrid crRNAs complexed with Cas12a and activated with WT ssDNA activators. Chimeric crRNA was designed by changing 12-nt near the PAM-proximal 5'-end of the crRNA to DNA (12D8R crRNA) and changing the PAM distal 8-nt end of the crRNA to DNA (12R8D crRNA). WT crRNA is represented in graphs WT crRNA is represented on graphs b-d by triangles, 12R8D crRNA is represented by squares, and 12D8R crRNA is represented by circles. FIGS. 9B-9D are graphs of relative RFU values of in vitro *trans-cleavage* assay with Cas12a orthologs (Lb, As, and Er) complexed with WT crRNA, 12D8R crRNA, and 12R8D crRNAs. FIGS. 9E-9G are graphs illustrating fold change at 60 min is represented for each crRNA and three Cas proteins. The reactions contained 25 nM ssDNA

GFP WT activator, 60 nM Cas12a, and 12 nM crRNA (WT, 12R8D, 12D8R). Reactions were incubated for 60 min at 37°C. Error bars represent SD (n=3).

FIGS. 10A-10C illustrate reverse transcription-free RNA detection with Cas12a and 'split activator' mechanism. Chimeric crRNAs include 12D8R and 12R8D crRNAs as well as WT crRNA. Combinations of activators include ssDNA and RNA targeting the PAM proximal and distal locations on the crRNA. FIGS. 10A-10C are heat maps representing the fold changes of *in vitro trans-cleavage* assay with Cas12a orthologs complexed with WT and chimeric crRNAs. Combinatorial schemes for the 'split activator system' are seen in (a) (n=3).

FIGS. 11A-11F illustrate detection of ssDNA or ssRNA sequences with SAHARA at different temperatures. FIGS. 11A-11C illustrate raw fluorescence data showing the *trans-cleavage* activity of SAHARA for the detection of an ssDNA or ssRNA sequence at either room temperature (RT) or 37°C for Lb (FIG. 11A), As (FIG. 11B), and Er (FIG. 11C) orthologs. Error bars represent S.D. (n=3). FIGS. 11D-11F are graphs showing background subtracted raw fluorescence intensity for the detection of ssDNA or ssRNA sequences at RT or 37°C for Lb (FIG. 11D), As (FIG. 11E), and Er (FIG. 11F) orthologs. Error bars represent S.D. (n=3).

FIGS. 12A-12B illustrate optimization of SAHARA with different divalent metal ions and concentrations. FIG. 12A illustrates the effect of different metal ions on the SAHARA with Lb, As, and Er Cas12a enzymes, and demonstrates that Mg ions have the greatest effect for increasing activity. Negative control represents a no-salt buffer. Each metal ion buffer contains 3 mM of the respective metal salt. The heat map indicates fold change at time t=60 min (n=3). FIG. 12B illustrates optimization of SAHARA with Mg ion concentration: The *trans-cleavage* activity of SAHARA with ErCas12a under increasing Mg²⁺ ion concentration is shown. The plot of fold change in RFU compared to NTC at t=60 min is shown. Error bars represent S.D. (n=3).

FIGS. 13A-13C illustrate optimization of SAHARA with pH conditions. FIGS. 13A-13C are bar graphs of the effect of buffer pH on the *trans-cleavage* activity of SAHARA indicating background subtracted RFU at time t=60 min for Lb (FIG. 13A), As (FIG. 13B), and Er (FIG. 13C) Cas12a orthologs at a different range of pH values. Error bars represent S.D. (n=3).

FIGS. 14A-14C are graphs illustrating the effect of S12 concentration ranging from 50 nM -780pM on the *trans-cleavage* activity of SAHARA is shown for Lb (FIG. 14A), As (FIG. 14B), and Er (FIG. 14C) Cas12a orthologs. The plot of RFU at t=60 min in the presence or absence of 25 nM target HCV-RNA and different S12 concentrations is shown. Error bars represent S.D. (n=3).

FIGS. 15A-15C are graphs illustrating the effect of S12 concentration ranging from 1.56 nM to 50 pM on the *trans-cleavage* activity of SAHARA is shown for Lb (FIG. 15A), As (FIG.

15B), and Er (FIG. 15C) Cas12a orthologs. A plot of RFU at t=60 min in the presence or absence of 25 nM target HCV-RNA and different S12 concentrations is shown. Error bars represent S.D. (n=3).

FIGS 16A-16C illustrate the effect of different lengths of PAM proximal (Pp) DNA activators and PAM-distal (Pd) RNA small target-activators on the activity of SAHARA with the three Cas12a orthologs: Lb (FIG. 16A), As (FIG. 16B), and Er (FIG. 16C).

DETAILED DESCRIPTION

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to particular embodiments described, as such may, of course, 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 be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit (unless the context clearly dictates otherwise), between the upper and lower limit of that range, and any other stated or intervening value in that stated range, is encompassed within the disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of genetics, biochemistry, molecular biology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

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 to perform the methods and use the compositions and compounds disclosed and claimed herein. Efforts have been made to ensure accuracy with respect to numbers (*e.g.*, amounts, temperature, *etc.*), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C, and pressure is at or near atmospheric. Standard temperature and pressure are defined as 20-25 °C and 1 atmosphere.

Before the embodiments of the present disclosure are described in detail, it is to be understood that, unless otherwise indicated, the present disclosure is not limited to particular materials, reagents, reaction materials, manufacturing processes, or the like, as such can vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting. It is also possible in the present disclosure that steps can be executed in different sequence where this is logically possible.

All publications and patents cited in this specification are cited to disclose and describe the methods and/or materials in connection with which the publications are cited. Publications and patents that are incorporated by reference, where noted, are incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference. Such incorporation by reference is expressly limited to the methods and/or materials described in the cited publications and patents and does not extend to any lexicographical definitions from the cited publications and patents. Any lexicographical definition in the publications and patents cited that is not also expressly repeated in the instant application should not be treated as such and should not be read as defining any terms appearing in the accompanying claims. Any terms not specifically defined within the instant application, including terms of art, are interpreted as would be understood by one of ordinary skill in the relevant art; thus, is not intended for any such terms to be defined by a lexicographical definition in any cited art, whether or not incorporated by reference herein, including but not limited to, published patents and patent applications. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure. Further, the dates of publication provided could be different from the actual publication dates that may need to be independently confirmed.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of cells. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise. In this disclosure, "consisting essentially of" or "consists essentially" or the like, when applied to methods and compositions encompassed by the present disclosure refers to compositions like those disclosed herein, but which may contain additional structural groups, composition components or method steps (or analogs or derivatives thereof as discussed above). Such additional structural groups, composition components or method steps, etc., however, do not materially affect the basic and novel characteristic(s) of the compositions or methods, compared to those of the corresponding compositions or methods disclosed herein. "Consisting essentially of" or "consists essentially" or the like, when applied to methods and compositions encompassed by the present disclosure have the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

Prior to describing the various embodiments, the following definitions are provided and should be used unless otherwise indicated.

Definitions

In describing and claiming the disclosed subject matter, the following terminology will be used in accordance with the definitions set forth below.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of cells. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

As used herein, "about," "approximately," and the like, when used in connection with a numerical variable, generally refers to the value of the variable and to all values of the variable that are within the experimental error (e.g., within the 95% confidence interval for the mean) or within +/- 10% of the indicated value, whichever is greater.

The terms “comprise”, “comprising”, “including”, “containing”, “characterized by”, and grammatical equivalents thereof are used in the inclusive, open sense, meaning that additional elements may be included. It is not intended to be construed as “consists of only.”

As used herein, “consisting of” and grammatical equivalent thereof exclude any element, step or ingredient not specified in the claim.

In this disclosure, “consisting essentially of” or “consists essentially” or the like, when applied to methods and compositions encompassed by the present disclosure refers to compositions like those disclosed herein, but which may contain additional structural groups, composition components or method steps (or analogs or derivatives thereof as discussed above). Such additional structural groups, composition components or method steps, etc., however, do not materially affect the basic and novel characteristic(s) of the compositions or methods, compared to those of the corresponding compositions or methods disclosed herein. “Consisting essentially of” or “consists essentially” or the like, when applied to methods and compositions encompassed by the present disclosure have the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

As used herein, “subject” refers to any living entity comprised of at least one cell. A living organism can be as simple as, for example, a single isolated eukaryotic cell or cultured cell or cell line, or as complex as a mammal, including a human being, and animals (e.g., vertebrates, amphibians, fish, mammals, e.g., cats, dogs, horses, pigs, cows, sheep, rodents, rabbits, squirrels, bears, primates (e.g., chimpanzees, gorillas, and humans).

As used herein, “cDNA” refers to a DNA sequence that is complementary to an RNA transcript in a cell. It is a man-made molecule. Typically, cDNA is made in vitro by an enzyme called reverse-transcriptase using RNA transcripts as templates.

As used herein with reference to the relationship between DNA, cDNA, cRNA, RNA, protein/peptides, and the like “corresponding to” or “encoding” (used interchangeably herein) refers to the underlying biological relationship between these different molecules. As such, one of skill in the art would understand that operatively “corresponding to” can direct them to determine the possible underlying and/or resulting sequences of other molecules given the sequence of any other molecule which has a similar biological relationship with these molecules. For example, from a DNA sequence an RNA sequence can be determined and from an RNA sequence a cDNA sequence can be determined.

As used herein, “deoxyribonucleic acid (DNA)” and “ribonucleic acid (RNA)” can generally refer to any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. RNA can be in the form of non-coding RNA such as tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), anti-sense RNA, RNAi (RNA interference construct), siRNA (short interfering RNA), microRNA (miRNA), or ribozymes, aptamers, guide RNA (gRNA), CRISPR RNA (crRNA), Trans-activating crRNA (tracrRNA), or coding mRNA (messenger RNA).

As used herein, the terms “guide polynucleotide,” “guide sequence,” “guide RNA” or “sgRNA” can refer to any polynucleotide sequence (typically an RNA sequence) having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. The degree of complementarity between a guide polynucleotide and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting examples of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g. the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies, ELAND (Illumina, San Diego, Calif.), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). A guide polynucleotide (also referred to herein as a guide sequence and includes single guide sequences (sgRNA)) can be about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, 90, 100, 110, 112, 115, 120, 130, 140, or more nucleotides in length. The guide polynucleotide can include a nucleotide sequence that is complementary to a target DNA sequence.

This complementary portion of the guide sequence can be referred to as the complementary region of the guide RNA or often the “CRISPR RNA (crRNA).” The guide sequence also has a region that associates with the CRISPR associated (Cas) enzyme, a region that is referred to as the tracr RNA in systems such as Cas 9 where the crRNA and tracr RNA are separate. In systems such as with CRISPR/Cas12a, which is a single guide RNA systems, the whole guide RNA is referred to as the “crRNA”, and the portion that associates with the Cas enzyme is often referred to as the scaffold portion. In such systems, the target binding region may be referred to as the complementary region, target region, variable region or spacer region. Since the present disclosure primarily discusses Cas12a systems, the guide sequence will often be referred to herein as simply the “crRNA.” The guide sequence can also

include one or more miRNA target sequences coupled to the 3' end of the guide sequence. The guide sequence can include one or more MS2 RNA aptamers incorporated within the portion of the guide strand that is not the complementary portion. As used herein the term guide sequence can include any specially modified guide sequences, including but not limited to those configured for use in synergistic activation mediator (SAM) implemented CRISPR (Nature 517, 583–588 (29 January 2015) or suppression (Cell Volume 154, Issue 2, 18 July 2013, Pages 442–451). A guide polynucleotide can be less than about 150, 125, 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guide polynucleotide to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide polynucleotide to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide polynucleotide to be tested and a control guide polynucleotide different from the test guide polynucleotide, and comparing binding or rate of cleavage at the target sequence between the test and control guide polynucleotide reactions. Other assays are possible, and will occur to those skilled in the art.

The term “activator” or “activator sequence” as used herein refers to a short polynucleotide sequence that is complementary to and capable of binding a region of a CRISPR RNA (crRNA), typically in a region of the crRNA called the spacer or variable region, which is a portion of a crRNA designed to bind a target (as opposed to the scaffold region of the crRNA which is conserved). In Cas12a systems, the crRNA includes the spacer region at the 3' end of the crRNA and a conserved scaffold portion of the crRNA is at the 5' the end of the crRNA. The scaffold portion provides a scaffold for the Cas enzyme that will cut the target strand near a PAM sequence (as short polynucleotide sequence on the non-target strand of a double stranded DNA (dsDNA) target). Thus, the portion of the crRNA spacer region that is closer to the 5' end and scaffold portion and thus nearer to where the PAM region of a target polynucleotide would lie, is referred to herein as the “PAM-proximal region” or “PAM-proximal end” of the crRNA, and the portion of the crRNA spacer region further from the scaffold region and closer to the 3' end of the crRNA is referred to herein as the “PAM-distal region” or “PAM-distal end” of the crRNA. Activator sequences as referred to herein can be complementary to the entire or majority of the complementary region of the crRNA or can be a shorter activator

sequence that can be complementary to and thus capable of binding to PAM proximal or PAM distal regions of the crRNA. In embodiments activators can be a single or dsDNA sequence. In some embodiments of the present disclosure the activator is a ss or dsDNA sequence that is configured to bind the PAM-proximal end of the crRNAs and is referred to herein as a DNA activator.

As used herein, the term “split activator” refers to two or more smaller activator sequences each complementary to a portion of the complementary region of the crRNA.

As used herein, “nucleic acid,” “nucleotide sequence,” and “polynucleotide” can be used interchangeably herein and can generally refer to a string of at least two base-sugar-phosphate combinations and 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 single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein can refer to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions can be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. “Polynucleotide” and “nucleic acids” also encompasses such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, inter alia. For instance, the term polynucleotide as used herein can include DNAs or RNAs as described herein that contain one or more modified bases. Thus, DNAs or RNAs including unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. “Polynucleotide”, “nucleotide sequences” and “nucleic acids” also includes PNAs (peptide nucleic acids), phosphorothioates, and other variants of the phosphate backbone of native nucleic acids. Natural nucleic acids have a phosphate backbone, artificial nucleic acids can contain other types of backbones, but contain the same bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are “nucleic acids” or “polynucleotides” as that term is intended herein. As used herein, “nucleic acid sequence” and “oligonucleotide” also encompasses a nucleic acid and polynucleotide as defined elsewhere herein.

As used herein, “isolated” means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. A non-naturally occurring polynucleotide, peptide,

polypeptide, protein, antibody, or fragments thereof, do not require "isolation" to distinguish it from its naturally occurring counterpart.

As used herein, "polypeptides" or "proteins" refers to amino acid residue sequences. Those sequences are written left to right in the direction from the amino to the carboxy terminus. In accordance with standard nomenclature, amino acid residue sequences are denominated by either a three letter or a single letter code as indicated as follows: Alanine (Ala, A), Arginine (Arg, R), Asparagine (Asn, N), Aspartic Acid (Asp, D), Cysteine (Cys, C), Glutamine (Gln, Q), Glutamic Acid (Glu, E), Glycine (Gly, G), Histidine (His, H), Isoleucine (Ile, I), Leucine (Leu, L), Lysine (Lys, K), Methionine (Met, M), Phenylalanine (Phe, F), Proline (Pro, P), Serine (Ser, S), Threonine (Thr, T), Tryptophan (Trp, W), Tyrosine (Tyr, Y), and Valine (Val, V). "Protein" and "Polypeptide" can refer to a molecule composed of one or more chains of amino acids in a specific order. The term protein is used interchangeable with "polypeptide." The order is determined by the base sequence of nucleotides in the gene coding for the protein. Proteins can be involved in the structure, function, and regulation of various functions.

As used herein with reference to the relationship between DNA, cDNA, crRNA, RNA, protein/peptides, and the like "corresponding to" or "encoding" (used interchangeably herein) refers to the underlying biological relationship between these different molecules. As such, one of skill in the art would understand that operatively "corresponding to" can direct them to determine the possible underlying and/or resulting sequences of other molecules given the sequence of any other molecule which has a similar biological relationship with these molecules. For example, from a DNA sequence an RNA sequence can be determined and from an RNA sequence a cDNA sequence can be determined. With respect to the present disclosure in the context of a guide sequence or crRNA and a target polynucleotide sequence and/or an activator sequence, "corresponding sequence" can also refer to the complementary sequence capable of binding/hybridizing to the reference sequence.

As used herein, the term "encode" refers to principle that DNA can be transcribed into RNA, which can then be translated into amino acid sequences that can form proteins.

As used herein, the terms "optional" or "optionally" means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

As used herein, the term "specific binding" or "preferential binding" can refer to non-covalent physical association of a first and a second moiety wherein the association between the first and second moieties is at least 2 times as strong, at least 5 times as strong as, at least 10 times as strong as, at least 50 times as strong as, at least 100 times as strong as, or

stronger than the association of either moiety with most or all other moieties present in the environment in which binding occurs. Binding of two or more entities may be considered specific if the equilibrium dissociation constant, K_d , is 10^{-3} M or less, 10^{-4} M or less, 10^{-5} M or less, 10^{-6} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less, 10^{-10} M or less, 10^{-11} M or less, or 10^{-12} M or less under the conditions employed, e.g., under physiological conditions such as those inside a cell or consistent with cell survival. In some embodiments, specific binding can be accomplished by a plurality of weaker interactions (e.g., a plurality of individual interactions, wherein each individual interaction is characterized by a K_d of greater than 10^{-3} M). In some embodiments, specific binding, which can be referred to as “molecular recognition,” is a saturable binding interaction between two entities that is dependent on complementary orientation of functional groups on each entity. Examples of specific binding interactions include primer-polynucleotide interaction, aptamer-aptamer target interactions, antibody-antigen interactions, avidin-biotin interactions, ligand-receptor interactions, metal-chelate interactions, hybridization between complementary nucleic acids, etc.

As used herein, “kit” means a collection of at least two components constituting the kit. Together, the components constitute a functional unit for a given purpose. Individual member components may be physically packaged together or separately. For example, a kit comprising an instruction for using the kit may or may not physically include the instruction with other individual member components. Instead, the instruction can be supplied as a separate member component, either in a paper form or an electronic form which may be supplied on computer readable memory device or downloaded from an internet website, or as recorded presentation.

As used herein, “instruction(s)” means documents describing relevant materials or methodologies pertaining to a kit. These materials may include any combination of the following: background information, list of components and their availability information (purchase information, etc.), brief or detailed protocols for using the kit, trouble-shooting, references, technical support, and any other related documents. Instructions can be supplied with the kit or as a separate member component, either as a paper form or an electronic form which may be supplied on computer readable memory device or downloaded from an internet website, or as recorded presentation. Instructions can comprise one or multiple documents and are meant to include future updates.

As used herein, the term “biocompatible,” with respect to a substance or fluid described herein, indicates that the substance or fluid does not adversely affect the short-term viability or long-term proliferation of a target biological particle within a particular time range.

Reference throughout this specification to “one embodiment”, “an embodiment”, “another embodiment”, “some embodiment,” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment,” “in an embodiment,” “in another embodiment”, or “in some embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but they may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some, but not other, features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention. For example, in the appended claims, any of the claimed embodiments can be used in any combination

Discussion

In accordance with the purpose(s) of the present disclosure, as embodied and broadly described herein, embodiments of the present disclosure, in some aspects, relate to compositions, devices, methods and/or systems for the detection of polynucleotides, including RNA, in a sample without the use of reverse transcription and with the use of CRISPR/Cas12a systems and activators. Compositions, devices, kits, and methods of the present disclosure are described briefly below and in the accompanying figures. Embodiments of such devices, kits and methods facilitate/provide the ability to detect RNA in a sample without the use of reverse transcription prior to detection. Embodiments of the present disclosure, also provide approaches for multiplexed detection of more than one different target polynucleotide in a system by using multiple crRNA's designed for different targets or by multiplexing a Cas12a split-activator system with a Cas13 RNA detection system.

Overview

The breakthrough of CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) systems has transformed the slow-progressing field of genome engineering with diverse applications in biology, agriculture, biotechnology, diagnostics, and treatment of genetic disorders. While manipulating and testing short DNA activators designed to interact with different regions of the crRNA, unique properties of Cas12a, a type V-A CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) -associated enzyme were discovered.

CRISPR is an adaptive immune system encoded within prokaryotes that have evolved to counter invasion by foreign nucleic acids such as bacteriophages and plasmids^{1,2}. Upon infection, the invading DNA sequences are captured and integrated into the host genome between an array of repeat sequences. The captured DNA sequences are called 'spacers' and they provide a genetic memory of prior infections³. For prokaryotic immunity, the CRISPR locus is transcribed and processed to generate multiple mature CRISPR RNAs (crRNA), each encoding a unique spacer sequence. Cas (CRISPR-associated) proteins are RNA-guided endonucleases that when complexed with the mature crRNAs can enable the silencing of any genetic material that is complementary to the crRNA sequence.

There are several diverse naturally occurring CRISPR/Cas systems found in prokaryotes^{4,5}. Among these, Cas12a is a class II, type V RNA-guided DNA endonuclease⁶. Since its discovery, it has been widely used for genome editing as well as molecular diagnostic applications⁷⁻¹¹. Structural and biochemical studies have shown that Cas12a can catalyze the cleavage of DNA substrates¹²⁻¹⁴ but there are no reports of targeted RNA cleavage by Cas12a. A special characteristic of the type V Cas12-family enzymes is their ability to initiate rapid and indiscriminate cleavage of any non-specific single-stranded DNA (ssDNA) molecules in their vicinity after target-specific recognition and cleavage^{15,16}. This unique catalytic property, known as *trans-cleavage*, has been harnessed to engineer CRISPR-based diagnostic tools that rely on the cleavage of FRET reporters, or other probes capable of producing a detectable signal or molecule, upon target recognition¹⁷.

So far, Cas12a-based tools have been limited to the detection of DNA substrates, unless they are coupled with additional steps involving reverse transcription or strand displacement. Recently, it was discovered that CRISPR-Cas12a can tolerate DNA/RNA heteroduplexes, but only when RNA is located at the non-target strand¹⁸. This system was used to detect RNA targets with Cas12a by creating a heteroduplex using a reverse transcription step without amplification. A reverse-transcription step is inconvenient because it adds to the time, cost, error, and complexity of the assay.

The other alternative is to use an RNA-targeting enzyme such as Cas12g¹⁹ or Cas13a-d²⁰⁻²²; however, these systems can only detect RNA and not DNA. It has been previously shown that DNA-cleaving enzymes such as Cas9 can be manipulated to also cleave RNA through the addition of a PAMmer sequence²³. However, similar approaches have not yet been investigated with *trans-cleaving* Cas enzymes like Cas12, primarily because the PAM recognition mechanism is very different for Cas9 and Cas12 enzymes²⁴. To date, there is no single

CRISPR-Cas system identified that can innately tolerate both DNA and RNA substrates to trigger *trans-cleavage*.

In contrast to Cas9, which uses two different active sites to generate a blunt double-stranded DNA break²⁵, Cas12a uses a single active site to make staggered cuts on the two strands of a dsDNA^{6,12,15}. After cleaving the target DNA, Cas12a releases the PAM-distal cleavage product while retaining the PAM-proximal cleavage product bound to the crRNA^{24,26}. This maintains Cas12a in a catalytically competent state, in which the active site of RuvC remains exposed to the solvent which then leads to *trans-cleavage* of neighboring single-stranded DNA molecules in a nonspecific manner.

As demonstrated in the Example below, the present disclosure describes for the first time that Cas12a can also tolerate RNA substrates, and not just DNA, at the PAM-distal end of the crRNA, for initiating *trans-cleavage*. This has demonstrated that while the PAM proximal seed region of the crRNA has a preference for DNA substrates, the PAM distal end of the crRNA can tolerate RNA along with DNA substrates in multiple Cas12a orthologs. Thus, by supplying a small piece of ssDNA or a PAM-containing dsDNA at the seed region of the crRNA RNA substrates can be detected at the non-seed region of the crRNA with multiple Cas12a orthologs. This property has been harnessed to develop a tool named, in the Example below, Split Activators for Highly Accessible RNA Analysis (SAHARA).

The system and method of the present disclosure provides RT-free detection of picomolar levels of DNA as well as RNA without amplification. These methods were used for RT-free detection of HCV and miRNA-155 (miR-155) RNA targets. As compared to the conventional CRISPR-Cas12a, SAHARA has improved specificity as can be performed at room temperature, and its activity can be turned ON or OFF using the seed region binding DNA activator as a switch. The switch function of the DNA activators was also utilized to provide systems and methods for multiplexed and simultaneous detection of different DNA and RNA targets. The systems and methods of the present disclosure can also be coupled with Cas13b to perform multiplexed detection of different RNA targets using different types of reporter molecules. Some of the elements of the systems, methods, and kits of the present disclosure will be described in greater detail below.

Systems, compositions, and Methods for reverse transcription-free CRISPR-based detection of RNA

Embodiments of the present disclosure provides a Reverse Transcription-free method for detecting RNA with Cas12a. The systems and methods of the present disclosure can also

detect DNA targets as well as both DNA and RNA targets and/or two different DNA or RNA targets.

Detection of the target sequence is done by using a split-activator system in which a short single or double stranded DNA sequence or “activator” is exogenously supplied to bind to the seed region of the crRNA, the PAM-proximal region, nearer the scaffold region of the crRNA. The target polynucleotide of interest is detected by designing the 3'-end, or PAM-distal end, of the crRNA to bind to the target. As demonstrated in the Example below, several variants of Cas12a demonstrate trans cleavage activity when both the exogenously supplied short DNA activator as well as a polynucleotide of interest, including RNA targets, bind to the crRNA simultaneously. This system/method thus allows RNA detection directly without any reverse transcription step.

This approach can be applied to detect any single-stranded RNA target such as HIV, SARS-CoV-2, microRNAs, mRNAs etc. The variations of microRNA (miRNA) expression can be valuable biomarkers in disease diagnosis and prognosis. However, current miRNA detection techniques mainly rely on reverse transcription, which is time consuming, contamination prone, and susceptible to sample loss. The methods and systems of the present disclosure can help overcome these limitations.

In some embodiments, patient samples or cells containing the target polynucleotide of interest can be lysed to extract their genomic content. This nucleic acid extract can then be subjected to a CRISPR/Cas12a based trans cleavage assay consisting of a Cas12a enzyme, fluorescent reporter molecules (“probes”), a short DNA activator molecule, and a crRNA that partially binds to the target and partially binds to the short DNA molecule that is exogenously supplied. The presence or absence of the target will determine whether trans-cleavage-based fluorescence increases in the sample is observed or not. The intensity of fluorescence in the CRISPR assay will be used to provide a diagnosis. Instead of fluorescence, a lateral flow strip-based assay can also be used, and such variations will be appreciated by one of skill in the art.

Thus, in embodiments, systems of the present disclosure can include the following elements: a Cas12a enzyme, probes (e.g., reporter molecules) that produce a detectable signal upon cleavage, a crRNA designed to partially bind to the target of interest at the PAM-distal region of the crRNA, and an engineered DNA activator molecule capable of binding with a PAM-proximal region of the crRNA, such that when the DNA activator molecule binds the crRNA and the crRNA binds the target polynucleotide in a sample, this crRNA/activator/target complex is able to form an activated CRISPR/Cas complex with the Cas12a enzyme to activate the trans cleavage activity of the Cas12a enzyme to cleave the probes to produce a signal indicating

presence of the target. Various components of the system and methods of the present disclosure are described below.

Cas 12a enzymes

CRISPR-associated (Cas) enzymes (also known as CRISPR effector protein) are enzymes which can bind to a guide RNA (sgRNA) and to a complementary target polynucleotide sequence, forming a CRISPR/Cas complex, and can cleave the target sequence (cis cleavage). Some Cas enzymes possess both cis- and trans-cleavage activity, where trans-cleavage activity is activated upon binding of the CRISPR/Cas complex with the target sequence. Activation of the trans cleavage activity allows cleavage of probes also included in the reaction mixture, such that the probes produce a detectable signal or molecule that indicates the presence of the target sequence.

The methods and systems of the present disclosure employ class II, type V RNA-guided endonucleases called Cas12a CRISPR-associated (Cas) enzymes. In embodiments, the Cas12a enzyme can be selected from Cas12a enzymes such as, but not limited to ArCas12a, AsCas12a, BfCas12a, BoCas12a, BsCas12a, CMacas12a, CmtCas12a, ErCas12a, FnCas12a, HkCas12a, LbCas12a, Lb2Cas12a, Lb5Cas12a, MbCas12a, Mb2Cas12a, Mb3Cas12a, MiCas12a, Pb2Cas12a, PcCas12a, PdCas12a, PrCas12a, PxCas12a, TsCas12a. In some embodiments the Cas12a enzymes of the methods and systems of the present disclosure include one or more of LbCas12a, ErCas12a, and/or AsCas12a. Variants of these Cas12a enzymes with similar or enhanced endonuclease activity may also be used in the methods of the present disclosure.

Guide RNA (crRNA)

The guide polynucleotide for Cas12a enzymes is a single guide RNA and does not require both an crRNA and tracrRNA, but is just a single crRNA that includes both a target binding ("variable" or "spacer" region) and a conserved scaffold region that is configured to complex with the Cas12a enzyme upon binding of a target sequence to form a CRISPR/Cas complex. In embodiments, the target polynucleotide can be a DNA or RNA associated with a specific condition/disease, such as cancer, genetic disease, or infectious agent (e.g., bacteria, virus, fungal, etc.). In embodiments, the target polynucleotide is a virus, such as, but not limited to SARS-CoV-2, HIV, HCV, Chagas, malaria, and the like. Thus, in embodiments the crRNA can be complimentary to and configured to bind a complimentary region on the target DNA or RNA of interest to detect the specific condition/disease, such as cancer, genetic disease, infection agent (e.g., bacteria, virus, etc.) and the like.

In the systems and methods of the present disclosure the crRNA's are designed such that the variable region has 2 different portions, a PAM-proximal end closer to the scaffold region and 5' end of the crRNA and a PAM-distal end closer to the 3' end of the crRNA. These two portions or regions of the crRNA are configured to bind different components of the system of the present disclosure. The PAM-distal end of the crRNA is configured to bind to the target polynucleotide. The PAM-proximal end of the crRNA is configured to bind to a DNA activator sequence. Activator sequences of the present disclosure will be described in greater detail below, but briefly these DNA activators are relatively short sequences of double stranded DNA (dsDNA) or single stranded DNA (ssDNA) configured to bind to the PAM-proximal end of the crRNA in order to "activate" it to be able to form a CRISPR/Cas complex with the Cas enzyme in the presence of the target polynucleotide. In embodiments of the present disclosure, the crRNA binds the target polynucleotide (DNA or RNA) at the PAM-distal end of the crRNA, but will not become fully active until binding of a DNA activator at the PAM-proximal end.

The typical length of a crRNA for complexing with a Cas12a enzyme is about 35 to 50 base pairs, but may be somewhat longer or shorter. In embodiments the length of the crRNA is about or more than about 20, 25, 30, 35, 40, 40, 50, 55, or 60 nucleotides (nt) in length, and in some embodiments the length of the crRNA is less than about 60, 60, 40, 30, 25, or 20 nucleotides in length. In embodiments of the present disclosure the mature crRNA is about 30 to 50 nt long, such as about 40-44 nt long. In embodiments, the length of the scaffold region is about 15 to 30 base pairs (e.g., about 19-21) and the length of the total variable region (PAM-distal and PAM proximal) is about 14 to 30 (e.g., about 20-24) base pairs. In embodiments of the present disclosure, the PAM-distal end of the crRNA that is configured to bind the target polynucleotide is from about 6 to 15 nts in length and is capable of binding short or long target polynucleotides. In embodiments, target polynucleotides can be about 6 nt or longer. In embodiments the PAM-distal end is designed to bind a DNA target polynucleotide (e.g., double or single-stranded DNA), and in other embodiments, the PAM-distal end is designed to bind an RNA target polynucleotide. Thus, in embodiments, the crRNA can bind and detect an RNA target polynucleotide without reverse transcription.

In embodiments, the PAM-proximal end of the crRNA that is configured to bind a DNA activator is from about 6 to 15 nt in length. In embodiments, when the target polynucleotides is DNA, a shorter activator (e.g., about 6 to 15 nt) can be used, and thus the PAM-proximal end of the crRNA configured to bind the activator can be from about 6 to 15 nt. In embodiments where the target polynucleotide is RNA, a slightly longer activator (e.g., about 10-15 nt) may be needed for activation of trans cleavage activity, and thus the PAM-proximal end of the crRNA

configured to bind the activator can be from about 10 to 15 nt. In some embodiments, the crRNA may also have a short DNA extension sequence at the 3' end as described in WO 2021/092519 A1, incorporated by reference herein. Additional optional features of the crRNAs of the present disclosure are provided in the Example, below.

Probe

As used herein, a "probe" refers to a polynucleotide-based molecule that can be cleaved by an activated CRISPR-associated (Cas) enzyme with a trans-cleavage activity to produce a detectable signal or a detectable molecule. A detectable signal may be any signal that can be detected using optical, fluorescent, chemiluminescent, electrochemical or other detection methods known in the art. The probe comprises an oligonucleotide element. In one embodiment, a first end of the oligonucleotide element in the probe is linked to a fluorophore; and a second end of the oligonucleotide element in the probe is linked to a quencher of the fluorophore. In one embodiment, the probe further comprises biotin. In some embodiments of the present disclosure, the probes are configured to be cleaved by a Cas12a enzyme of the present disclosure that is in an activated CRISPR/Cas complex (e.g., Cas12a complexed with a crRNA, target polynucleotide, and activator where needed), such that the detectable signal or molecule can be produced upon binding of the crRNA/Cas complex to the target polynucleotide.

Quenching of the fluorophore can occur as a result of the formation of a non-fluorescent complex between the fluorophore and another fluorophore or nonfluorescent molecule. This mechanism is known as ground state complex formation, static quenching, or contact quenching. Accordingly, the oligonucleotide element may be designed so that the fluorophore and quencher are in sufficient proximity for contact quenching to occur. Fluorophores and their cognate quenchers are known in the art and can be selected for this purpose by one having ordinary skill in the art.

Upon activation of the Cas12a enzyme disclosed herein (e.g., by recognition of the target sequence, binding of an activator, and formation of an active crRNA/Cas complex), the trans cleavage activity of the Cas12a enzyme is activated and the oligonucleotide-based probe is cleaved, thereby severing the proximity between the fluorophore and quencher needed to maintain the contact quenching effect. Accordingly, detection of the fluorophore may be used to determine the presence of a target molecule in a sample. In one embodiment, the fluorophore is selected from the group consisting of FITC, HEX and FAM, and the quencher is selected from the group consisting of BHQ1, BHQ2, MGBNFQ, and 3IABkFQ. In one embodiment, a first end of the oligonucleotide element in the probe is linked to a fluorophore; a second end of the oligonucleotide element in the probe is linked to a quencher; and the probe further comprises

biotin. In embodiments, the probe is selected from HEX-polyT-Quencher (HEX-FQ) and FAM-polyT-Quencher, which are shown in the examples below to work well in the one pot assays of the present disclosure. In one embodiment, a fluorophore-quencher probe is within the crRNA.

A detectable molecule may be any molecule that can be detected by methods known in the art. In one embodiment, the detectable molecule is one member of a binding pair and can be detected by binding to another member of the binding pair. Examples of binding pairs include, but are not limited to, antibody-antigen pairs, enzyme-substrate pairs, receptor-ligand pairs, and streptavidin-biotin.

In one embodiment, the oligonucleotide element in the probe is ssDNA, since Cas12a trans cleavage activity preferentially cuts ssDNA. Since Cas12 enzymes preferentially cleaves DNA with an A/T rich sequence, in embodiments the oligonucleotide element of the probe includes at least 55%, 60%, 65%, 70%, 75%, 80%, 90%, or 95% of A and/or T. In embodiments the oligonucleotide element of the probe is a ssDNA and is about 80% of A and/or T. In some embodiments the oligonucleotide element is TA-rich or TA-only, and is about 2-10 nucleotides in length. In one embodiment, the ssDNA of the oligonucleotide element of the probe consists of A and/or T. In one embodiment, the oligonucleotide element is TTATT. In some embodiments, the oligonucleotide element is primarily or only T “poly T”, and in some embodiments it is polyT and about 2-10 nucleotides in length. In embodiments it is an “8-mer poly T” (TTTTTTTT).

In one embodiment, the probe can be, but is not limited to a HEX-FQ reporter or a FAM-FQ reporter, such as HEX-TTATT-FQ or FAM-TTATT-FQ. In another embodiment, the probe comprises HEX or FAM-polyT-Quencher (HEX/FAM-FQ). In embodiments, the probe can also be and FITC probe or a Cy5 probe, such as, but not limited to FITC-polyT-Quencher and Cy5-PolyT-quencher.

In embodiments of methods and systems of the present disclosure, 2 or more types of probes might be provided, one configured to be cleaved by an activated Cas12a complex of the present disclosure and to produce a first detectable signal, and a second type of probe configured to be cleaved by a different Cas enzyme (e.g., a Cas13b enzyme) to produce a second detectable signal, where the first and second detectable signals are distinguishable.

Activators

The methods and systems of the present disclosure also include activators, short single or double-stranded DNA sequences configured to bind the crRNA along with another activator (split activators) or a target sequence to “activate” a crRNA/Cas complex. In embodiments the activator can be any DNA or RNA sequence complementary and capable of binding a portion of the spacer region of the crRNA, but as described in the Examples below, it was found that DNA

activators (double or single stranded DNA activators) configured to bind the PAM-proximal portion/end of the crRNA are especially effective for activating the CRISPR/Cas complex even when the PAM-distal end of the crRNA is bound to an RNA target. Thus, such DNA activators can provide CRISPR/Cas systems that can directly detect an RNA target without the need for a reverse transcription step.

Thus, in embodiments the activator is a DNA activator having a sequence configured to bind the PAM[proximal end of the crRNA, leaving the PAM-distal end of the crRNA to bind a target sequence, where the target sequence can be either an RNA sequence or a DNA sequence. In embodiments, the target sequence can be a second short activator sequence, such that the two activators bind to form a full activator (e.g., binding to all or most of the variable region of the crRNA), but in other embodiments the target sequence may be a sequence for detection in a sample, and thus may be a longer RNA or DNA sequence.

In embodiments of the present disclosure the DNA activator can be double stranded DNA or single stranded DNA. If the activator is dsDNA, the activator includes a PAM sequence on the non-target strand, but since a ssDNA does not have a non-target strand, a PAM sequence is not needed. Thus the activators of the present disclosure can also provide for PAM-less target binding and activation of trans-cleavage activity of Cas12a. In embodiments the PAM sequence is a short T-A rich sequence. In embodiments, the PAM is 4 nucleotides and comprises A and T. In embodiments, the PAM sequence is TTTV, where V is a nucleotide selected from A, C and G (e.g., TTTA, TTTC, TTTCG).

In embodiments the activator is about 6 to 15 nucleotides in length. Since the total variable/spacer region of a crRNA for Cas12a can be about 14-30 nt long, typically 20-24 nucleotides in length, typically a DNA activator for binding the PAM proximal region of a crRNA (and leaving room on the crRNA for a target binding PAM-distal region) is about 6-15 nt in length, though longer or shorter sequences may be possible. In embodiments, if the target polynucleotide is an RNA, a slightly longer activator and corresponding PAM-proximal region are needed. Thus, in embodiments where the target polynucleotide is RNA, the DNA activator (and corresponding PAM-proximal region of the crRNA) can be about 10 to 15 nt in length. For target sequences that are DNA, a slightly shorter activator and PAM-proximal region can be effective; thus, in embodiments where the target polynucleotide is DNA, the DNA activator (and corresponding PAM-proximal region of the crRNA) can be about 6 to 15 nucleotides in length.

When the DNA activator binds to the crRNA and the other portion of the crRNA binds a target, this activates formation of an activated CRISPR/Cas complex to enable trans cleavage activity of the Cas12a enzyme to cleave probes to generate a detectable signal, indicating the

presence of the target sequence. In some embodiments, multiple different activators may be provided, each configured to bind a different crRNA to allow for detection of multiple targets.

Methods for detecting target polynucleotides

Methods of the present disclosure employ Cas12a Cas enzymes, engineered crRNAs, DNA activators, and probes for detection of polynucleotide targets in a sample, including detection of RNA targets without the need for reverse transcription and PAM-less detection of ssDNA targets.

Embodiments of methods of detecting a polynucleotide include combining a sample (e.g., in a reaction vessel) with a Cas12a enzyme, a crRNA having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide. The method further includes combining with the Cas12 enzyme and the crRNA, a plurality of probes of the present disclosure, where each probe has an oligonucleotide element configured to be cleaved by the activated Cas12a enzyme upon formation of an activated CRISPR/Cas complex to generate a CRISPR-generated detectable signal or detectable molecule. These components can be combined in a reaction vessel or other reaction substrate and incubated before or after addition of DNA activator of the present disclosure to the reaction mixture. As described above, the DNA activator is a single or double stranded DNA sequence configured to bind the PAM-proximal end of the crRNA such that an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide. The DNA activator can be added after the other components and act as a molecular “switch” to turn on the CRISPR-mediated detection. The method further includes detecting the CRISPR-generated detectable signal or detectable molecule if the target is present in the sample.

As described in the examples below, the activator-mediated detection of targets was more robust upon addition of magnesium (Mg^{2+}) ions. Thus, some embodiments include adding Mg^{2+} ions to the reaction mixture. In embodiments, the concentration of Mg^{2+} is about 1.5 to 30 mM. In embodiments, the method includes maintaining the pH of the reaction is maintained at about 5.5-9, such as by addition of a reaction buffer. Another advantage of the methods of the present disclosure is that detection is robust at room temperature, so heating is not required. In embodiments, the reaction vessel is incubated/maintained at about room temperature. In embodiments the temperature of the reaction is conducted at a temperature of about 20-45 °C.

In some embodiments the methods include detecting a target RNA sequence without reverse transcription prior to detection. In other embodiments, the target polynucleotide is a single stranded DNA sequence and does not include a PAM sequence.

The methods can include various DNA activators of the present disclosure as described above. For instance, in methods where the DNA activator is a single stranded DNA sequence, the activator does not include a PAM sequence, but in methods where the DNA activator is a double stranded DNA sequence, the activator includes a PAM sequence on the non-target strand. In embodiments, the PAM sequence comprises at least 75% A and T. In embodiments, the PAM sequence is TTTV, where V is A, G, or C (e.g., TTTA, TTTG, TTTC). As described above, the DNA activator can be from about 6 to 15 nt in length. In embodiments for detecting a target RNA, the DNA activator is about 10 nt or more, and in embodiments for detecting a target DNA, the DNA activator is about 6 nt or more.

It was also found in the Example that a fairly low concentration of DNA activator could activate Cas12a mediated trans cleavage in the presence of the target sequence. In some embodiments, the concentration of DNA activator is about 50 pM or more.

Methods of the present disclosure also include methods for detecting multiple target polynucleotides in the same sample or at the same time by utilizing more than one target-specific crRNA corresponding to each different target polynucleotide. Each different crRNA has a specific PAM-distal end configured to bind a specific target. In such embodiments, as long as the PAM-distal end of each different type of crRNA is specific for its corresponding target, the PAM-proximal end of the crRNAs can be the same, and the same activators can be used for detection of each target polynucleotide. In this manner, different crRNA's specific for a different target polynucleotide could each be placed in a different reaction well, along with probes and sample, then the same activator can be added to each reaction well, and detection of the signal would indicate the presence of the target corresponding with the specific crRNA. However, in other embodiments, each crRNA can also be designed to have different sequences in the PAM-proximal end that are each bound by a specifically designed DNA activator, such that each target polynucleotide has a different set of crRNAs and activators. There are advantages to each approach. The above methods can be used to simultaneously detect two or more different targets, where the targets can RNA, DNA or a combination of DNA targets and RNA targets.

In other methods of the present disclosure, the activator-mediated detection of the present disclosure can be used for multiplexed detection of different targets using different Cas systems. For instance, two different sequences can be detected by using the Cas12a enzyme, split-activator approach of the present disclosure, along with a Cas13b detection approach. For

instance methods of the present disclosure for multiplexed detection of two different RNA target polynucleotides can include a first crRNA of the present disclosure having a PAM-distal end configured to bind a first RNA target, with a DNA activator of the present disclosure configured to bind the PAM-proximal end of the crRNA to produce an activated CRISPR/Cas complex with Cas12a in the presence of the first RNA target and to cleave a first set of probes configured to be cleaved by the Cas12a enzyme. The method then further includes adding a second crRNA configured to bind a second RNA target and form a complex with a Cas13b enzyme and adding a second plurality of probes configured to be cleaved by the Cas13b enzyme upon formation of an activated CRISPR/Cas complex and generate a second detectable signal or molecule that is distinguishable from the first. Such methods also include detecting a signal where detecting the first detectable signal indicates the presence of the first target RNA, detecting the second detectable signal indicates the presence of the second target RNA, and detecting both the first and second detectable signal indicates the presence of both targets. In other methods, the first crRNA can be configured to detect a DNA target and complex with the DNA activator and Cas12a, while the second crRNA can be configured to detect an RNA target and complex with the Cas12b enzyme. Various combinations can be provided within the scope of the methods of the present disclosure.

Methods of the present disclosure also include methods for detecting and/or distinguishing mutant variants of a target polynucleotide, such as single point mutations. As described in the Example below, it was found that the split activator approach of the present disclosure was sensitive to mutations (e.g., point mutations) that occurred on the target sequence in positions corresponding to positions in the region of the crRNA that are located near the “split” between the activator-binding PAM-proximal region of the crRNA and the target-binding PAM-distal region of the crRNA. This region of about 1-4 nucleotides that bridge the split between the PAM-proximal region and PAM-distal region (e.g., 1-4 nt at the 5' end of the PAM-distal region and possibly 1-2 nt at the 3' end of the PAM-proximal region) are referred to herein as the “bridge region” of the crRNA. If a target polynucleotide has a point mutation that corresponds to a nucleotide in the bridge region, the crRNA is sensitive to this mutation and will not bind the target as well and thus not activate the CRISPR/Cas complex and trans cleavage activity, resulting in lower signal. Thus, crRNAs can be designed such that the PAM-distal end that binds the target polynucleotide corresponds to a region of the target polynucleotide suspected of being a site of point mutations. The crRNA is designed such the site of suspected mutation in the target strand corresponds to the nucleotides in the bridge region of the crRNA (e.g., near the 5' end of the PAM-distal region) such that a target with point mutations in the

target strand can be distinguished from a wild type target by detecting a change in trans cleavage activity (as determined by probe signal).

As described in the Example, a point mutation on the target strand in a position that binds the PAM distal end of the crRNA within a few nucleotides of the interface between the target-binding PAM-distal end and the activator binding PAM-proximal end, was demonstrated to result in significantly reduced trans-cleavage activity. Thus, with mutations in these positions, the crRNA/activator/target complex either could not be formed and recruit the CAS12a for formation of an active CRISPR/Cas complex or could not function sufficiently to generate a robust signal. Thus, methods of the present disclosure can be used to detect mutant variants of a target sequence by detecting a loss of trans cleavage activity when a target has a mutation of one or more nucleotides in the target polynucleotide in a position corresponding to a nucleotides in the crRNA bridge region.

Systems for detecting target polynucleotides

The present disclosure also provides systems for detecting target polynucleotides in a sample using the Cas12a enzymes, crRNAs, DNA activators, and probes of the present disclosure. In embodiments, the systems of the present disclosure can be used to carry out the methods describe above for CRISPR/Cas mediated detection of one or more target polynucleotides in a sample, including detection of RNA without the need for reverse transcription.

Embodiments of systems for detecting a target polynucleotide in a sample include the following components: a Cas12a enzyme, a crRNA having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, where the PAM-distal end of the crRNA is configured to bind to the target polynucleotide, a plurality of probes of the present disclosure each configured to be cleaved by the Cas23a enzyme upon formation of an activated CRISPR/Cas complex to generate a CRISPR-generated detectable signal or molecule, and a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA such that an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide. In embodiments, the activator can be either a single stranded or double stranded DNA sequence. As described above, in embodiments where the DNA activator is a ssDNA, it does not include a PAM sequence, and in embodiments where the DNA activator is a dsDNA it includes a PAM sequence on the non-binding strand. In embodiments the PAM sequence is T-A rich, in embodiments the PAM sequence is TTTA.

Systems of the present disclosure can also include additives for optimal performance, such as Mg^{2+} ions, pH buffers and the like. In embodiments, the DNA activator can be about 6-24 nucleotides (nt) in length, where the activator is about 6 nt or more for use to detect a DNA target and about 10 nt or more for use to detect an RNA target.

Systems of the present disclosure can also include components for use in detecting multiple different target polynucleotides in the same sample. For instance, when the system includes a target-specific crRNA corresponding to each target polynucleotide, each crRNA has a specific PAM-distal end configured to bind a specific target. However, in such systems, each crRNA may have the same sequence at the PAM proximal end configured to bind the same DNA activator. While separate DNA activators can be designed for each crRNA, in many situations, it may be convenient to use the same activator, and use the activator as a switch to “turn on” detection by activating the CRISPR/Cas complex to produce a detectable signal with the PAM-distal end of the crRNA binds to the corresponding target polynucleotide. Thus, such systems can include multiple different crRNA's each specific for a different target.

Systems of the present disclosure for multiplexed detection of two or more RNA targets and/or a combination of RNA and DNA targets may also include both Cas12a and Cas13b enzymes along with two different sets probes with distinguishable detectable signals, where one set of probes has an oligonucleotide element configured to be cleaved by an activated cas12a enzyme and the other set of probes has an oligonucleotide element configured to be cleaved by an activated Cas13b enzyme. Such systems also include at least a first crRNA configured to bind a first target, a DNA activator, form an activated complex with Cas12a and cleave a first set of probes, and a second crRNA configured to bind a second target, form an activated complex with Cas13b, and cleave a second set of probes. Wherein the first set of probes and second set of probes produce distinguishable detectable signals. In embodiments the first crRNA is configured to bind a DNA target or an RNA target and the second crRNA is configured to bind an RNA target.

The elements of the system of the present disclosure can also be included in commercial kits with instructions for use to detect a target. For instance, such kits may include the system components described above and instructions for combining the crRNA, and probes with a sample and DNA activators. The instructions may also include instructions relating to temperature, pH and the addition of metal ions to modulate performance. Some kits of the present disclosure may have the system components included in a packaging with container, test strips and the like with instructions for collecting and adding a sample, as well as

instructions for incubation and detection. Some variations will be understood by those of skill in the art.

Additional details regarding the methods, compositions, systems, and articles of the present disclosure are provided in the example and figures. The specific examples below are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present disclosure to its fullest extent.

It should be emphasized that the embodiments of the present disclosure, particularly, any “preferred” embodiments, are merely possible examples of the implementations, merely set forth for a clear understanding of the principles of the disclosure. Many variations and modifications may be made to the above-described embodiment(s) of the disclosure without departing substantially from the spirit and principles of the disclosure. All such modifications and variations are intended to be included herein within the scope of this disclosure, and protected by the following claims.

Various aspects and embodiments of the present disclosure

The present disclosure further includes the following aspects and embodiments.

Aspect1: A method of detecting a target polynucleotide in a sample, the method comprising:

combining the sample in a reaction vessel with the following:

a Cas12a CRISPR-associated (Cas) enzyme;

a CRISPR RNA (crRNA) having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide;

a plurality of probes, each probe comprising an oligonucleotide element labeled with a detectable label, wherein the probe is configured to be cleaved by the Cas12a enzyme upon formation of an activated CRISPR/Cas complex thereby generating a CRISPR-generated detectable signal or detectable molecule; and

a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA, wherein the activator is a single stranded or double stranded DNA sequence, wherein an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide;

incubating the reaction vessel; and

detecting the CRISPR-generated detectable signal or detectable molecule if the target polynucleotide is present in the sample.

Aspect 2: The method of aspect 1, wherein the DNA activator is added to the reaction vessel after combining the sample, the crRNA and the plurality of probes.

Aspect 3: The method of aspect 1 or 2, further comprising adding Mg^{2+} ions to the reaction vessel.

Aspect 4: The method of aspect 3, wherein the concentration of Mg^{2+} ions is about 1.5 to 30 mM.

Aspect 5: The method of any of aspects 1-4, comprising buffering the pH of the contents of the reaction to a pH of about 5.5-9.

Aspect 6: The method of any of aspects 1-5, wherein the reaction vessel is incubated at about room temperature.

Aspect 7: The method of any of aspects 1-5, wherein the reaction vessel is incubated at a temperature of about 20 to 45°C.

Aspect 8: The method of any of aspects 1-7, wherein the target polynucleotide is an RNA sequence, and the method does not include reverse transcription prior to detection.

Aspect 9: The method of any of aspects 1-8, wherein the DNA activator is a single stranded DNA sequence and does not include a PAM sequence

Aspect 10: The method of any of aspects 1-8, wherein the DNA activator is a double stranded DNA sequence and includes a PAM sequence on the non-target strand.

Aspect 11: The method of any of aspects 1-10, wherein the concentration of DNA activator is about 50pM or more.

Aspect 12: The method of aspect 8, wherein the target polynucleotide is RNA having a length of about 6 nucleotides (nt) or more and the DNA activator is about 10 to 15 (nt) in length.

Aspect 13: The method of any of aspects 1-7 or 9-11, wherein the target polynucleotide is DNA having a length of about 6 nt or more, and the DNA activator is about 6 to 15 nt in length.

Aspect 14: The method of any of the foregoing aspects for detecting multiple different target polynucleotides in the same sample, further comprising adding a target-specific crRNA corresponding to each target polynucleotide, wherein each crRNA has a specific PAM distal end configured to bind a specific target and each crRNA having the same sequence at the PAM proximal end configured to bind the DNA activator, such that the binding of the activator to the PAM proximal end of the crRNA acts as a switch to activate the CRISPR/Cas complex to produce a detectable signal with the PAM-distal end of the crRNA binds to the corresponding target polynucleotide.

Aspect 15: The method of any of the foregoing aspects for detecting a mutant variant of the target polynucleotide, the method comprising:

providing a crRNA having a sequence corresponding to portion of a target strain such that a single point mutation on the target polynucleotide is located at a position corresponding to about 1-4 nucleotides on the crRNA in a bridge region of the PAM-proximal end and the PAM-distal end of the crRNA; and

detecting a loss of trans cleavage activity when the target polynucleotide has a single point mutation at a position corresponding to a nucleotide in the crRNA bridge region.

Aspect 16: The method of any of the foregoing aspects for multiplexed detection of two different RNA target polynucleotides, wherein the PAM-distal end of the crRNA is configured to bind a first RNA target, the method further comprising adding to the reaction vessel:

a second crRNA configured to bind a second RNA target and to form a complex with a Cas13b enzyme,

a Cas13b enzyme; and

a second plurality of probes each comprising an oligonucleotide element labeled with a second detectable label, wherein the second probe is configured to be cleaved by the Cas13b enzyme upon formation of an activated CRISPR/Cas complex thereby generating a second CRISPR-generated detectable signal or detectable molecule that is distinguishable from the CRISPR-generated detectable signal or detectable molecule generated by the Cas12a enzyme

Aspect 17: A system for detecting a target polynucleotide in a sample, the system comprising:

a Cas12a CRISPR-associated (Cas) enzyme;

a CRISPR RNA (crRNA) having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide;

a plurality of probes, each probe comprising an oligonucleotide element labeled with a detectable label, wherein the probe is configured to be cleaved by the Cas12a enzyme upon formation of an activated CRISPR/Cas complex thereby generating a CRISPR-generated detectable signal or detectable molecule; and

a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA, wherein the activator is a single stranded or double stranded DNA sequence, wherein an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide.

Aspect 18: The system of aspect 17, wherein the DNA activator is a single stranded DNA sequence and does not include a PAM sequence.

Aspect 19: The system of aspect 17, wherein the DNA activator is a double stranded DNA sequence and includes a PAM sequence on the non-binding strand.

Aspect 20: The system of aspect 19, wherein the PAM sequence is ttvv.

Aspect 21: The system of any of aspects 17-20, further comprising Mg^{2+} ions.

Aspect 22: The system of any of aspects 17-21, wherein the target polynucleotide is RNA, and the DNA activator is about 10 to 24 nucleotides (nt) in length.

Aspect 23: The system of any of aspects 17-21, wherein the target polynucleotide is DNA, and the DNA activator is about 6 to 24 nt in length.

Aspect 24: The system of any of aspects 17-23 for detecting multiple different target polynucleotides in the same sample, wherein the system comprises a target-specific crRNA corresponding to each target polynucleotide, wherein each crRNA has a specific PAM distal end configured to bind a specific target and each crRNA having the same sequence at the PAM proximal end configured to bind the DNA activator, such that the binding of the activator to the PAM proximal end of the crRNA acts as a switch to activate the CRISPR/Cas complex to produce a detectable signal when the PAM-distal end of the crRNA binds to the corresponding target polynucleotide.

Aspect 25: A kit for detecting a target polynucleotide in a sample, the kit comprising: the system of any of aspect 17-24 and instructions for use.

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 to perform the methods and use the compositions and compounds disclosed herein. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, *etc.*), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C, and pressure is at or near atmospheric. Standard temperature and pressure are defined as 20 °C and 1 atmosphere.

It should be noted that ratios, concentrations, amounts, and other numerical data may be expressed herein in a range format. It is to be understood that such a range format is used for convenience and brevity, and thus, should be interpreted in a flexible manner to include not only the numerical values explicitly recited as the limits of the range, but also to include all the individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly recited. To illustrate, a concentration range of "about 0.1% to about 5%" should be interpreted to include not only the explicitly recited concentration of about

0.1 wt% to about 5 wt%, but also include individual concentrations (e.g., 1%, 2%, 3%, and 4%) and the sub-ranges (e.g., 0.5%, 1.1%, 2.2%, 3.3%, and 4.4%) within the indicated range. In an embodiment, the term “about” can include traditional rounding according to significant figures of the numerical value. In addition, the phrase “about ‘x’ to ‘y’” includes “about ‘x’ to about ‘y’”.

SEQUENCES:

The following list and tables provide sequences of nucleic acids and polypeptides described in the present disclosure and/or used in the Example.

Table 1: List of crRNA used in Example 1 (5'→3'). The spacer region of SAHARA crRNAs is colored to indicate the positions bound by S12 activators (single underline) and the target DNA or RNA (double underline):

Name	Sequence	SEQ ID NO:
crGFP-3'DNA7 (ENHANCE)	UAAUUUCUACUAAGUGUAGAUCUCAG GGCGGACUGGGUGCUTATTATT	1
crGFP-WT	UAAUUUCUACUAAGUGUAGAUCUCAG GGCGGACUGGGUGCU	2
crGFP-SH	UAAUUUCUACUAAGUGUAGAUGAUUA <u>GCAUUAACUCAGGGCGGAC</u>	3
cr155-Head-SH	UAAUUUCUACUAAGUGUAGAUCUCAG <u>GGCGGACGAUUAGCAUUA</u>	4
cr155-Tail-SH	UAAUUUCUACUAAGUGUAGAUCUCAG <u>GGCGGACACCCCUAUCAC</u>	5
cr155-Head-SH v2	UAAUUUCUACUAAGUGUAGAU <u>AUGGU</u> <u>GAGCAAGGAUUAGCAUUA</u>	6
crHCV-Head-SH	UAAUUUCUACUAAGUGUAGAUCUCAG <u>GGCGGACGUACCACAAGGC</u>	7
crHCV-Mid-SH	UAAUUUCUACUAAGUGUAGAUCUCAG <u>GGCGGACGAUGCACGGUCU</u>	8
crHCV-Tail-SH	UAAUUUCUACUAAGUGUAGAUCUCAG <u>GGCGGACGAGGUUUAGGAU</u>	9
crHCV-Tail-SH-25%GC	UAAUUUCUACUAAGUGUAGAU <u>AUCAU</u> <u>AUCUGAAGAGGUUUAGGAU</u>	10
crHCV-Tail-SH-33%GC	UAAUUUCUACUAAGUGUAGAU <u>AUCAG</u> <u>AUCUGAAGAGGUUUAGGAU</u>	11
crHCV-Tail-SH-50%GC	UAAUUUCUACUAAGUGUAGAUCUCAG <u>AUCGGAAGAGGUUUAGGAU</u>	12

Table 2: List of Target Activators used in Example 1 (5'→3').

Name	Sequence	SEQ ID NO:
GFP-20-nt	AGCACCCAGTCCGCCCTGAG	13
GFP-20-nt RNA	AGCACCCAGUCCGCCCUAG	14
GFP-Pp-6-nt	CCTGAG	
GFP-Pp-8-nt	GCCCTGAG	
GFP-Pp-10-nt	CCGCCCTGAG	15
GFP-Pp-12-nt	GTCCGCCCTGAG	16
GFP-Pp-14-nt	CAGTCCGCCCTGAG	17
GFP-Pp-16-nt	CCCAGTCCGCCCTGAG	18
GFP-Pp-18-nt	CACCCAGTCCGCCCTGAG	19
GFP-Pd-6-nt	AGCACC	
GFP-Pd-8-nt	AGCACCCA	
GFP-Pd-10-nt	AGCACCCAGT	20
GFP-Pd-12-nt	AGCACCCAGTCC	21
GFP-Pd-14-nt	AGCACCCAGTCCGC	22
GFP-Pd-16-nt	AGCACCCAGTCCGCCC	23
GFP-Pd-18-nt	AGCACCCAGTCCGCCCTG	24
GFP-Pp-dsDNA-10-nt	CCGCCCTGAGTAAAGCG (TS) GCGAAATGAGTCCCGCC (NTS)	25 26
GFP-Pd-dsDNA-10-nt	AGCACCCAGTTAAAGCG (TS) GCGAAATTGACCCACGA (NTS)	27 28
GFP-Pp-RNA-10-nt	CCGCCCUGAG	29
GFP-Pd-RNA-10-nt	AGCACCCAGU	30
GFP-Pp-RNA-12-nt	GUCCGCCCUAG	31

GFP-ssDNA-Pp-NTS-12-nt	CTCAGGGCGGAC	32
GFP-PS-ssDNA	GTCCGCCCTGAGTAAAGCG	33
GFP-PS-ssDNA NTS	CGCTTTACTCAGGGCGGAC	34
HCV polypeptide precursor RNA	GCCUUGUGGUACUGCCUGAUAGGGUGCUUG CGAGUGCCCCGGGAGGUCUCGUAGACCGUG CAUCAUGAGCACAAAUCCUAAACCUC	35
miRNA-155 target RNA	UUAAUGCUAAUCGUGAUAGGGGU	36
GFP-RNA 730-nt	GAGAGCCGCCACCAUGGUGAGCAAGGGCGA GGAGCUGUUCACCGGGGUGGUGCCCAUCCU GGUCGAGCUGGACGGCGACGUAAACGGCCA CAAGUUCAGCGUGUCCGGCGAGGGCGAGGG CGAUGCCACCUACGGCAAGCUGACCCUGAAG UUCAUCUGCACCACCGGCAAGCUGCCCGUGC CCUGGCCCACCCUCGUGACCACCCUGACCUA CGGCGUGCAGUGCUUCAGCCGCUACCCCGA CCACAUGAAGCAGCACGACUUCUUAAGUCC GCCAUGCCC GAAGGCUACGUCCAGGAGCGCA CCAUCUUCUUAAGGACGACGGCAACUACAA GACCCGCGCCGAGGUGAAGUUCGAGGGCGA CACCCUGGUGAACCGCAUCGAGCUGAAGGGC AUCGACUUAAGGAGGACGGCAACAUCUGG GGACAAGCUGGAGUACAACUACAACAGCCA CAACGUCUAUAUCAUGGCCGACAAGCAGAAG AACGGCAUCAAGGUGAACUUAAGAUCGCGC ACAACAUCGAGGACGGCAGCGUGCAGCUCGC CGACCACUACCAGCAGAACACCCCCAUCGGC GACGGCCCCGUGCUGCUGCCCGACAACCACU ACCUGAGCACCCAGUCCGCCUGAGCAAAGA CCCCAACGAGAAGCGCGAUCACAUGGUCCUG CUGGAGUUCGUGACCGCCGCCGGGAUCACU CUCGGCAUGGACGAGCUGUACAAG	37
HCV full-length WT	GAGTCCCGCCTGCTCCAAATCCTA	38
HCV SAHARA WT	CTCCAAATCCTA	39

Table 3: List of 'seed-region' binding S12-activators used in Example 1 (5'→3'):

Name	Sequence	SEQ ID NO:
S12 for crGFP-SH	TTAATGCTAATCTAAAGCG (TS) CGCTTTAGATTAGCATTAA (NTS)	40 41
S12 for: crHCV-Head-SH; crHCV-Tail-SH; crHCV- Mid-SH, cr155-Head-SH, cr155-Tail-SH	GTCCGCCCTGAGTAAAGCGA (TS) TCGCTTTACTCAGGGCGGAC (NTS)	42 43
S12 for cr155-Head v2 SH	CTTGCTCACCATTAAACAC (TS) GTGTTTAATGGTGAGCAAG (NTS)	44 45
S12 for crHCV-Tail 25%	TTCAGATATGATTAAACAC (TS) GTGTTTAATCATATCTGAA (NTS)	46 47
S12 for crHCV-Tail 33%	TTCAGATCTGATTAAACAC (TS) GTGTTTAATCAGATCTGAA (NTS)	48 49
S12 for crHCV-Tail 50%	TTCCGATCTGAGTAAACAC (TS) GTGTTTACTCAGATCGGAA (NTS)	50 51
S12 AAAT PAM	TTAATGCTAACATTTGCG (TS) CGCAAATGTTAGCATTAA (NTS)	52 53
S12 VVN PAM	TTAATGCTAACNBBBGCG (TS) CGCVVNGTTAGCATTAA (NTS)	54 55

Note: TS = "target strand"; NTS = "non-target strand"

Table 4: List of protein sequences used in Example 1:

Name (SEQ ID NO:)	Sequence
LbCas12a (SEQ ID NO: 56)	MSKLEKFTNCYSLSKTLRFKAIPVGKTQENIDNKRLLVEDEKRAEDYKGVKKLLDRY YLSFINDVLHSLKLNLYISLFRKKTRTEKENKELENLEINLRKEIAKAFKGNIEGYKS LFKKDIIETILPEFLDDKDEIALVNSFNGFTTAFTGFFDNRENMFSEEAKSTSIAPRCIN ENLTRYISNMDIFEKVD AIFDKHEVQEIKEKILNSDYDVEDFFEGEFFNFVLTQEGIDV YNAIIGGFVTESGEKIKGLNEYINLYNQKTKQKLPKFKPLYKQVLS DRESLSFYGEGY TSDEEVLEVFRNTLNKNSEIFSSIKKLEKLFKNFDEYSSAGIFVKNGPAISTISKDIFGE WNVIRDKWNAEYDDIHLKKKAVVTEKYEDDRRKSFKKIGSFSLEQLQEYADADLSV EKLKEIIQKVDEIYKVYGSSEKLFDAFVLEKSLKKNDVAVAIMKDLLDSVKSFENYIK AFFGEGKETNRDES FYGDFVLAYDILLKVDHIYDAIRNYVTQKPYSKDKFKLYFQNPQ FMGGWDKDKETDYRATILRYGSKYYLAIMDKKYAKCLQKIDKDDVNGNYEKINYKLL PGPNKMLPKVFFSCKWMAYYNPSEDIQKIYKNGTFKKGDMFNLNDCHKLIDFFKDSI SRYPKWSNAYDFNFSETEKYKDIAGFYREVVEEQGYKVSFESASKKEVDKLVVEEGKL YMFQIYNKDFSDKSHGTPNLHTMYFKLLFDENNHHGQIRLSGGAELFMRRASLKKEEL VVHPANSPIANKNPDNPKTTTTLSYDVYKDKRFSEDQYELHIPIAINKCPKNIFKINTE VRVLLKHDDNPYVIGIDRGERNLLYIVVVDGKGNIVEQYSLNEIINNFGIRIKTDYHSL LDKKEKERFEARQNWTSIENIKELKAGYISQVVKICELVEKYDAVIALEDLNSGFKN SRVKVEKQVYQKFEKMLIDKLNMYMVDKSNPCATGGALKGYQITNKFESFKSMSTQ NGFIFYIPAWLTSKIDPSTGFVNLLKTKYTSIADSKKFISSFDRIMYVPEEDLFEFALDY KNFSRTDADYIKWKLYSYGNRIRIFRNPKNNVFDWEEVCLTSAYKELFNKYGINY QQGDIRALLCEQSDKAFYSSFMALMSLMLQMRNSITGRTDVDFLISPVKNSDGIFYD SRNYEAQENAILPKNADANGAYNIARKVLWAIGQFKKAEDEKLDKVKIAISNKEWLEY AQTSVKH
AsCas12a (SEQ ID NO: 57)	MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQGFIEEDKARNDHYKELKPIIDRIYK TYADQCLQLVQLDWENLSAAIDSYRKEKTEETRNLALIEEQATYRNAIHDFIGRTDNL TDAINKRHAIEIYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYEN RKNVFS AEDISTAIPHRIQDNFPKFENCHIFTRLITAVPSLREHFENVKKAIGIFVST SIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTEKIKGLNEVLNLAIQKNDETAHII ASLPHRFIPLFKQILSDRNTLSFILEEFKSDEEVIQSFCKYKTLRNENVLETAEALFNE LNSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEKVQRSLKH EDINLQEIIISAAGKELSEAFKQKTSEILSHAHAAALDQPLPTTLKKQEEKEILKSQDLSLL GLYHLLDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFK LNFQMPTLASGWDVNKEKNNGAILFVKNGLYLGIMPKQKGRYKALSFEPTTEKTSE

	GFDKMYDYFPDAAKMIPKCSTQLKAVTAHFQTHHTTPILLSNNFIEPLEITKEIYDLNN PEKEPKKFQTAYAKKTGDQKGYREALCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQY KDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNKDFAKGHHGKPNLHT LYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQKTPIP DTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIHKDRRFTSDKFFFHVPITLN YQAANSPSKFNQRVNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSNLIQQF DYQKKLDNREKERVAAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVLENL NFGFKSKRTGIAEKAVYQQFEKMLIDKLNCLVLKDYPAEKVGGVLNPYQLTDQFTSF AKMGTQSGFLFYVPAPYTSKIDPLTGFVDPFVWKTIKNHESRKHFLEGFDLHYDVK TGDFILHFKMNRNLSFQRGLPGFMPAWDIVFEKNETQFDAKGTPIAGKRIVPVIEH RFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDSDHAIDTMVALIRSVLQMR NSNAATGEDYINSPVRDLNGVCFDSRFQNPWPMDADANGAYHIALKGQLLLNHLK ESKDLKLQNGISNQDWLAYIQELRN
ErCas12a (SEQ ID NO: 58)	NNGTNNFQNFQISSLQKTLRNALIPTETTQQFIVKNGIIEDELRGENRQILKDIMDDY YRGFISSETLSSIDDIDWTSLEFEKMEIQLKNGDNKDTLIKEQTEYRKAIHKKFANDDRFK NMFSAKLISDILPEFVIHNNNYSASEKEEKTQVIKLSRFATSFKDIFYKNRANCFSAD DISSSSCHRIVNDNAEIFFSNALVYRRIVKSLSNDDINKISGDMKDSLKEMSLEEIYSY EKYGEFITQEGISFYNDICGKVNSFMNLYCQKNKENKNLYKLQKLHKQILCIADTSYE VPYKFESDEEVYQSVNGFLDNISSKHIVERLRKIGDNYNGYNLDKIYIVSKFYESVSQ KTYRDWETINTALEIHYNILPGNGKSKADKVKKAVKNDLQKSITEINELVSNYKLCS DDNIKAETIHEISHILNNFEAQELKYNPEIHLVESELKASELKNVLDVIMNAFWCSV FMTEELVDKDNFYAELEEIYDEIYPVISLYNLVRNYVTQKPYSTKKIKLNFGIPTLAD GWSKSKEYSNNAILMRDNLYYLGIFNAKNKPDKKIIEGNTSENKGDYKKMIYNLLPG PNKMIPKVFLSSKTGVETYKPSAYILEGYKQNKHIKSSKDFDITFCHDLIDYFKNCIAIH PEWKNFGFDFSDTSTYEDISGFYREVELQGYKIDWTYISEKDIDLLQEKGQLYLFQIY NKDFSKKSTGNDNLHTMYLKNLFSEENLKDIVLKLNGEAEIFFRKSSIKNPIIHKKSIL VNRTYEAEEKDQFGNIQIVRKNIPENIQELYKYFNDKSDKELSDEAAKLKNVVGHH EAATNIVKDYRYTYDKYFLHMPITINFKANKTGFINDRILQYIAKEKDLHVIGIDRGERN LIYVSVIDTCGNIVEQKSFNIVNGYDYQIKLKQREGARQIARKEWKEIGKIKEIKEGYLS LVIHEISKMVIKYNAIIVMEDLSYGFKKGRFKVERQVYQKFETMLINKLNLYLVFKDISIT ENGGLLKGYQLTYIPDKLKNVGHQCGCIFYPAAAYTSKIDPTTGfVNIKFkdLTvDA KREFIKKFDSIRYDSEKNLFCFTFDYNNFITQNTVMSKSSWSVYTYGVRIKRRFVNG RFSNESDTIDITKDMEKTLEMTDINWRDGHDLRQDIIDYEIVQHIFEIFRLTVQMRNSL SELED RDYDRLISPVLNENNIFYDSAKAGDALPKDADANGAYCIALKGLYEIKQITEN WKEDGKFSRDKLKISNKDWFDFIQNKRYL

EXAMPLES

Now having described the embodiments of the disclosure, in general, the examples describe some additional embodiments. While embodiments of the present disclosure are described in connection with the example and the corresponding text and figures, there is no intent to limit embodiments of the disclosure to these descriptions. On the contrary, the intent is to cover all alternatives, modifications, and equivalents included within the spirit and scope of embodiments of the present disclosure.

EXAMPLE 1—Reverse Transcription-Free RNA Detection with CRISPR/Cas12a Using Split-Activators

Introduction

CRISPR-Cas12a RNA-guided complexes are widely utilized for diagnostic purposes through nucleic acid detection, which typically involves binding of a target sequence by crRNA and recruitment of the Cas enzyme for cleavage of DNA substrates. The present example demonstrates that while the PAM-proximal “seed” region of the crRNA exclusively recognizes DNA for initiating *trans-cleavage*, the PAM-distal region of the crRNA can tolerate both RNA and DNA substrates. By manipulating this property of CRISPR-Cas12a, the present example provides systems and methods to detect RNA target sequences at the PAM-distal region of the crRNA by merely supplying an “activator”, a short ssDNA or a PAM-containing dsDNA, to the seed region. In the present example this method is named Split Activators for Highly Accessible RNA Analysis or “SAHARA.” SAHARA allows reverse transcription-free detection of RNA with Cas12a.

The example also describes modulation of SAHARA by controlling Mg²⁺ concentration and pH. SAHARA was also demonstrated to work robustly at room temperature. SAHARA displays a significant improvement in the specificity for target recognition as compared to the WT CRISPR-Cas12a, at certain positions along the crRNA. We employed SAHARA to perform amplification-free detection of picomolar concentrations of miR-155 as well as Hepatitis C Virus RNA. Finally, the example shows that the DNA substrate binding to the ‘seed-region’ of the crRNA can be used as a switch to control the *trans-cleavage* activity of Cas12a for the detection of DNA and RNA substrates. This phenomenon further enables multiplexed detection of distinct DNA and RNA targets using a pool of crRNA/Cas12a complexes. SAHARA is a unique CRISPR-Cas-based detection system that can identify both DNA as well as RNA substrates in a multiplexed fashion without any additional steps.

Materials and Methods

Plasmid construction

Plasmids expressing Lb, As, and ErCas12a enzymes were constructed as described in Nguyen et.al⁴²(incorporated by reference herein). Briefly, plasmids expressing LbCas12a and AsCas12a were obtained from Addgene and directly used for protein expression. For ErCas12a, a plasmid containing the human codon-optimized Cas12a gene was obtained from Addgene, then was PCR amplified using Q5 Hot Start high fidelity DNA polymerase (New England Biolabs, Catalog #M0493S), and subcloned into a bacterial expression vector (Addgene plasmid #29656). The product plasmids were then transformed into Rosetta™(DE3) pLysS Competent Cells (Millipore Sigma, Catalog #70956) following the manufacturer's protocols.

Protein expression and purification:

For protein production, bacterial colonies containing the protein-expressing plasmid were plated on an agar plate and grown at 37°C overnight. Individual colonies were then picked and inoculated for 12 hours in 10 mL of LB Broth (Fisher Scientific, Catalog #BP9723-500). The culture was subsequently scaled up to a 1.5 mL TB broth mix and grown until the culture reached an OD = 0.6 to 0.8. The culture was then placed on ice before the addition of Isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was then continued to grow overnight at 16°C for 14-18 hours.

The overnight culture was pelleted by centrifuging at 10,000xg for 5 minutes. The cells were then resuspended in lysis buffer (500 mM NaCl, 50 mM Tris-HCl, pH = 7.5, 20 mM Imidazole, 0.5 mM TCEP, 1 mM PMSF, 0.25 mg/mL Lysozyme, and DNase I). The cell mixture was then subjected to sonication followed by centrifugation at 39800xg for 30 minutes. The cell lysate was filtered through a 0.22 μ m syringe filter (Cytiva, Catalog #9913-2504) and then run through into 5 ml HisTrap FF (Cytiva, Catalog #17525501, Ni²⁺ was stripped off and recharged with Co²⁺) pre-equilibrated with Wash Buffer A (500 mM NaCl, 50 mM Tris-HCl, pH = 7.5, 20 mM imidazole, 0.5 mM TCEP) connected to BioLogic DuoFlow™ FPLC system (Bio-rad). The column was eluted with Elution Buffer B (500 mM NaCl, 50 mM Tris-HCl, pH = 7.5, 250 mM imidazole, 0.5 mM TCEP). The eluted fractions were pooled together and transferred to a 10 kDa – 14 kDa MWCO dialysis bag. Homemade TEV protease (plasmid was obtained as a gift from David Waugh, Addgene #8827, and purified in-house)⁽⁴⁴⁾ was added to the bag, submerged in Dialysis Buffer (500 mM NaCl, 50 mM HEPES, pH 7, 5 mM MgCl₂, 2 mM DTT) and dialyzed at 4°C overnight.

The protein mixture was taken out of the dialysis bag and concentrated down to around 10 mL using a 30 kDa MWCO Vivaspin® 20 concentrator. The concentrate was then equilibrated with 10 mL of Wash Buffer C (150 mM NaCl, 50 mM HEPES, pH = 7, 0.5 mM TCEP) before injecting into 1 mL Hitrap Heparin HP column pre-equilibrated with Wash Buffer C operated in the BioLogic DuoFlow™ FPLC system (Bio-rad). The protein was eluted from the column by running a gradient flow rate that exchanges Wash Buffer C and Elution Buffer D (2000 mM NaCl, 50 mM HEPES, pH = 7, 0.5 mM TCEP). Depending on how pure the protein samples were, additional size-exclusion chromatography may have been needed. In short, the eluted protein from the previous step was run through a HiLoad® 16/600 Superdex® (Cytiva, Catalog #28989335). Eluted fractions with the highest protein purity were selected, pooled together, concentrated using a 30 kDa MWCO Vivaspin® 20 concentrator, snap-frozen in liquid nitrogen, and stored at -80°C until use.

Target DNA, RNA, and guide preparation:

All DNA and RNA oligos as well as the chimeric DNA/RNA hybrid crRNAs were obtained from Integrated DNA Technologies (IDT). Single-stranded oligos were diluted in 1xTE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). Complementary oligos for synthesizing dsDNA were first diluted in nuclease-free duplex buffer (30 mM HEPES, pH 7.5; 100 mM potassium acetate) and mixed in 1:5 molar ratio of target:non-target strand. Both strands were then subjected to denaturation at 95°C for 4 mins and gradient cooling at a rate of 0.1 °C/s to 25°C.

For generating the 730-nt long GFP target sequence, Addgene plasmid pCMV-T7-EGFP (BPK1098) (Addgene plasmid # 133962, a gift from Benjamin Kleinstiver) was obtained and PCR amplified using Q5 Hot Start high fidelity DNA polymerase (New England Biolabs, Catalog #M0493S) from position 376-1125. The PCR amplified product was *in-vitro* transcribed using the HiScribe T7 High Yield RNA synthesis kit (NEB #E2040S) following the manufacturer's protocol. The transcribed product was treated with DNase I for 30 min at 37°C and then purified using RNA Clean and Concentrator Kit (Zymo Research #R1016).

Preparation of metal ion buffers:

The different metal ion buffers were prepared by first creating a master mix of the following components: 50 mM NaCl, 10 mM Tris-HCl, and 100 µg/ml BSA. To this master mix, chloride salts of different monovalent, divalent, and trivalent cations (NH₄⁺, Rb⁺, Mg²⁺, Zn²⁺, Co²⁺, Cu²⁺, Ni²⁺, Ca²⁺, Mn²⁺, and Al³⁺) were diluted to a final concentration of 10 mM. The pH of the buffer was adjusted to 7.9 by adding 1M NaOH.

CRISPR-Cas12a reaction for fluorescence-based detection:

All fluorescence-based detection assays were carried out in a low-volume, flat-bottom, black 384 well-plate. The crRNA-Cas12a conjugates were assembled by mixing them in NEB 2.1 buffer and nuclease-free water followed by incubation at room temperature for 10 min. The assembled crRNA-Cas12a mixes were then added to 250-500 nM FQ reporter and the necessary concentration of the target activator in a 40- μ l reaction volume. The 384 well-plate was then incubated in a BioTek Synergy fluorescence plate reader at 37°C for 1 hour. Fluorescence intensity measurements for a FAM reporter were measured at the excitation/emission wavelengths of 483/20 nm and 530/20 nm every 2.5 min. A final concentration of 30 nM Cas12a, 60 nM crRNA, and 25 nM of target activator are used in all the assays unless otherwise specified.

Limit of detection calculation:

To find the limit of detection (LoD), the *trans-cleavage* assay was carried out with several different dilutions of the activator. The LoD calculations were based on the following formula⁴³:

$$LoD = \frac{3.3 \times Std\ of\ RFU\ in\ the\ absence\ of\ activator}{Slope\ of\ RFU\ vs.\ Activator\ concentration}$$

Results:

In contrast to Cas9, which uses two different active sites to generate a blunt double-stranded DNA break²⁵, Cas12a uses a single active site to make staggered cuts on the two strands of a dsDNA^{6,12,15}. After cleaving the target DNA, Cas12a releases the PAM-distal cleavage product while retaining the PAM-proximal cleavage product bound to the crRNA^{24,26}. This maintains Cas12a in a catalytically competent state, in which the active site of RuvC remains exposed to the solvent which then leads to *trans-cleavage* of neighboring single-stranded DNA molecules in a nonspecific manner. It has been shown that crRNA-target DNA hybrids of length 14-nt or less do not trigger any cis- or trans- cleavage activity, and a crRNA-DNA hybrid of at least 17-nt is typically for stable Cas12a binding and cleavage. These observations suggest that the interaction of Cas12a with crRNA-target DNA hybrid at positions 14-17nt influences initiation of cleavage^{24,27,28}.

In this Example, we demonstrate that Cas12a can also tolerate RNA substrates, and not just DNA, at the PAM-distal end of the crRNA, for initiating *trans-cleavage*. This observation highlights the possibility that Cas12a also accepts crRNA-RNA hybrids at positions 14-17 nt and not just crRNA-DNA. In essence, have found that while the PAM proximal seed region of the crRNA strictly tolerates DNA substrates, the PAM distal end of the crRNA can tolerate RNA

along with DNA substrates in multiple Cas12a orthologs. Thus, by supplying an activator in the form of a small piece of ssDNA or a PAM-containing dsDNA at the seed region of the crRNA we can detect RNA substrates at the non-seed region of the crRNA with multiple Cas12a orthologs. In this Example we refer to this new method and system as Split Activators for Highly Accessible RNA Analysis (SAHARA).

This Example describes achievement of RT-free detection of picomolar levels of DNA as well as RNA without amplification using SAHARA. We applied SAHARA for the detection of HCV and miRNA-155 (miR-155) RNA targets. We showed that compared to the conventional CRISPR-Cas12a, SAHARA has improved specificity as can be performed at room temperature, and its activity can be turned ON or OFF using the seed region binding DNA activator as a switch. We took advantage of this switch to perform multiplexed and simultaneous detection of different DNA and RNA targets. We also coupled SAHARA with Cas13b to perform multiplexed detection of different RNA targets using different types of reporter molecules. These key findings provided insights into the substrate requirements for the *trans-cleavage* activity of Cas12a, and have utilized them to develop SAHARA, a valuable and versatile tool that can simultaneously detect both DNA and RNA substrates.

LbCas12a, AsCas12a, and ErCas12a are orthologs of Cas12a nucleases that are derived from *Lachnospiraceae* bacterium ND2006, *Acidaminococcus* sp, and *Eubacterium rectale* and are simply referred to here as Lb, As, and Er, respectively²⁹⁻³². The mature crRNAs for each ortholog are 41-44 nt in length, each containing 19-21 nt of the scaffold sequence and the remaining 20-24 nt of the spacer⁶.

To test the effect of activator truncation on the *trans-cleavage* activity of each ortholog, we designed a crRNA targeting a short 20-nt ssDNA activator resembling green fluorescence protein (GFP). In earlier work, we demonstrated that modifying the crRNA by adding a short 7-nt DNA sequence at its 3'-end (termed ENHANCE crRNA) significantly boosts the *trans-cleavage* activity of LbCas12a¹⁸ (see also 2021/092519 A1, incorporated herein by reference). We therefore initially designed the ENHANCE version of the crRNA for this study. For this crRNA, we designed several short ssDNA target activators of lengths ranging from 6-20-nt that were complementary to either the PAM-proximal (Pp) seed region of the crRNA or the PAM-distal (Pd) end of the crRNA (FIG. 1A).

We performed *in vitro trans-cleavage* assays with varying lengths of activators with three different orthologs of Cas12a. We observed that the *trans-cleavage* activity is extremely sensitive to truncations of the ssDNA activators across the tested orthologs (FIGS. 1B-D). Compared to the full-length 20-nt activator, the *trans-cleavage* activity for a 16-nt activator

diminishes by as much as 50-70 fold, and activity is completely lost for shorter activators, <12-nt in length, in all three Cas12a orthologs, tested. Unlike LbCas12a and ErCas12a, AsCas12a also showed an infinitesimal *trans-cleavage* activity for the activator of length 14-nt. These results corroborate an earlier study showing that a crRNA-target DNA interaction longer than 14-nt is typically important to initiate the indiscriminate *trans-cleavage* activity of Cas12a²⁷.

Next, we tested to check if the simultaneous addition of two truncated split-activator sequences, binding to different regions of the crRNA and mimicking a full-length target would be able to regain the lost *trans-cleavage* activity. For this, we tested different truncated activators of length equal to or less than 14-nt in a combinatorial fashion (FIGS. 1 E-G). We were surprised to observe that while the individual truncated activators failed to trigger any *trans-cleavage* activity, a split-activator combination of two activators was able to partially regain the diminished activity provided that the combined length of both the activators was greater than or equal to 20-nt. We also observed that the longer the length of the activator binding to the PAM-proximal 'seed region' of the crRNA, the greater the recovery in the *trans-cleavage* activity of Cas12a. Thus, the activator combination of 14-nt (Pp) + 6-nt (Pd) shows higher activity than the combination of 6-nt (Pp) + 14-nt (Pd) even though the total length of both activators and the binding sequence in both cases is identical. These observations reinforce the idea that the seed region of the crRNA plays a role in modulating the *trans-cleavage* activity of Cas12a orthologs. Interestingly, for ErCas12a, a 12-nt Pp activator showed a higher activity across the board for different lengths of the Pd activators, indicating that the 12-nt truncated activator at the Pp region might be ideal for recovering *trans-cleavage* activity in ErCas12a.

After observing the *trans-cleavage* behavior of Cas12a orthologs towards truncated ssDNA activators added simultaneously, we investigated how sensitive the Pd and Pp regions were to RNA and dsDNA substrates. To test this, we designed 10-nt ssDNA, dsDNA, and RNA activators complementary to either the Pp or the Pd regions of the crRNA (FIG. 2A). Cas12a orthologs are known to require a protospacer-adjacent motif (PAM) sequence to be present at 5'-end of the non-target strand of a dsDNA target to initiate binding and cleavage^{6,24}. Therefore, we added a PAM sequence to the dsDNA activator binding at the Pp region. However, the dsDNA activator binding at the Pd region did not contain any PAM sequence.

We tested the detection of different ssDNA, dsDNA, and RNA activators in a combinatorial fashion (FIGS. 2 B-D). Firstly, upon switching from ssDNA to RNA at the Pp seed region, we observed the *trans-cleavage* activity to completely vanish for Lb and Er orthologs of Cas12a irrespective of the type of substrate that was supplied at the Pd. This demonstrates a strict DNA substrate requirement at the seed region for Lb and Er orthologs. On the contrary,

AsCas12a seemed to accept even RNA substrates at the Pp region, hinting at an underlying RNase activity that is distinct from other Cas12a orthologs. All three Cas12a were observed to tolerate RNA substrates at the Pd region of the crRNA, provided a short piece of DNA substrate is supplied at the Pp region. The DNA binding at the Pp can be ssDNA or dsDNA.

When the Pp activator is dsDNA, it was observed that the *trans-cleavage* activity is significantly boosted for As and Er with a PAM-containing dsDNA at the Pp region instead of a ssDNA (See FIGS. 8A-8D). In order to test the effect of the PAM sequence in double-stranded DNA binding at the Pp-end or Pd-end of the crRNA in a split-activator fashion, we designed PAM-containing as well as PAM-less (No PAM) double-stranded activator binding to either the Pp or the Pd (FIG. 8A). We then tested the detection of these double-stranded activators in a combinatorial fashion and observed activity (FIGS. 8B-8D). We observed the split activator system to have the best activity when a PAM-containing dsDNA binds at the Pp and the No-PAM dsDNA binds at the Pd. We also observed that there is no activity when a No-PAM dsDNA binds at the Pp-end, indicating that a PAM is crucial for dsDNA binding at the Pp end. AsCas12a was observed to have the most promiscuous activity and was able to activate a small amount of *trans-cleavage* even after binding to the short PAM-containing dsDNA activator supplied at Pp.

We also tested the effect of different lengths of PpDNA activator and Pd RNA target on the *trans cleavage* activity. To test the limits of the DNA and RNA activator binding at the Pp and Pd end of the crRNA, we designed multiple activators of different length ranging from 6nt-14nt and binding to either the Pp end (DNA activator) or the Pd end (RNA "target") of the crRNA. We tested these activators in a combinatorial fashion and observed that a combination of 12-nt DNA activator at the Pp end and 8-nt RNA target/activators at the Pd end works robustly across the three orthologs tested (FIG. 16A-16C). The RNA detection ability of SAHARA diminished if the length of the Pp binding DNA is reduced below 10-nt, suggesting that a minimum of 10-nt DNA binding near the PAM-proximal region facilitates the RNA detection activity of SAHARA.

We also wondered whether our observations above were influenced by the use of an ENHANCE crRNA. To test this, we compared the *trans-cleavage* activity of wild-type (WT) crRNA against an ENHANCE crRNA for the detection of a 10-nt ssDNA or ssRNA sequence at the Pd region, while a 10-nt PAM-containing dsDNA was supplied at the Pp end. It has been shown that crENHANCE increases collateral cleavage by up to 3.5 fold for LbCas12a but is variable with other Cas enzymes. While these ENHANCE extensions have been reported by our group to detect RNA as DNA/RNA heteroduplex, they have not been studied for direct detection

of ssRNA. Both ssDNA and ssRNA had a significantly greater activity with the WT crRNA for LbCas12a, while AsCas12a and ErCas12a were unaffected by the different crRNA designs. Of the three Cas proteins, we observed that both the WT as well as EN versions of ErCas12a highly exceeds its counterparts in RFU change, with nearly 5-fold the activity for ssRNA detection compared to LbCas12a (FIGS. 2F-H).

Chimeric DNA-RNA hybrid guides have been previously used to increase the sensitivity, and reduce the off-target effects for both Cas9 and Cas12 nucleases^{18,33,34}. We questioned if the use of a chimeric DNA-RNA hybrid guide RNA would help with the detection of split-activator sequences. To investigate this, we designed two chimeric crRNAs by changing either 12-nt at the Pp region of the crRNA to DNA (crRNA-12D8R) or 8-nt of the Pd region of the crRNA to DNA (crRNA-12R8D) (FIGS. 9A-G). However, our results indicated that the chimeric DNA-RNA hybrid guides did not perform as well as non-hybrid guides in the detection of split-activators (FIGS. 10A-D).

While it was clear that short RNA activators could be detected in a split-activator manner, this study aims to aid in the optimization of point-of-care detection for a variety of targets and lengths. Therefore, we designed two RNA targets: a short RNA of length 20-nt and a longer RNA of length ~730-nt that contained within it the same sequence as the 20-nt RNA (FIG. 3A). We designed two crRNAs to target both RNA fragments. The first crRNA was programmed to detect the full 20-nt of both activators and the other was designed to accommodate a 12-nt dsDNA in the PAM-proximal region (S12) and only 12-nt of the RNA target. The spacer regions of the aforementioned crRNAs were 20-nt and 24-nt respectively, with the latter being extended to 24-nt to leave room sterically for target binding.

Except for AsCas12a (FIG. 3C), which tends to have nonspecific collateral cleavage, it was observed that Cas12a proteins are unable to detect full-length RNA activators on their own. We also noted that AsCas12a exhibited a significant amount of *trans-cleavage* activity with only an S12 activator, once again revealing its greater tolerance to truncated activators (FIG. 3C). For Lb and Er, supplying an S12 piece robustly amplified detection of the short RNA activator compared to the WT (FIGS. 3B-D). Remarkably, ErCas12a had even greater detection for the long activator using SAHARA. Lb was also observed to have significant activity with the long activator, but still less so than for its detection of the 20-nt RNA oligonucleotide.

We observed that supplying a completely complimentary 12-nt dsDNA (S12) to the Pp region allows for the detection of RNA by Lb and Er. However, to confirm this observation, we tested a 12-nt scrambled dsDNA oligo (SR-Scr) and compared RNA detection with this, a complementary Pp dsDNA (SR-S12), and a WT system that included no dsDNA supplementary

sequence (FIGS. 3E-G). Similar to the comparison of the short and long RNA sequences, the WT trial included a 20-nt crRNA and detected a fully complementary 20-nt RNA activator, while both trials involving a 12-nt dsDNA utilized a 24-nt crRNA to allow for binding space. Through this design, it was confirmed that the presence of a 12-nt dsDNA complementary to the seed region allows for the detection of short RNA activators by Lb and Er. None of the Cas enzymes exhibited detection with a scrambled substitute, while As again had noticeable activity detecting a full-length RNA activator.

To validate SAHARA with clinically relevant RNA targets, we designed multiple crRNAs targeting Hepatitis C Virus (HCV) and miRNA-155. For HCV, we synthesized a short target RNA resembling a polypeptide precursor gene that is conserved across multiple HCV genotypes. For this RNA, we designed 3 different SAHARA guides each targeting it at either the 5'-end (Head), 3'-end (Tail), or in the middle (Mid) (FIG. 4A). The SAHARA guides were designed to target 12-nt of the HCV RNA at the PAM distal end of the crRNA and were complementary to a 12-nt S12 activator at the PAM proximal end.

The miRNA-155 (or miR-155) is known to play a crucial role in breast cancer progression and is overexpressed in breast cancer tissues^{38,39}. Therefore, reliable detection of miR-155 is important for the early diagnosis of breast cancer. The mature miR-155 is ~23-nt in length. We synthesized the mature microRNA target and designed two SAHARA guides targeting it at either 12-nt at the 5'-end or 11-nt at the 3'-end, keeping the seed region of both guides constant (FIG. 4D).

For both miR-155 and HCV targets, we first tested detection with all the designed guides individually. While all the designed guides for both targets were functional and displayed *trans-cleavage* activity against their respective targets, we observed that some guides exhibited a lot higher activity than others. For miR-155, the 5'-end targeting guide showed an almost 5-fold higher activity than its 3'-end targeting counterpart (FIG. 4E). Similarly, for HCV, the tail targeting guide showed as much as a 6-fold increase in activity as compared to head-targeting and the mid-targeting guide (FIG. 4B).

Upon closer inspection of the crRNAs with their corresponding targets, we observed that the secondary structure of the RNA target determines the activity of SAHARA. Targets with a high amount of secondary structure are more inaccessible to bind to the Cas12a-crRNA complex, and are therefore harder to detect, while targets with relatively low or no secondary structure are detected easily. For miRNA-155, the fact that the tail guide only binds to 11-nt of the target as opposed to 12-nt binding in the head guide might also be playing a role in reducing its activity.

It has previously been shown with Cas13 enzymes that pooling together multiple crRNAs, each targeting a different region of the RNA, enhances the level of detection⁴⁰. We rationalized that a similar approach will work with SAHARA. We, therefore, pooled together the different crRNAs for HCV and miRNA-155 and performed detection of each target with their respective pooled crRNAs. In concordance with the previous reports, SAHARA also displayed a higher activity with pooled crRNAs with both miRNA-155 and HCV targets (FIGS. 4C, 4E). Although, the increase in activity was higher for HCV, with three crRNAs pooled together, than for miRNA-155, with only 2 crRNAs pooled.

Finally, we tested to check the sensitivity of SAHARA. For this, we created dilutions of miRNA-155 and HCV targets ranging from 25 nM - 10 fM and tested for the detection of each target at different concentrations with their corresponding pooled crRNAs (FIGS. 4D, 4F). We obtained a limit of detection of 132 pM for HCV and 767 pM for the miRNA-155 RNA target. These results are in concurrence with earlier studies showing similar limits of amplification-free detection of ssDNA and dsDNA with Cas12a⁴¹.

We hypothesized that SAHARA might be more sensitive in discriminating single point mutations in the target as compared to the WT CRISPR-Cas12a since SAHARA binds to significantly fewer nucleotides of the target. To test this, we designed 12-nt single-stranded DNA activators with single-point mutations for detection with SAHARA, as well as 20-nt single-stranded DNA activators with identical mutations for detection of WT CRISPR-Cas12a for comparison. We observed that the detection of single-point mutants with both SAHARA and WT CRISPR was position-dependent compared to the WT target. We observed that mutations in positions M01-M06 significantly decreased the SAHARA-mediated *trans-cleavage* activity for Lb and Er (FIGS. 5A, 5C, 5D, 5F). The same was true with positions M01-M04 with As but not with positions M05 and M06 (FIGS. 5B, 5E). Mutations in positions beyond M06 did not decrease the *trans-cleavage* activity for either Cas12a ortholog. Surprisingly, however, mutations at positions M09 and M10 led to a dramatic increase in the *trans-cleavage* activity, even beyond that of the WT activator for all three Cas12a orthologs. This was interesting because Cas12a is known to induce cis-cleavage of the target strand between positions M09 and M10. While all three orthologs showed increased activity with mutants M09 and M10, LbCas12a showed the highest rise in the catalytic activity with an over a 2-3-fold increase in *trans-cleavage* as compared to the WT activator (FIGS. 5 H-J).

To compare SAHARA and WT CRISPR for the detection of single-point mutants, we normalized the *trans-cleavage* activity for all the mutant activators with the unmutated WT activator. Our data indicated that SAHARA had improved specificity over WT-CRISPR for

mutations at the position M01-M06 for Lb and Er, as well as M01-M04 for AsCas12a. This implied that mutations closer to the boundary of the split-activators had a larger effect on the *trans-cleavage* activity of SAHARA as compared to mutations away from the boundary.

To verify the PAM-dependency of SAHARA for initiating *trans-cleavage*, we designed three different S12 activators each containing either a TTTA, AAAT, or VVVN PAM respectively (FIG. 6A). TTTA is one of the canonical PAM for Cas12a, AAAT is the anti-PAM sequence, and VVVN encompasses the space of all the PAM sequences that are not tolerated by Cas12a. We tested the detection of a short 20-nt RNA with these S12 activators. As expected, the TTTA-PAM S12 was able to mediate RNA detection with all three Cas12a orthologs (FIGS. 7 B-D). Not surprisingly, upon changing the PAM from TTTA to AAAT or VVVN, the *trans-cleavage* activity was completely diminished for Lb and As. Interestingly, however, Er Cas12a was able to detect RNA even with an AAAT or VVVN PAM, hinting at a broader PAM tolerance for *trans-cleavage* by Er than previously reported.

Next, we conjectured whether the GC content of the S12 activator plays a role in the RNA detection activity of SAHARA. To check this, we designed different crRNAs and S12 activators consisting of GC content ranging from 25-75%. All 3 Cas12a orthologs were able to tolerate changes in the GC content for the activity of SAHARA (FIGS. 6E-G). This implies that Cas12a orthologs can tolerate a wide range of GC content for RNA detection.

Finally, we tested to check the minimum concentration of S12 needed to initiate RNA detection with SAHARA. For this, we varied the concentration of S12 from 0-1.5 nM in increasing amounts and tested for the detection of a 25 nM RNA target. We tested various concentrations of S12 activators on *trans cleavage* activity (FIGS. 6H-6J; FIG. 14; FIG. 15). To study the effect of varying S12 concentration on SAHARA, we tested S12 concentrations ranging from 50 nM – 780 pM (FIG. 14) and 1560 pM – 50 pM (FIG. 15) in two independent experiments. We observed SAHARA to have robust activity down to S12 concentration of 780 pM, below which the activity decreased with lower concentrations of S12. However, even at S12 concentration as low as 50 pM, we observed a small amount of *trans-cleavage* activity. We observed an increase in the *trans-cleavage* activity of different Cas12a orthologs with increasing S12 concentration thereby suggesting that the activity of SAHARA is S12-dependent (FIGS. 6H-6J). Surprisingly, S12 concentration as low as 50 pM concentration was sufficient to initiate *trans-cleavage* activity for a CRISPR-Cas12a complex consisting of 30 nM Cas12a and 60 nM crRNA (FIGS. 6H-6J; FIGS. 14 and 15). This suggests that a very low amount of S12 is needed to initiate *trans-cleavage* activity of SAHARA. Interestingly, we observed that in the absence of S12 there was no *trans-cleavage* despite the presence of crRNA, Cas12a, and target. This

suggested that S12 is critical for the initiation of *trans-cleavage* activity by Cas12a orthologs and can be used as a switch to selectively turn the activity ON or OFF.

We envisioned leveraging the switch-like function of the seed-binding S12 DNA activator to selectively turn ON the *trans-cleavage* activity of an individual crRNA from a pool of multiple different crRNAs. To investigate this, we used three different crRNAs (crRNA-a, crRNA-b, and crRNA-c, FIG. 7A), each having a unique target and S12, and pooled them together. To demonstrate that we can simultaneously detect DNA and RNA with SAHARA, we used an ssDNA target A for crRNA-a while targets B and C were ssRNAs. We then performed the detection of each of the three targets with the pooled crRNAs and different Cas12a orthologs, in a combinatorial fashion, in the presence or absence of the corresponding S12. We also used a control wherein no S12 sequence was supplied to the crRNA-Cas-target mix.

Unsurprisingly, in the no S12 control, there was no *trans-cleavage* activity with any of the target combinations despite the crRNA-Cas complex and the target being mixed, further reinforcing the idea that S12 is critical for activity with SAHARA. Amazingly, in the presence of different S12s, only the mixtures with the corresponding target and crRNA displayed *trans-cleavage*. For instance, in the presence of S12a, only the reactions where target A was available (A only, A+B, and A+B+C) were active, whereas the reactions without target A (B only, C only, or B+C) were inactive despite the Cas, crRNA-b, crRNA-c, and their respective targets being mixed (FIGS. 7B-D). The data here shows strong evidence that the S12 DNA can be used to selectively activate specific crRNAs and can be used to control the *trans-cleavage* activity of CRISPR-Cas12a, thereby enabling simultaneous and multiplexable detection of both DNA and RNA targets.

Next, we postulated if we could perform multiplexed RNA detection by combining SAHARA with Cas13b and by using distinct DNA and RNA reporters consisting of orthogonal dyes to differentiate the signal obtained from SAHARA and Cas13. This is similar to the multiplexed detection with Cas13 and Cas12 demonstrated before²¹, but here we aimed to detect RNA substrates with both Cas12 and Cas13, and not DNA. To test this, we used Lb, As, and Er Cas12a orthologs in conjunction with PsmCas13b to detect two distinct RNA targets (T1 and T2). We designed Cas12 and Cas13 guide RNAs such that the guide for Cas12 was complementary to only the T1 target while the guide for Cas13 was complementary to only T2. We also used a FAM-containing DNA reporter and a HEX-containing RNA reporter to distinguish the signal of SAHARA from the signal obtained by Cas13b (FIG. 7E).

Upon performing the detection of the T1 and T2 targets individually and in conjunction, we observed that SAHARA produced *trans-cleavage* activity only in the presence of the T1

target, while Cas13b produced *trans-cleavage* only in the presence of T2 (FIGS. 7F-G). Furthermore, the *trans-cleavage* signal obtained from SAHARA and Cas13b could be distinguished from each other by using orthogonal fluorescent dyes such as FAM and HEX on different types of reporter molecules. Thus, it is feasible to combine SAHARA with Cas13b for multiplexed detection of distinct RNA targets.

In order to determine if the need for a heat source could be eliminated, we wondered if SAHARA would also function at room temperature as opposed to 37°C. While the catalytic activity of Cas12a enzymes is optimal at 37°C, the short DNA activators that are used in the SAHARA system can bind in a more stable manner at temperatures lower than 37°C. Therefore, to study how the activity of SAHARA varies as a function of temperature, we tested the detection of a ssDNA target and an ssRNA target with SAHARA, each at temperatures of 37°C and Room Temperature (RT) (FIGS. 11A-11F).

We observed that both the ssDNA as well as ssRNA targets can be detected at room temperature with SAHARA. However, the activity drops by 3-5-fold depending on the Cas12a ortholog being used, with Lb and Er exhibiting higher activity at room temperature than As (FIGS. 11A-11F). Nevertheless, our data indicates that SAHARA can function at room-temperature and therefore can be utilized in point-of-care diagnostic applications wherein having a heat-block is inconvenient or cost-prohibitive.

We also tested the effect of different metal ions on the activity of SAHARA. Cas12a effectors are metal-dependent endonucleases; therefore, the type and concentration of metal ions used in the reaction can have a significant effect on their activity. While Mg²⁺ ions have been used in Cas12a based applications, Mn²⁺ ions have also been shown to work well. To study the effect of different metal ions on the activity of SAHARA, we tested a range of different metal cations and discovered that most metal ions including (Zn²⁺, Cu²⁺, Co²⁺, Mn²⁺ etc.) actually inhibited the *trans-cleavage* activity of SAHARA, and only the presence of Mg²⁺ ions displayed a robust activity with SAHARA (FIG. 12A).

Of the different divalent metal ions tested, since Mg²⁺ ions displayed the highest activity with SAHARA *in-vitro*, we further characterized the effect of increasing concentration of Mg²⁺ ions on the activity. By varying the amount of Mg²⁺ ions in the Cas12a reaction, we observed a significant increase in the activity of SAHARA with increasing concentration up to about 15 mM. Further increasing the Mg²⁺ concentration beyond 15 mM seemed to slightly decrease the activity, suggesting around 15 mM to be a useful concentration of Mg²⁺ for SAHARA (FIG. 12B).

The pH of a reaction can have a notable effect on the charge of the enzyme and can subsequently affect the activity. Thus, we screened the activity of SAHARA in different pH

buffers ranging from 5.5-9.25 for the three Cas12a orthologs (FIGS. 13A-13C). We observed that SAHARA has optimal activity at about pH = 7.9 for all three orthologs, and the activity drops rather sharply upon further increasing or decreasing the pH (FIG.13). There is no observed activity at pH = 9.25 for any of the 3 orthologs tested.

Discussion

Engineering enzymes to improve their functionality above and beyond what they are naturally capable of has been a long-standing goal of molecular biology. Often, the engineering is done through man-made intervention after careful structural and biochemical analyses. Rarely, and even more excitingly, novel properties of already well-characterized enzymes are discovered, putting them under a new spotlight and giving them a fresh perspective. While CRISPR-Cas systems have been studied for almost a decade, *trans-cleaving* Cas enzymes such as Cas12 and Cas13 are relatively new. Despite their novelty, tremendous progress is being made towards studying and understanding their underlying mechanism, especially due to their utility in molecular diagnostics. Cas12a, in particular, has been extensively studied and is widely used in diagnostic platforms such as DETECTR and ENHANCE. However, Cas12a-based diagnostic methods are limited to DNA detection, since RNA substrates are not innately tolerated by Cas12a.

Here we have discovered that substrate tolerance of Cas12a enzymes is position-dependent concerning the crRNA, and that RNA substrates can be used to turn on the *trans-cleavage* activity of Cas12a by binding at the PAM-distal end of the crRNA, as long as an engineered DNA activator is supplied at the PAM-proximal end.

We first show that truncating the length of ssDNA activators binding to the crRNA drastically diminishes the *trans-cleavage* activity of Lb, As, and Er Cas12a. However, while individual truncated activators fail to initiate *trans-cleavage*, supplying two truncated activators, each binding to a different region of the crRNA in a split-activator fashion, partially regains the lost activity. Furthermore, upon changing the truncated activators from ssDNA to dsDNA or RNA, we demonstrated that while the PAM-proximal seed region of the crRNA exclusively tolerates DNA for initiating *trans-cleavage*, the PAM-distal end of the crRNA could tolerate RNA along with DNA. The exception here was AsCas12a, which was able to tolerate RNA even at the PAM-proximal end, unlike Lb or Er Cas12a. We also observed that using a PAM-containing dsDNA at the Pp-end greatly increases the *trans-cleavage* as compared to using an ssDNA for two of the three Cas12a orthologs (As and Er).

Surprised by our results, we engineered a system to detect longer lengths of RNA sequences at the PAM-distal end of the crRNA. To enable this, we designed long crRNAs containing 24-nt of the spacer. Within this spacer, 12-nt at the Pp end was complementary to a PAM-containing dsDNA that we call S12, while 12-nt at the distal end was complementary to the target of interest. We named this split-activator-based method of RNA detection with Cas12a as SAHARA. Upon testing the detection of short and long RNA substrates with WT CRISPR-Cas12a and SAHARA, we observed that WT Cas12a could not detect RNA at all, or in the case of As, had a very small level of RNA detection. With SAHARA, however, all three cas12a orthologs displayed robust RNA detection activity for different lengths of RNA. ErCas12a was observed to have the strongest activity for the detection of long RNA sequences.

Furthermore, we found that the presence of a PAM sequence on a dsDNA S12 activator can generate robust *trans-cleavage* activity and that changing the PAM sequence eliminates the activity for Lb and As Cas12a while reducing it for Er. Changing the GC content of the S12 activators seemed to have a negligible effect on the activity of all three orthologs, suggesting that a wide range of GC content can be used for the S12 activator. Remarkably, we found that the S12 concentration as low as 50 pM was sufficient to trigger the *trans-cleavage* of a 30 nM crRNA-Cas complex for detecting a 25 nM target. These results indicated that very low levels of the S12 DNA binding to the crRNA are sufficient for detection, however, the fluorescence intensity output is increased at higher concentrations. Notably, in the absence of the S12, there is no *trans-cleavage* activity with any of the three Cas12a orthologs, implying that the S12 can be used as a switch to control the *trans-cleavage* of Cas12a.

SAHARA was applied for the detection of clinically relevant targets such as Hepatitis C virus (HCV) and miRNA-155. In doing so, we found that the secondary structure of the target plays an important role in the RNA detection activity of Cas12a. Increased secondary structure in the RNA target makes it more inaccessible to bind to the crRNA and reduces the activity. We also observed that pooling together multiple crRNAs targeting different regions of the activator enhances the detection capability. Using this approach, we were able to detect picomolar levels of both miRNA-155 as well as HCV with SAHARA. We also found SAHARA to be highly specific to mutations at certain positions along the target. Mutations at positions closer to the interface of the S12 DNA and the bound target seemed to be especially detrimental in inhibiting activity. Finally, we used the switch-like behavior of the S12 activator to selectively turn ON the *trans-cleavage* activity of a single crRNA from a pool of multiple different crRNAs each targeting a mixture of DNA and RNA substrates. We were also able to combine SAHARA with Cas13b for multiplexed detection of different RNA targets using DNA and RNA reporters containing

orthogonal fluorescent dyes. Thus, SAHARA can be used as a unique diagnostic system that can detect both DNA as well as RNA targets simultaneously and in a multiplexable fashion.

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CLAIMS

We claim at least the following:

1. A method of detecting a target polynucleotide in a sample, the method comprising:
 - combining the sample in a reaction vessel with the following:
 - a Cas12a CRISPR-associated (Cas) enzyme;
 - a CRISPR RNA (crRNA) having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide;
 - a plurality of probes, each probe comprising an oligonucleotide element labeled with a detectable label, wherein the probe is configured to be cleaved by the Cas12a enzyme upon formation of an activated CRISPR/Cas complex thereby generating a CRISPR-generated detectable signal or detectable molecule; and
 - a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA, wherein the activator is a single stranded or double stranded DNA sequence, wherein an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide;
 - incubating the reaction vessel; and
 - detecting the CRISPR-generated detectable signal or detectable molecule if the target polynucleotide is present in the sample.
2. The method of claim 1, wherein the DNA activator is added to the reaction vessel after combining the sample, the crRNA and the plurality of probes.
3. The method of claim 1 or 2, further comprising adding Mg^{2+} ions to the reaction vessel.
4. The method of claim 3, wherein the concentration of Mg^{2+} ions is about 1.5 to 30 mM.

5. The method of any of claims 1-4, comprising buffering the pH of the contents of the reaction to a pH of about 5.5-9.
6. The method of any of claims 1-5, wherein the reaction vessel is incubated at about room temperature.
7. The method of any of claims 1-5, wherein the reaction vessel is incubated at a temperature of about 20 to 45°C.
8. The method of any of claims 1-7, wherein the target polynucleotide is an RNA sequence, and the method does not include reverse transcription prior to detection.
9. The method of any of claims 1-8, wherein the DNA activator is a single stranded DNA sequence and does not include a PAM sequence.
10. The method of any of claims 1-8, wherein the DNA activator is a double stranded DNA sequence and includes a PAM sequence on the non-target strand.
11. The method of any of claims 1-10, wherein the concentration of DNA activator is about 50pM or more.
12. The method of claim 8, wherein the target polynucleotide is RNA having a length of about 6 nucleotides (nt) or more and the DNA activator is about 10 to 15 (nt) in length.
13. The method of any of claims 1-7 or 9-11, wherein the target polynucleotide is DNA having a length of about 6 nt or mor, and the DNA activator is about 6 to 15 nt in length.

14. The method of claim 1 for detecting multiple different target polynucleotides in the same sample, further comprising adding a target-specific crRNA corresponding to each target polynucleotide, wherein each crRNA has a specific PAM distal end configured to bind a specific target and each crRNA having the same sequence at the PAM proximal end configured to bind the DNA activator, such that the binding of the activator to the PAM proximal end of the crRNA acts as a switch to activate the CRISPR/Cas complex to produce a detectable signal with the PAM-distal end of the crRNA binds to the corresponding target polynucleotide.

15. The method of claim 1 for detecting a mutant variant of the target polynucleotide, the method comprising:

providing a crRNA having a sequence corresponding to portion of a target strain such that a single point mutation on the target polynucleotide is located at a position corresponding to about 1-4 nucleotides on the crRNA in a bridge region of the PAM-proximal end and the PAM-distal end of the crRNA; and

detecting a loss of trans cleavage activity when the target polynucleotide has a single point mutation at a position corresponding to a nucleotide in the crRNA bridge region.

16. The method of claim 1 for multiplexed detection of two different RNA target polynucleotides, wherein the PAM-distal end of the crRNA is configured to bind a first RNA target, the method further comprising adding to the reaction vessel:

a second crRNA configured to bind a second RNA target and to form a complex with a Cas13b enzyme,

a Cas13b enzyme; and

a second plurality of probes each comprising an oligonucleotide element labeled with a second detectable label, wherein the second probe is configured to be cleaved by the Cas13b enzyme upon formation of an activated CRISPR/Cas complex thereby generating a second CRISPR-generated detectable signal or detectable molecule that is distinguishable from the CRISPR-generated detectable signal or detectable molecule generated by the Cas12a enzyme.

17. A system for detecting a target polynucleotide in a sample, the system comprising:

a Cas12a CRISPR-associated (Cas) enzyme;

a CRISPR RNA (crRNA) having a polynucleotide sequence with a PAM-distal end and a PAM-proximal end, wherein the PAM-distal end of the crRNA is configured to bind to the target polynucleotide;

a plurality of probes, each probe comprising an oligonucleotide element labeled with a detectable label, wherein the probe is configured to be cleaved by the Cas12a enzyme upon formation of an activated CRISPR/Cas complex thereby generating a CRISPR-generated detectable signal or detectable molecule; and

a DNA activator having a sequence configured to bind the PAM-proximal end of the crRNA, wherein the activator is a single stranded or double stranded DNA sequence, wherein an activated CRISPR/Cas complex is formed upon binding of the DNA activator to the PAM-proximal end of the crRNA and binding of the PAM-distal end of the crRNA to the target polynucleotide.

18. The system of claim 17, wherein the DNA activator is a single stranded DNA sequence and does not include a PAM sequence.

19. The system of claim 17, wherein the DNA activator is a double stranded DNA sequence and includes a PAM sequence on the non-binding strand.

20. The system of claim 19, wherein the PAM sequence is ttv.

21. The system of any of claims 17-20, further comprising Mg^{2+} ions.

22. The system of any of claims 17-21, wherein the target polynucleotide is RNA, and the DNA activator is about 10 to 24 nucleotides (nt) in length.

23. The system of any of claims 17-21, wherein the target polynucleotide is DNA, and the DNA activator is about 6 to 24 nt in length.

24. The system of claim 17 for detecting multiple different target polynucleotides in the same sample, wherein the system comprises a target-specific crRNA corresponding to each target polynucleotide, wherein each crRNA has a specific PAM distal end configured to bind a specific target and each crRNA having the same sequence at the PAM proximal end configured to bind the DNA activator, such that the binding of the activator to the PAM proximal end of the crRNA acts as a switch to activate the CRISPR/Cas complex to produce a detectable signal when the PAM-distal end of the crRNA binds to the corresponding target polynucleotide.

25. A kit for detecting a target polynucleotide in a sample, the kit comprising:
the system of any of claim 17-24 and instructions for use.

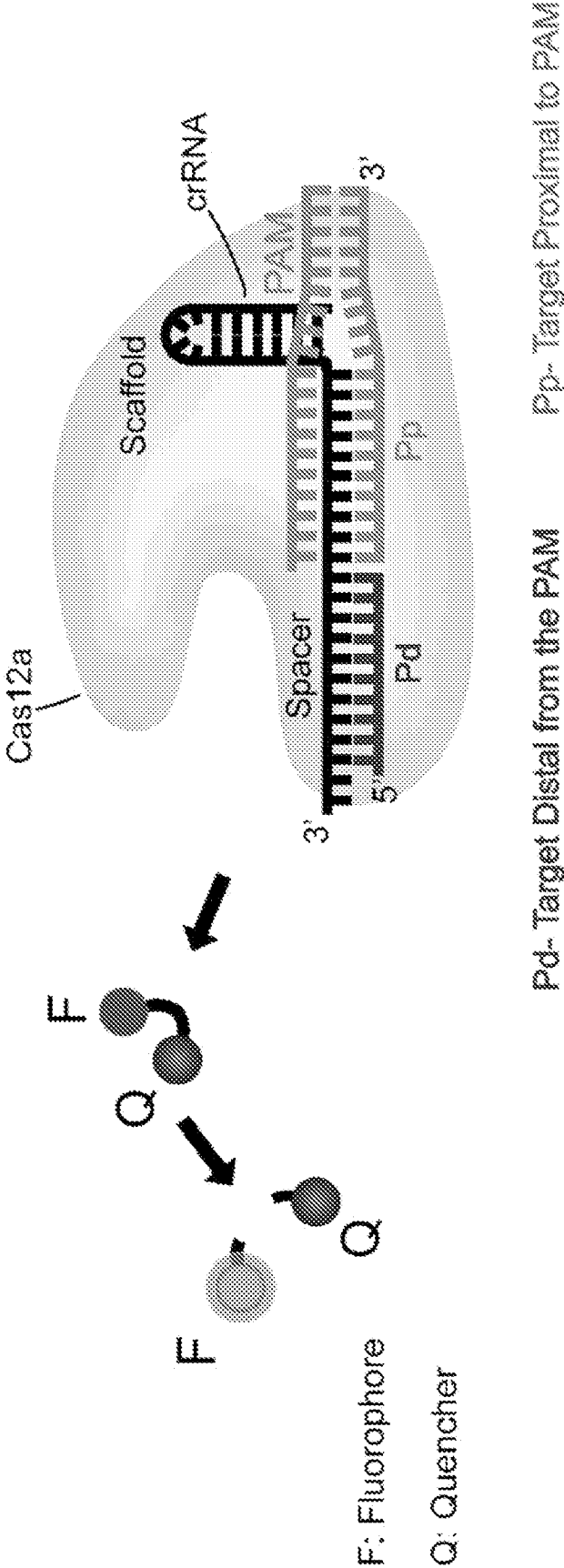
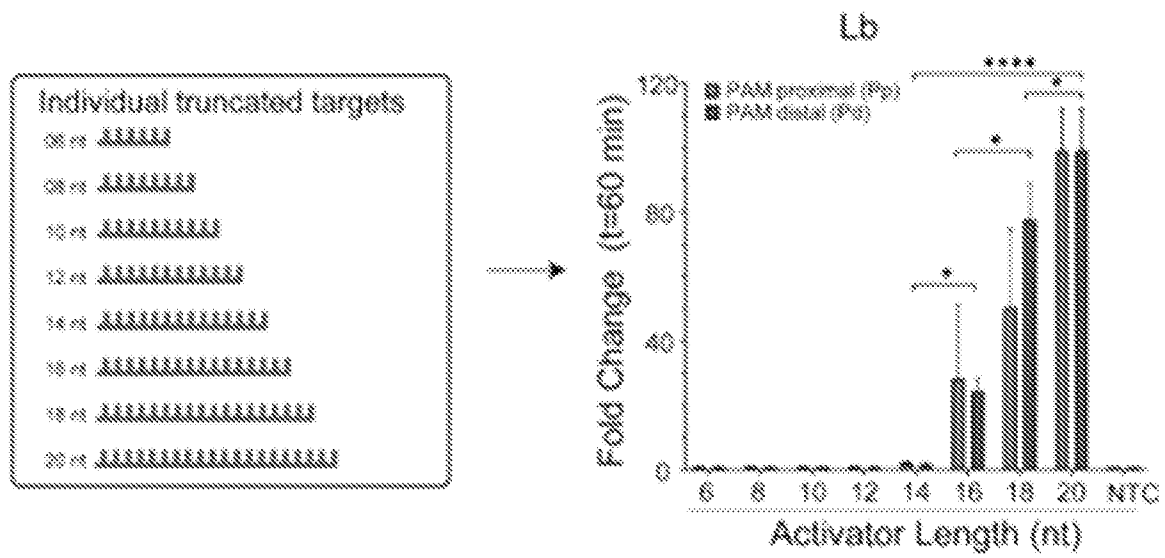
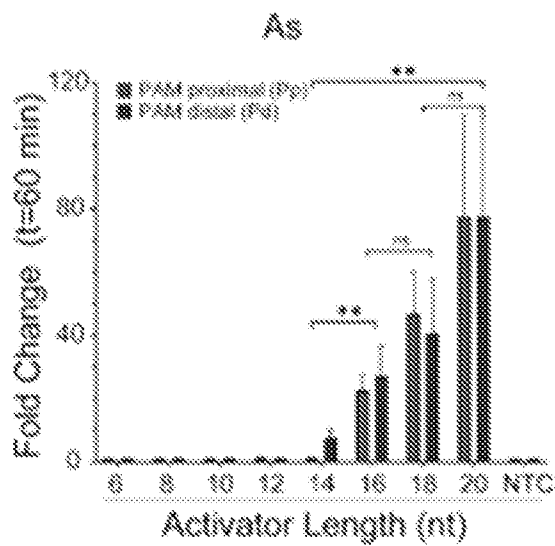
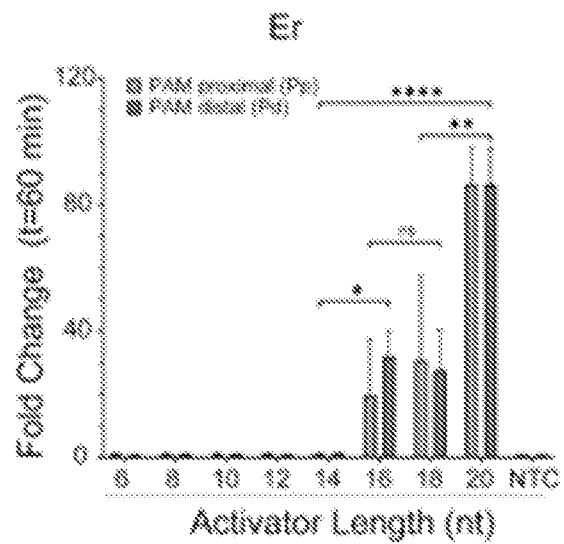
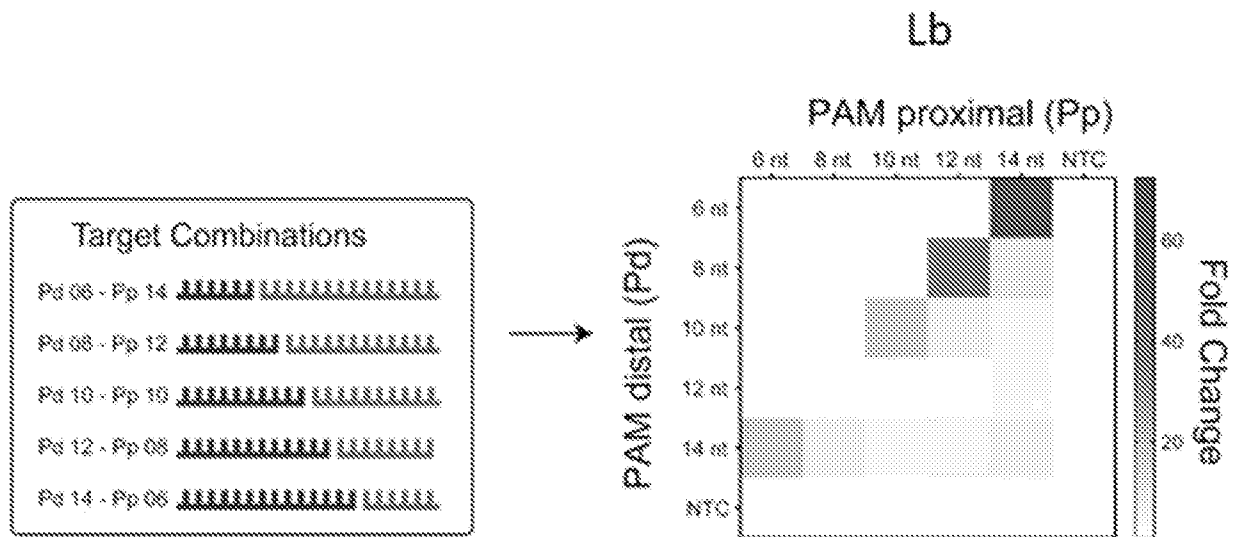
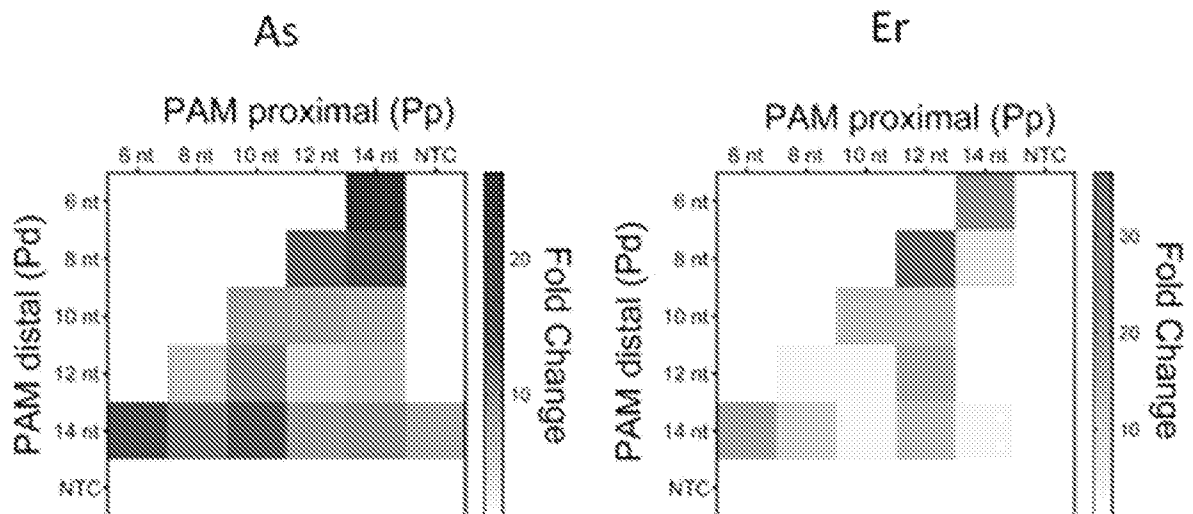


FIG. 1A

**FIG. 1B****FIG. 1C****FIG. 1D**

**FIG. 1E****FIG. 1F****FIG. 1G**

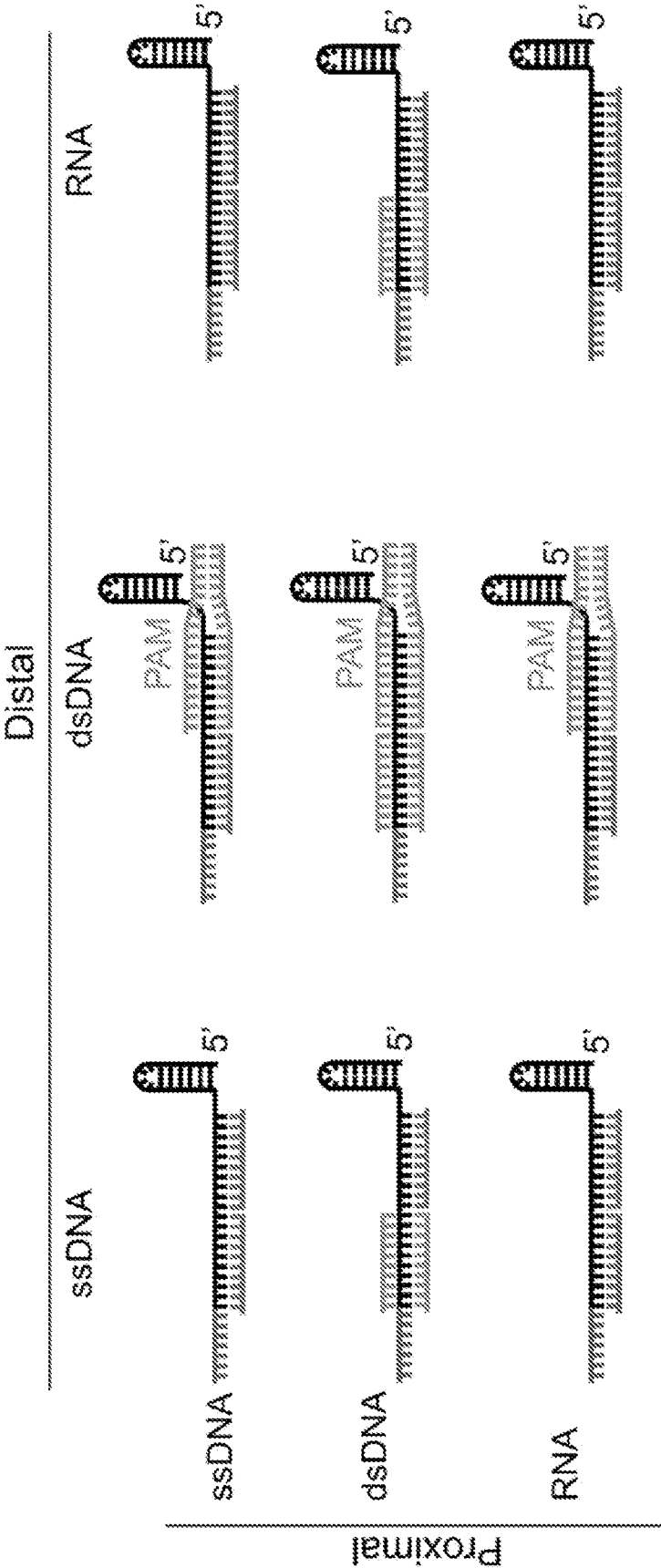


FIG. 2A

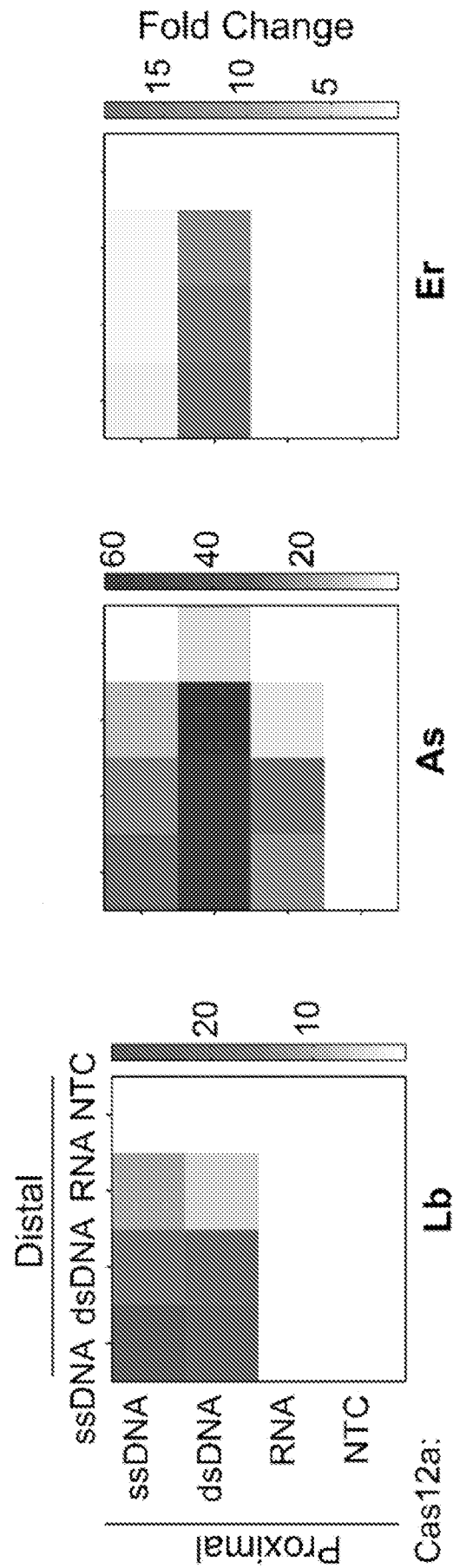


FIG. 2B

FIG. 2C

FIG. 2D

FIG. 2E

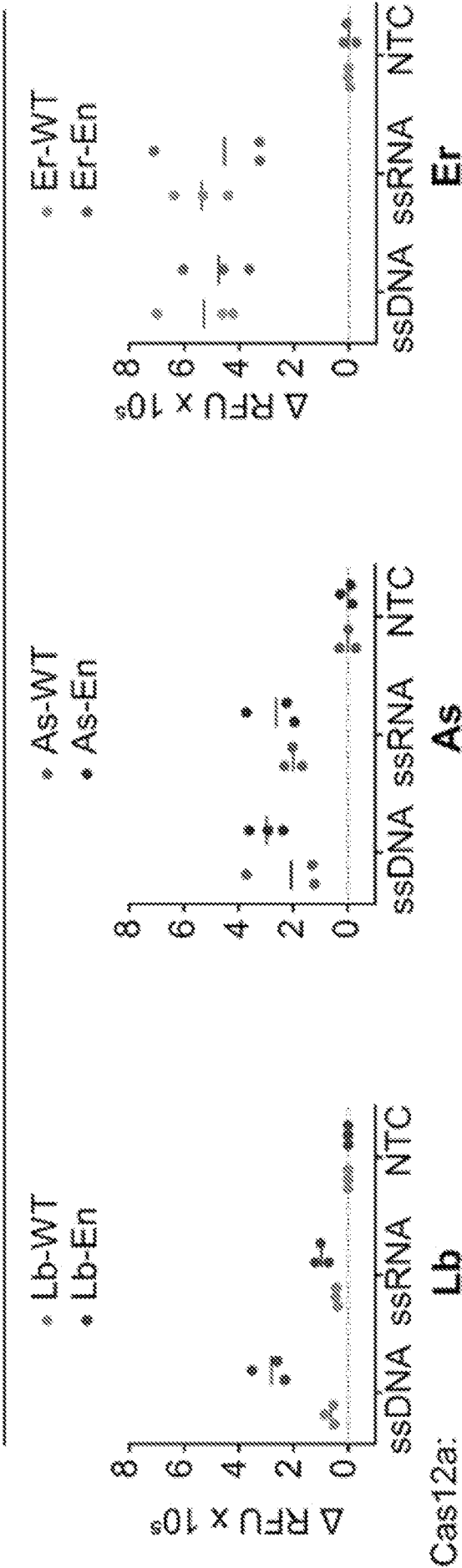
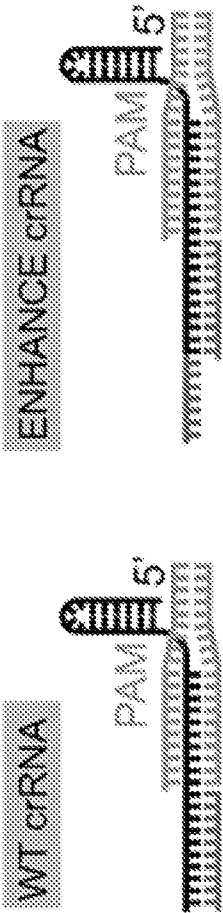


FIG. 2F

FIG. 2G

FIG. 2H

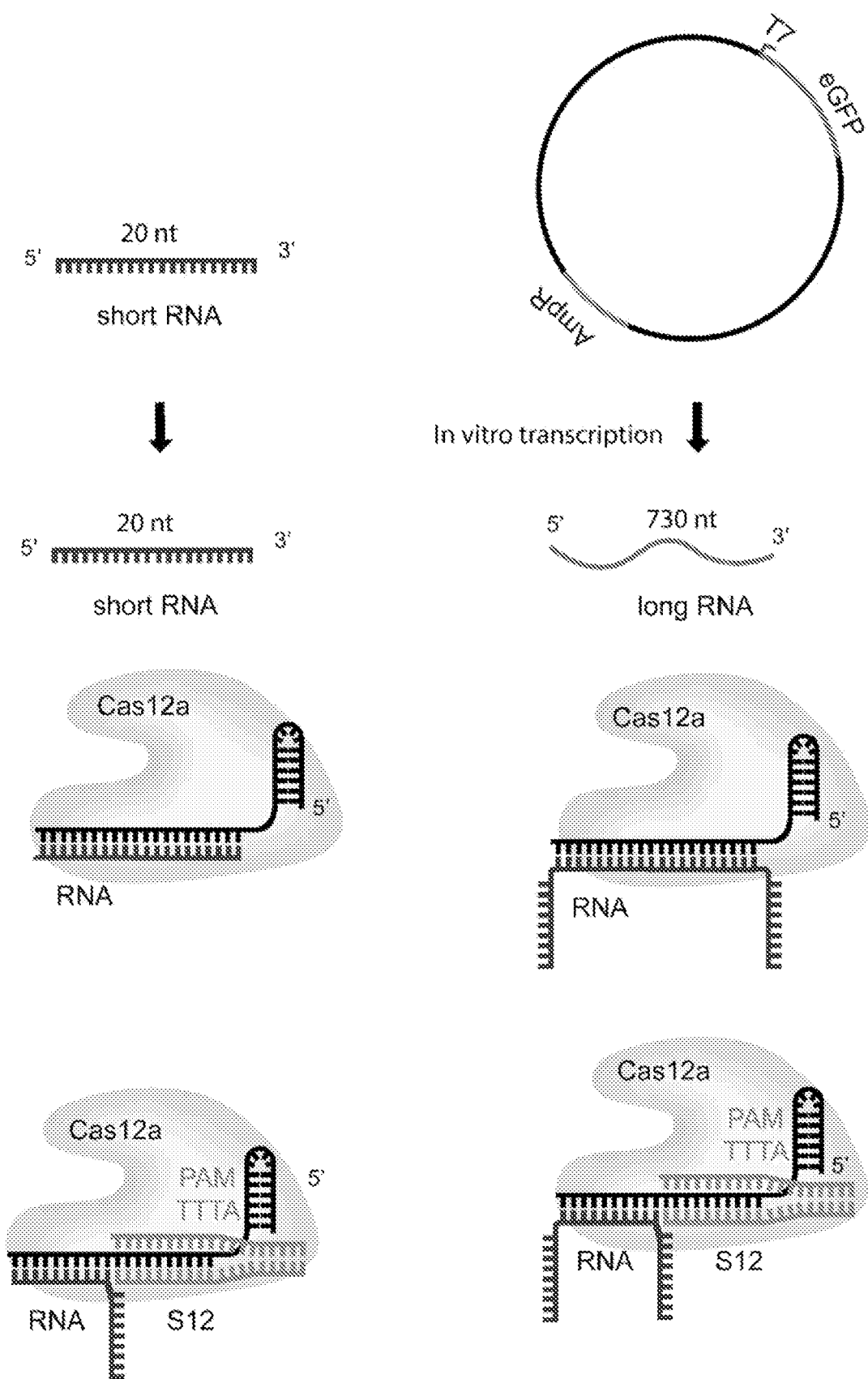


FIG. 3A

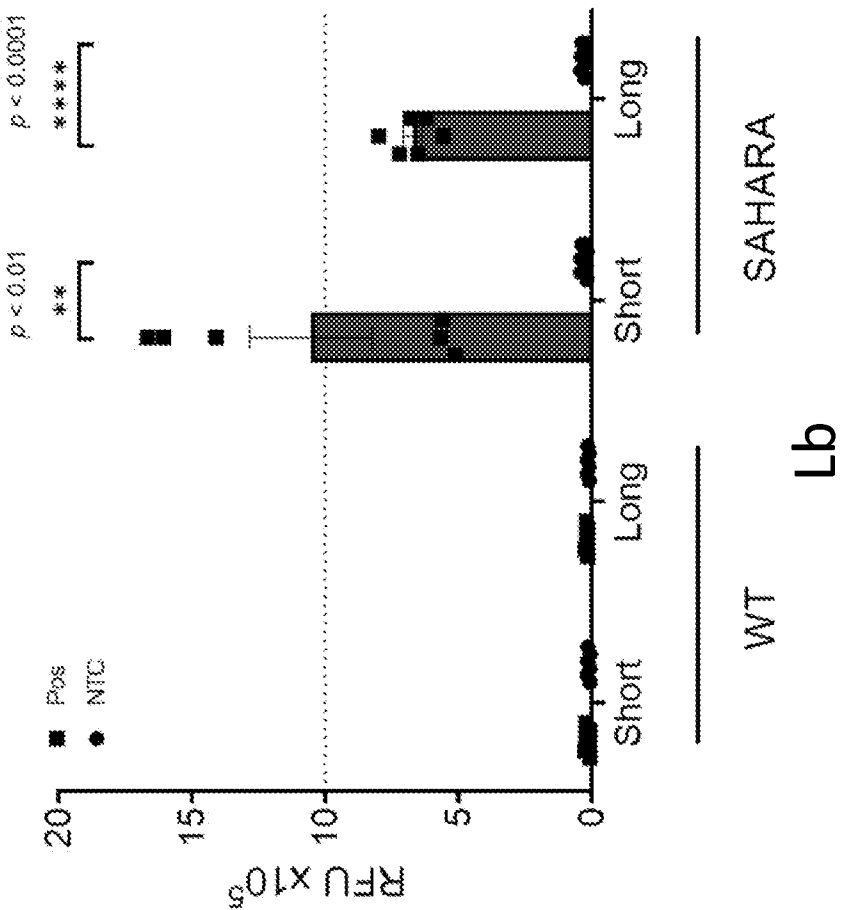
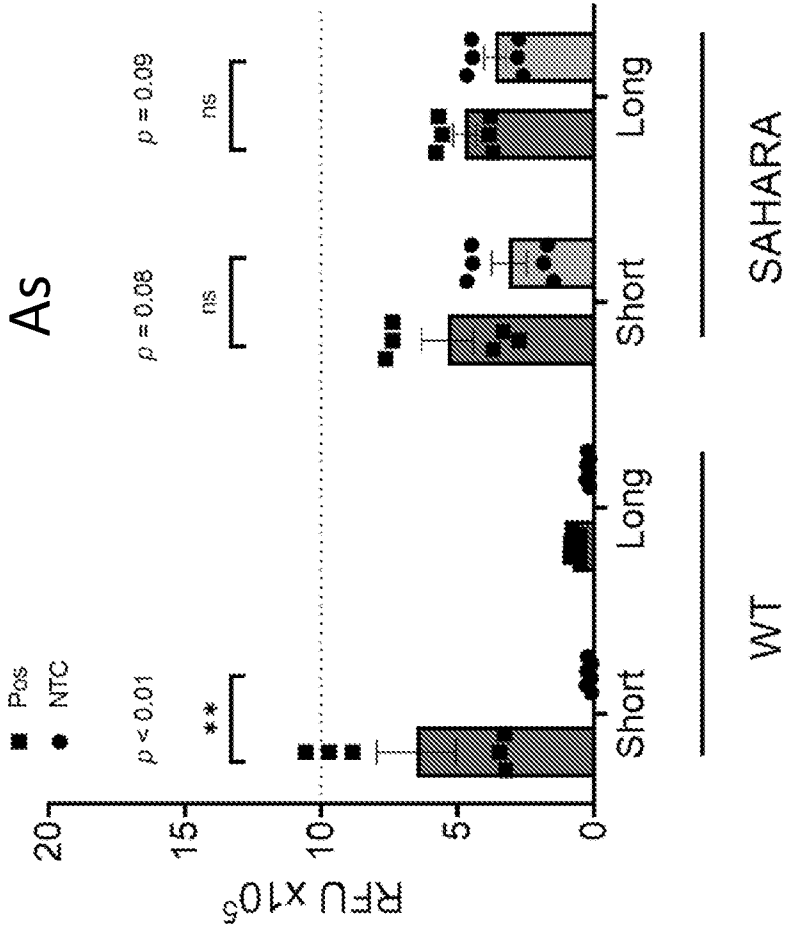
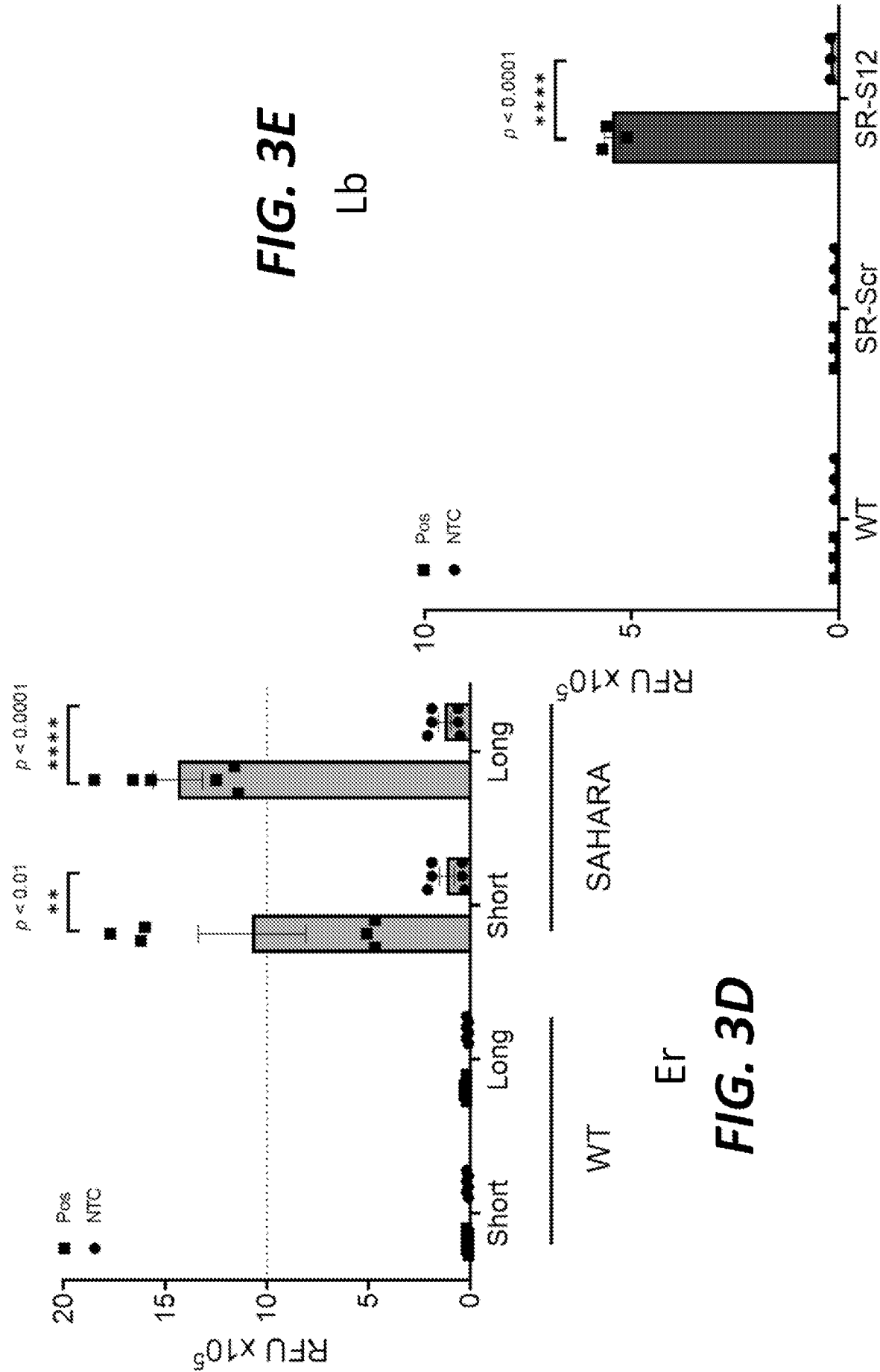
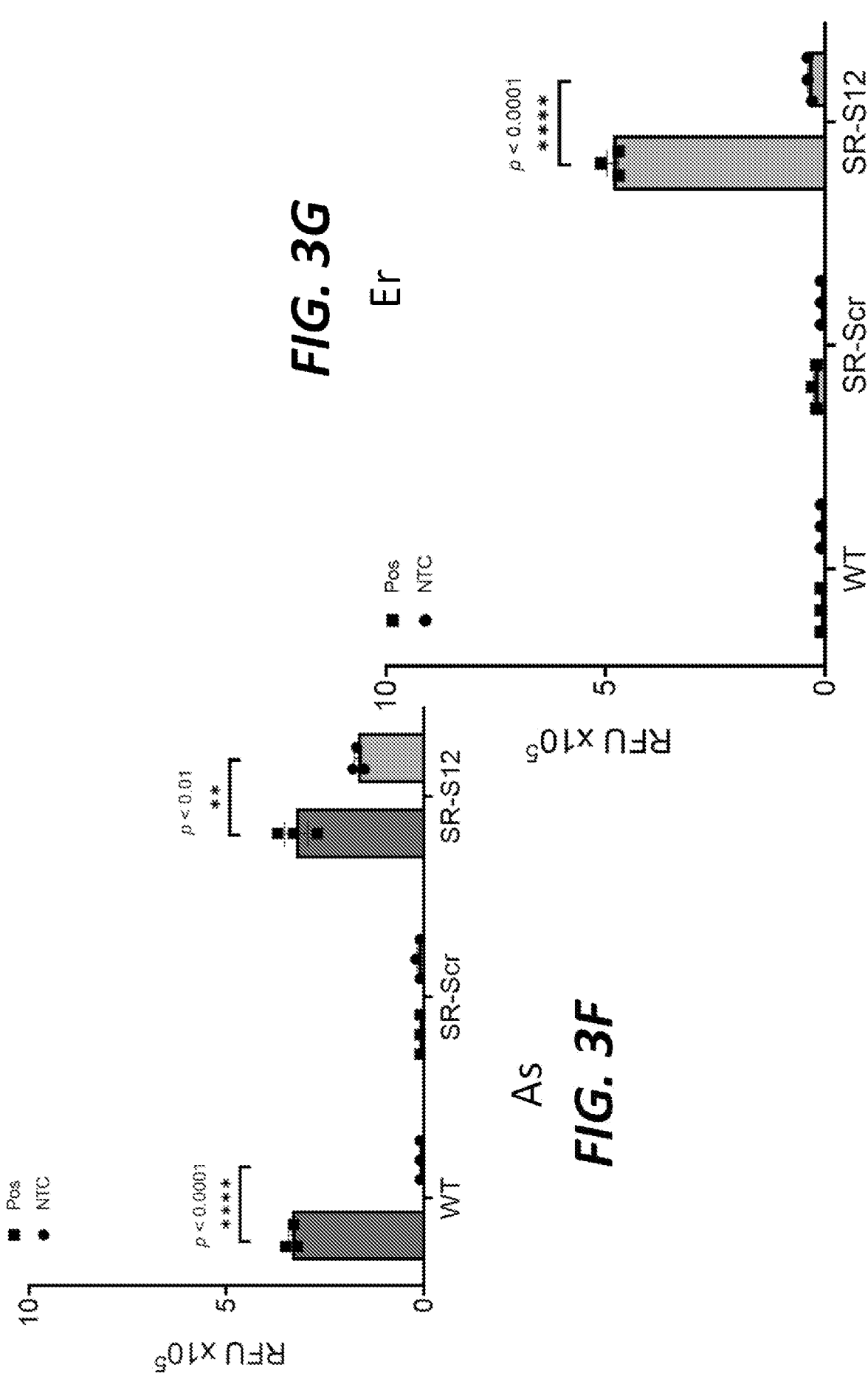


FIG. 3C







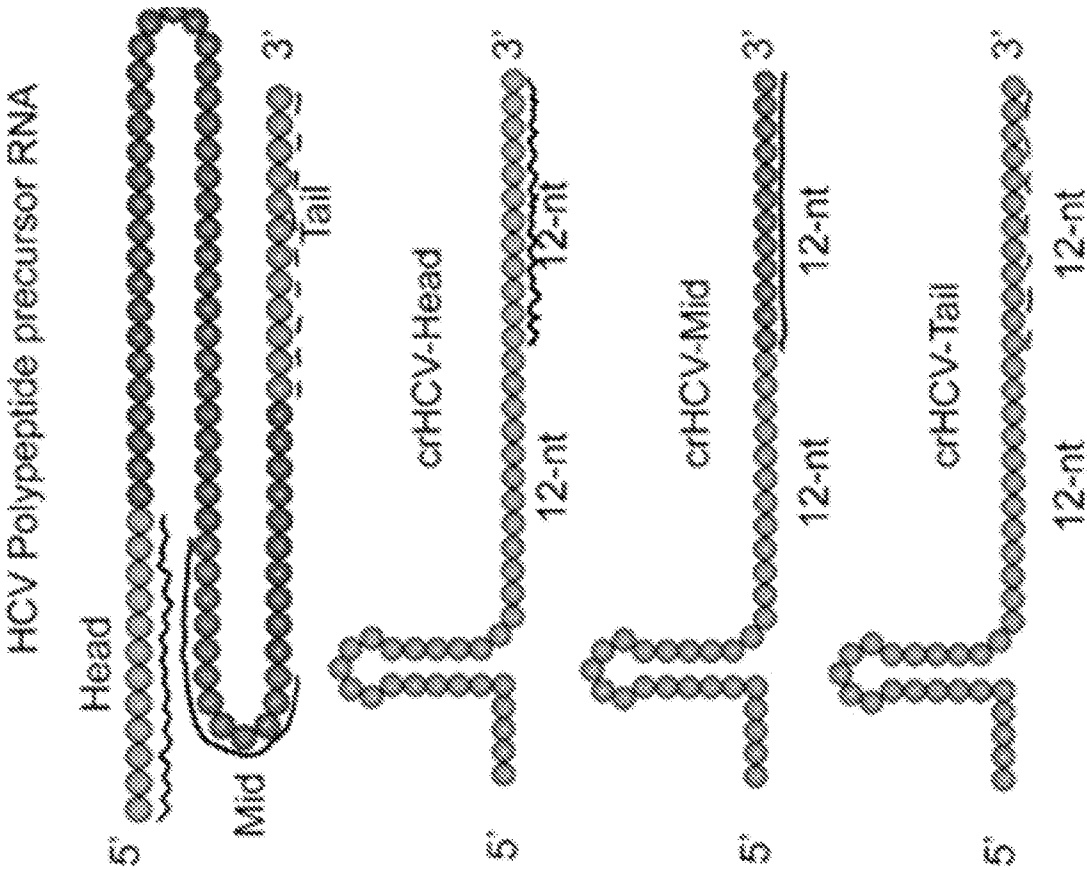


FIG. 4A

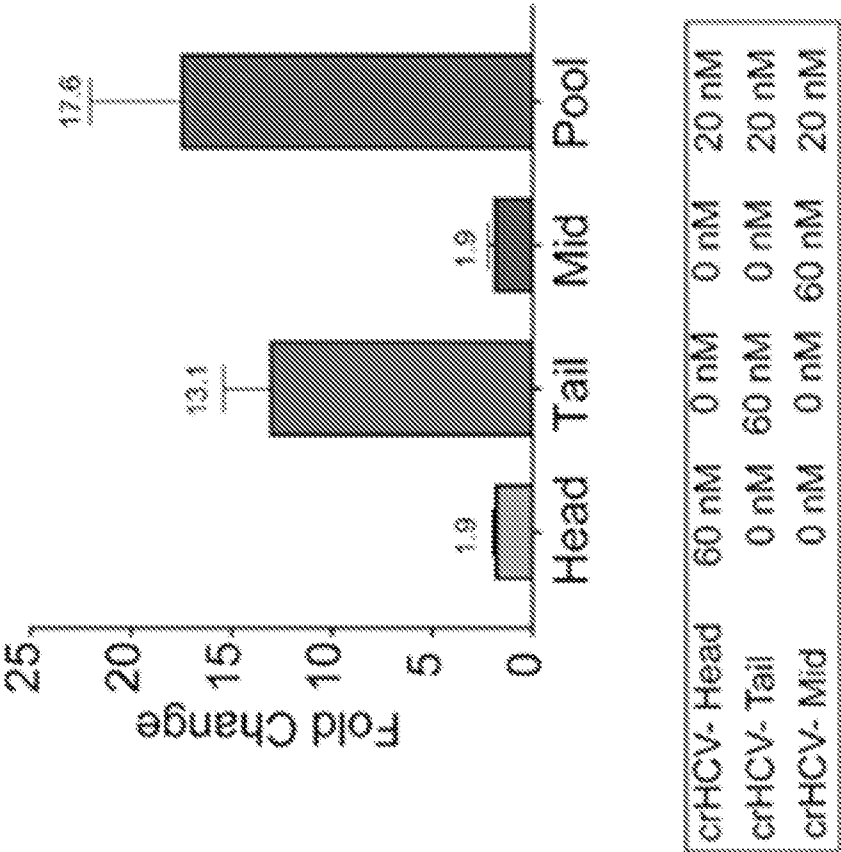


FIG. 4B

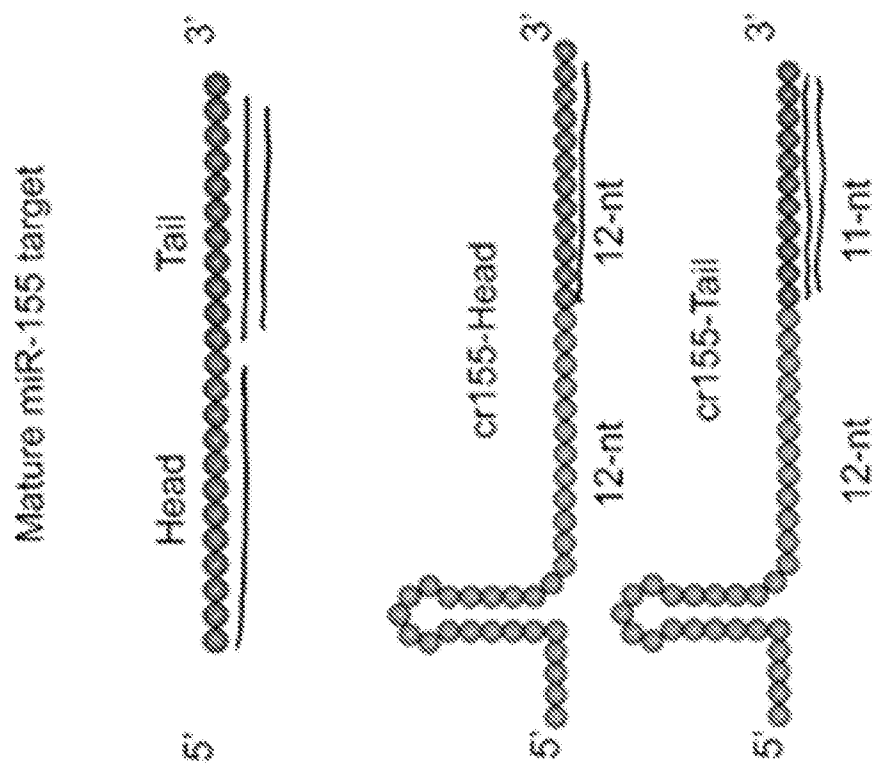


FIG. 4D

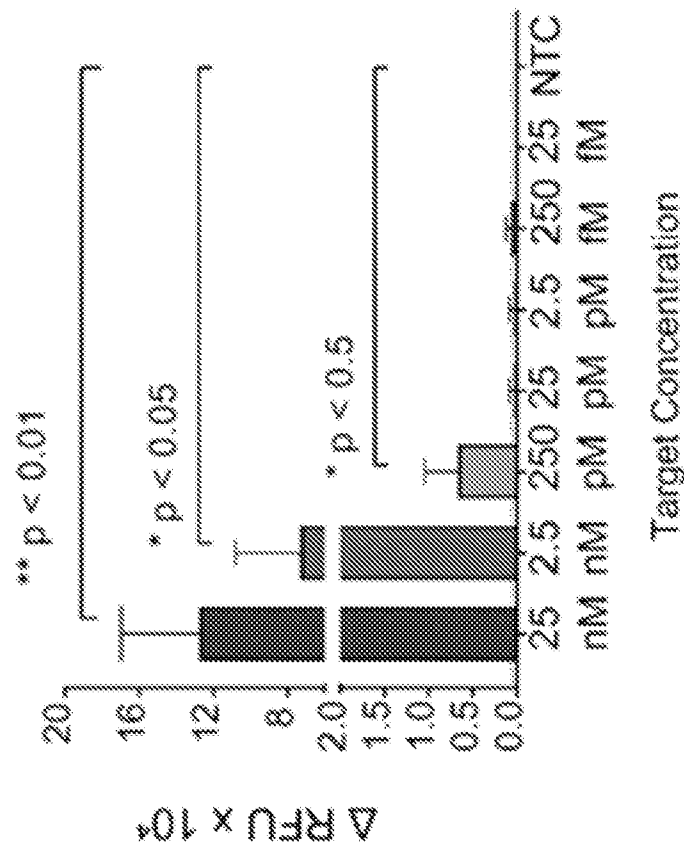
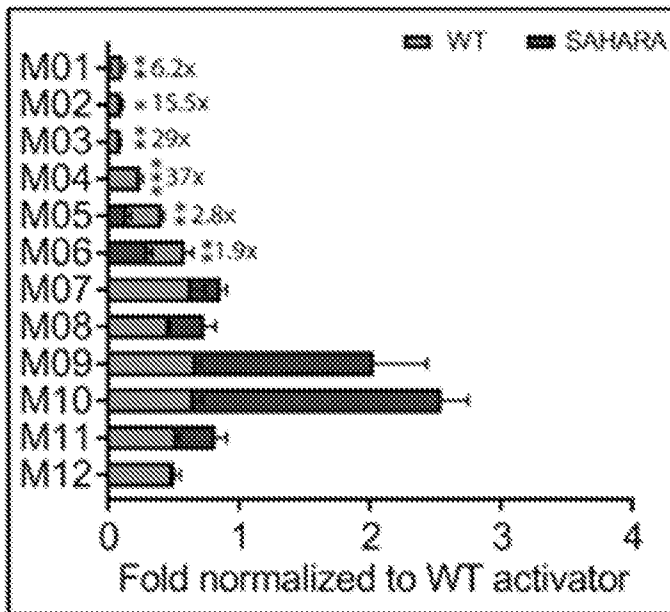
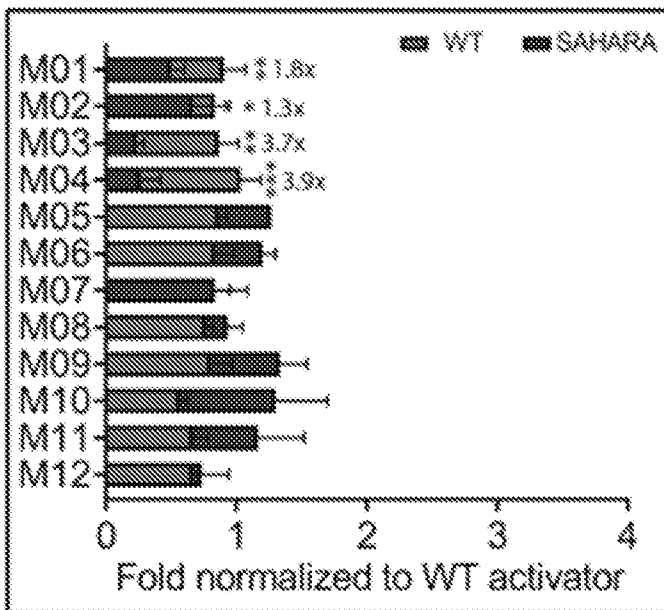


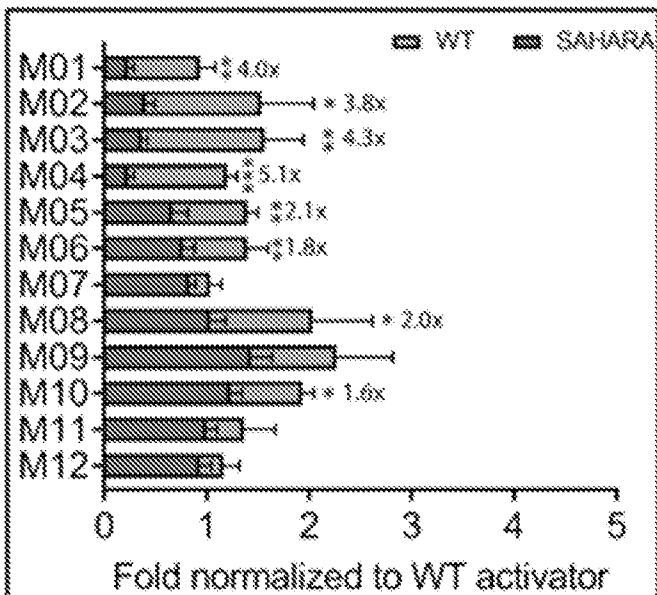
FIG. 4C

**FIG. 5A**

Lb

**FIG. 5B**

As

**FIG. 5C**

Er

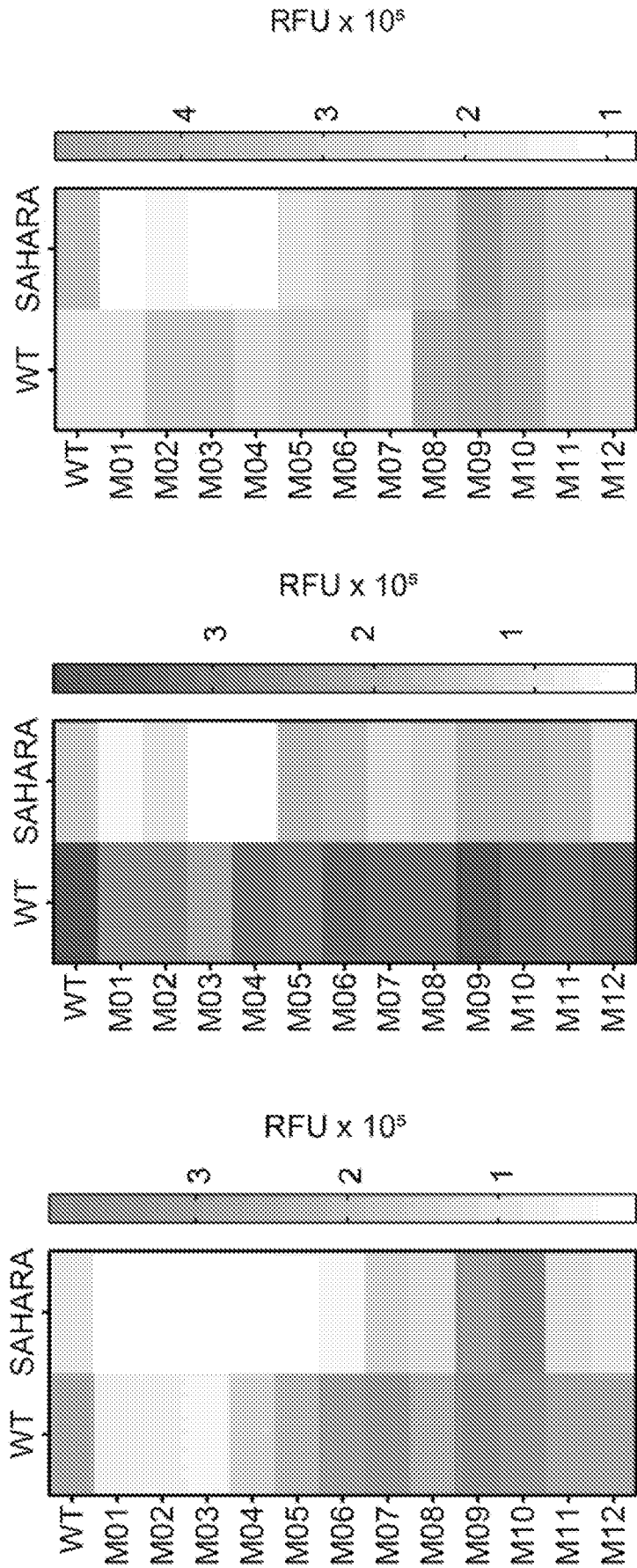


FIG. 5D

FIG. 5E

FIG. 5F

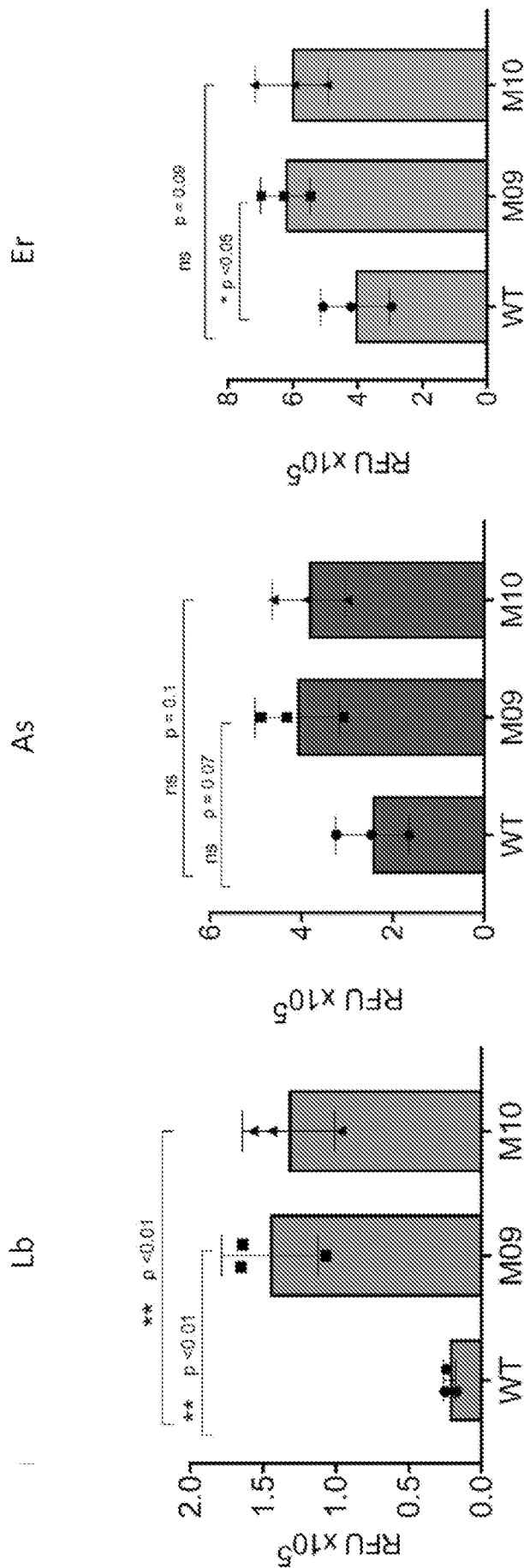
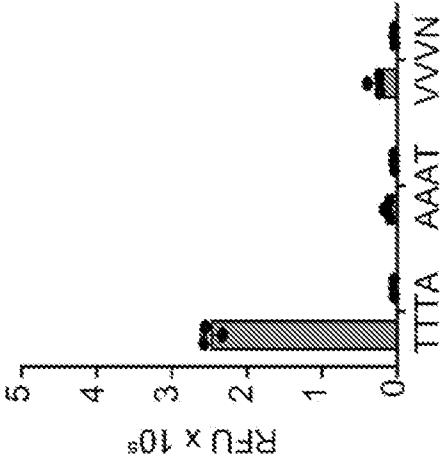
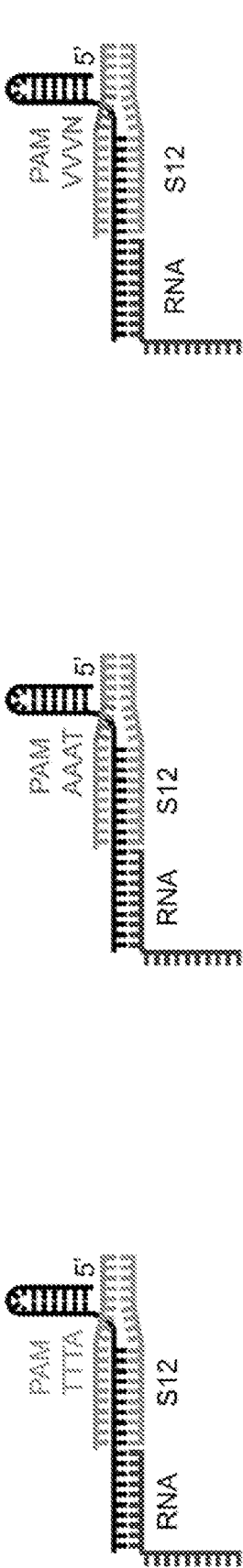


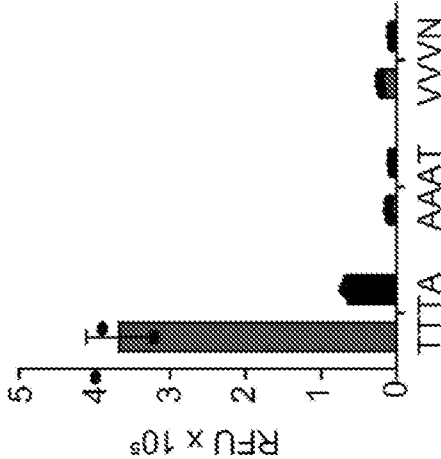
FIG. 5G **FIG. 5H** **FIG. 5I**

FIG. 6A



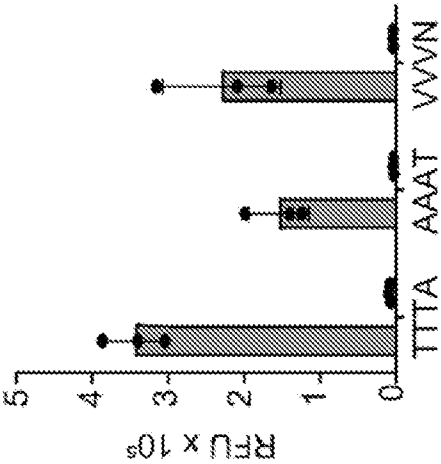
Lb

FIG. 6B



As

FIG. 6C



Er

FIG. 6D

FIG. 6E

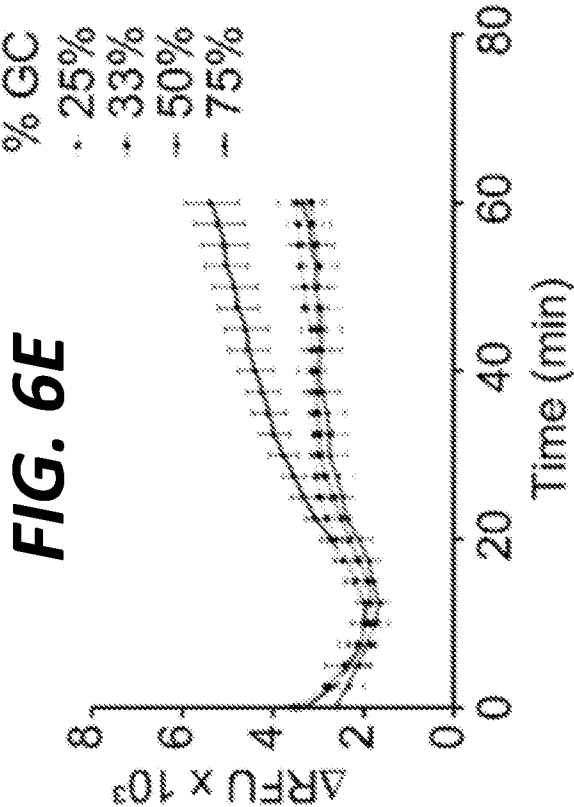


FIG. 6G

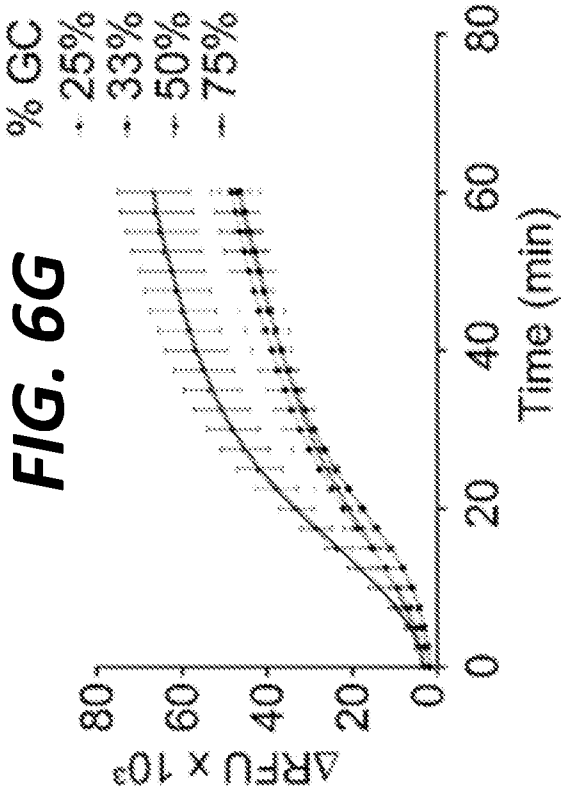
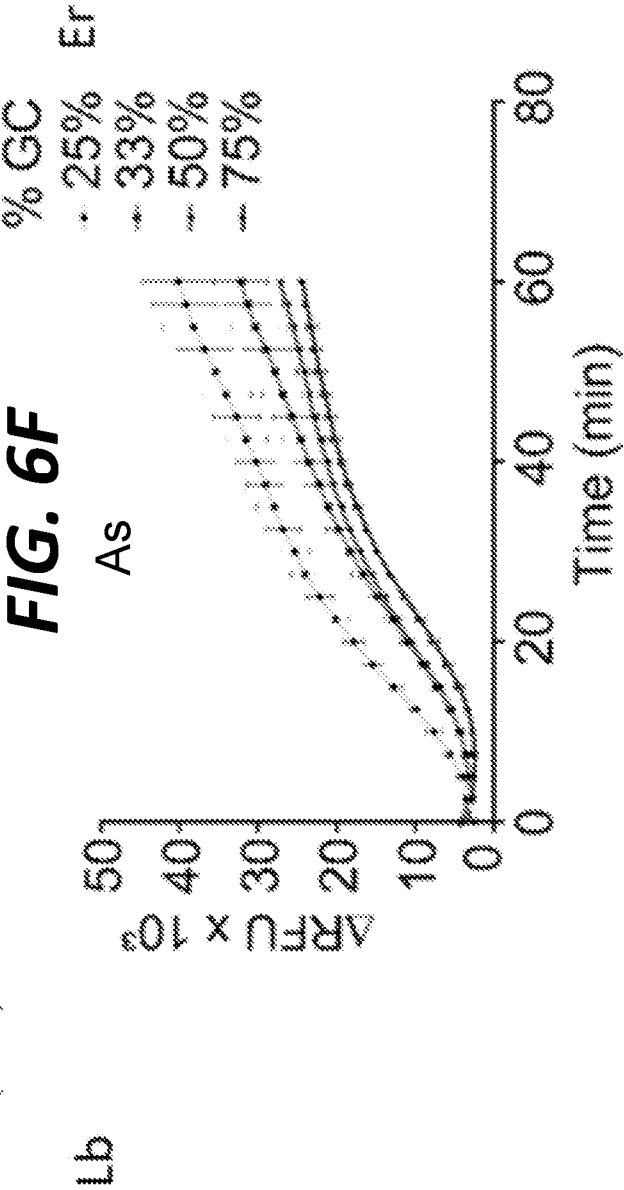


FIG. 6F



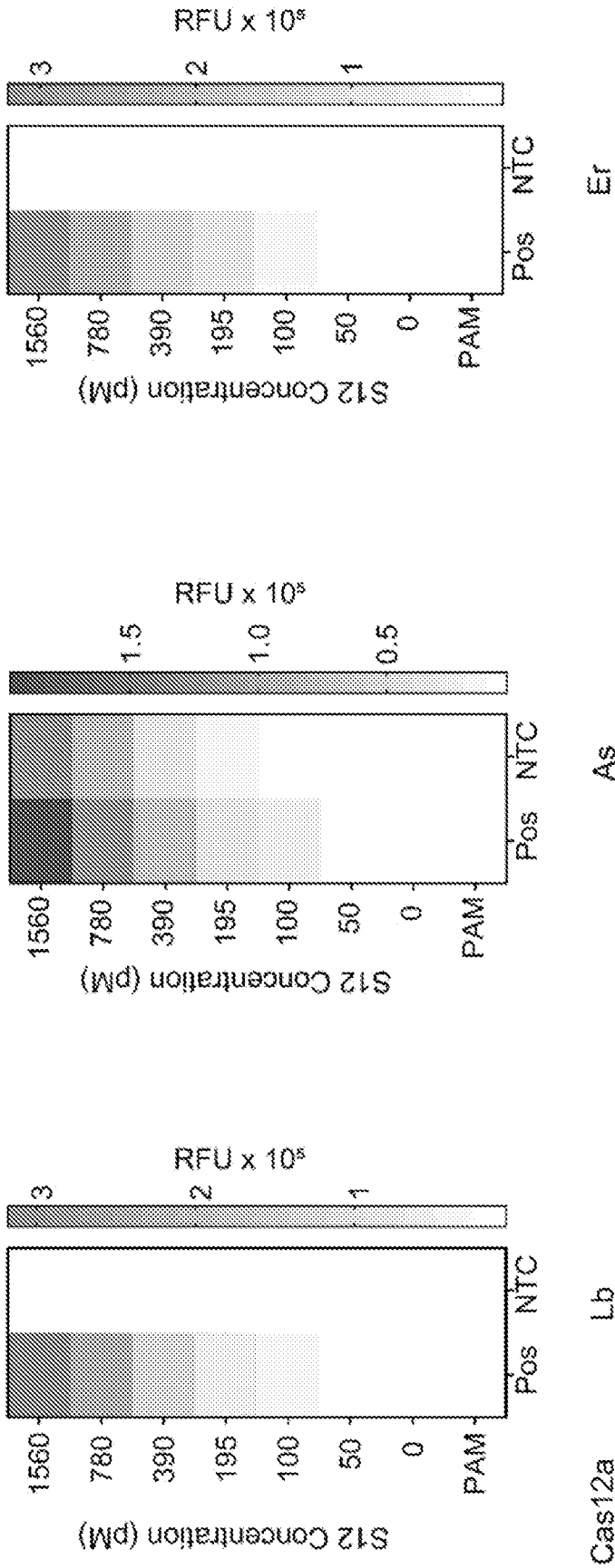
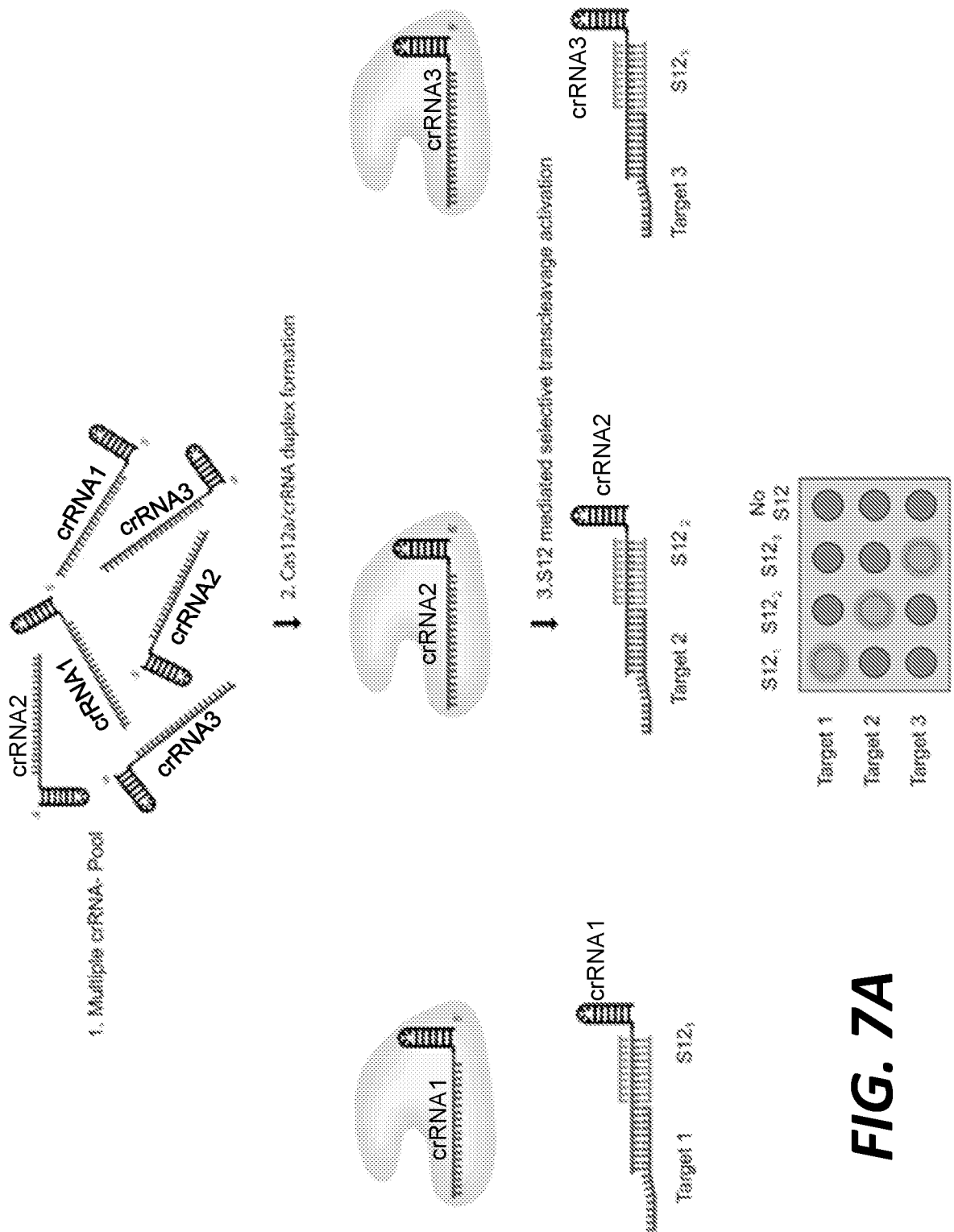


FIG. 6H

FIG. 6I

FIG. 6J



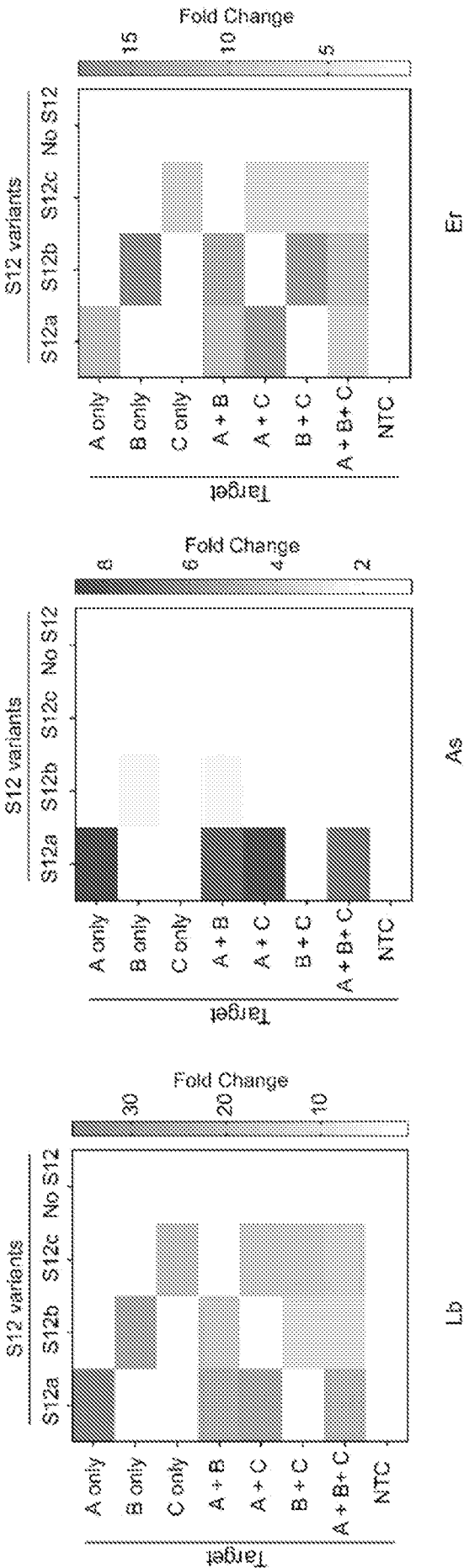


FIG. 7B

FIG. 7C

FIG. 7D

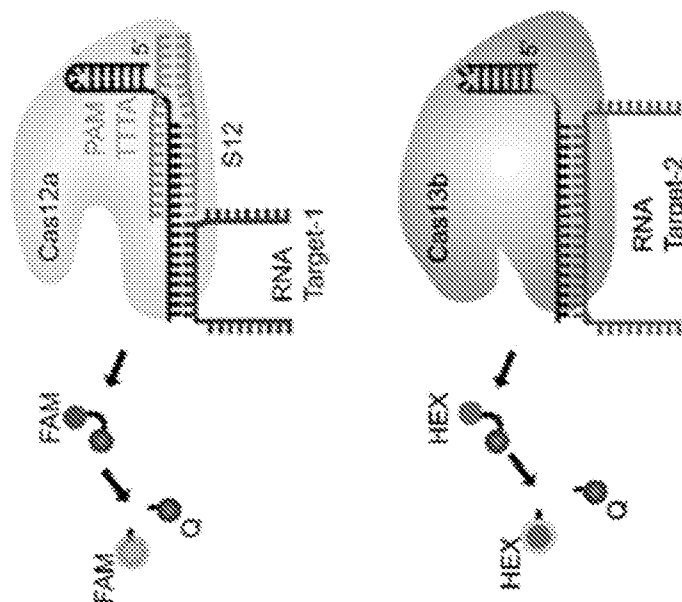
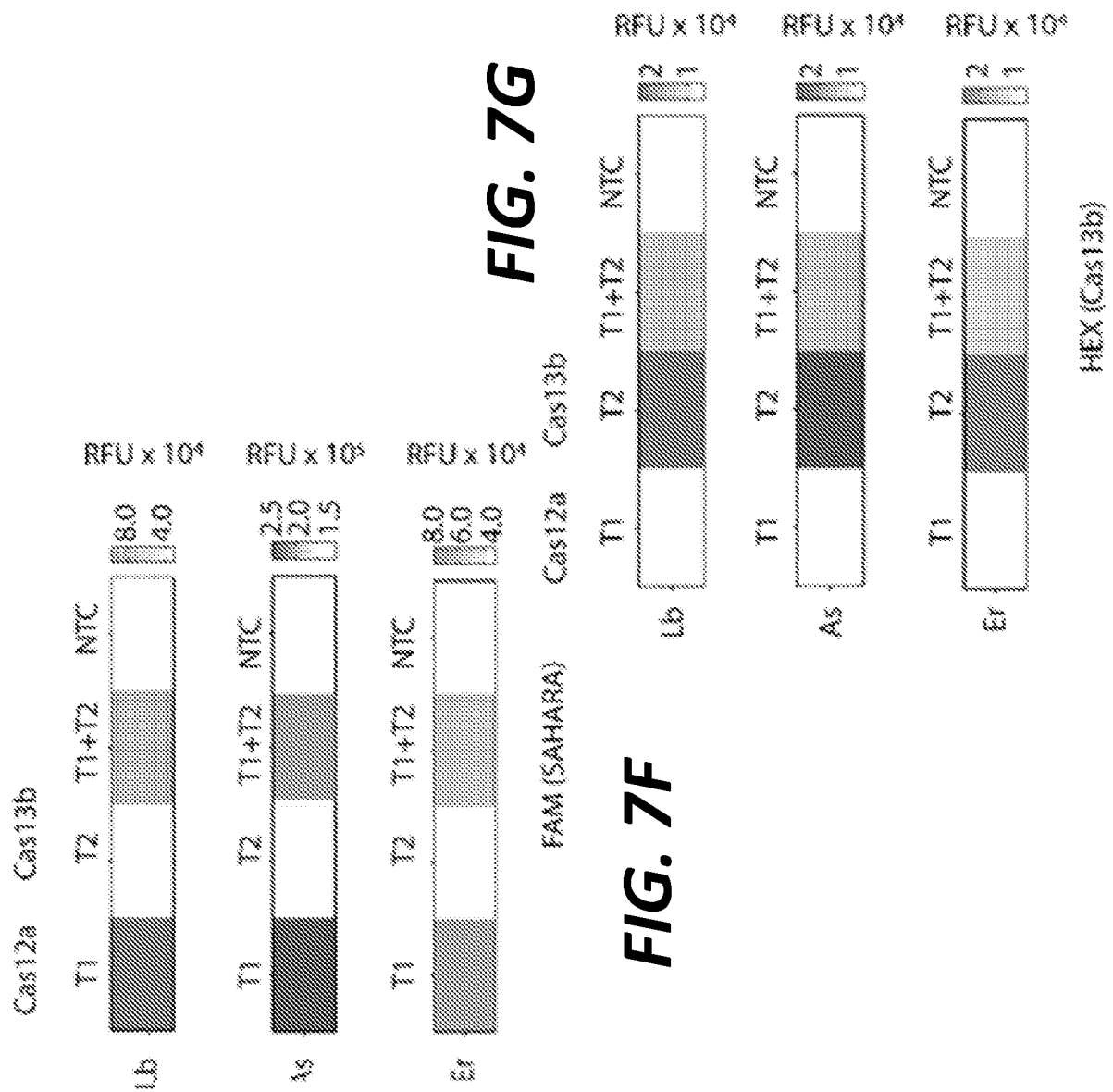


FIG. 7E

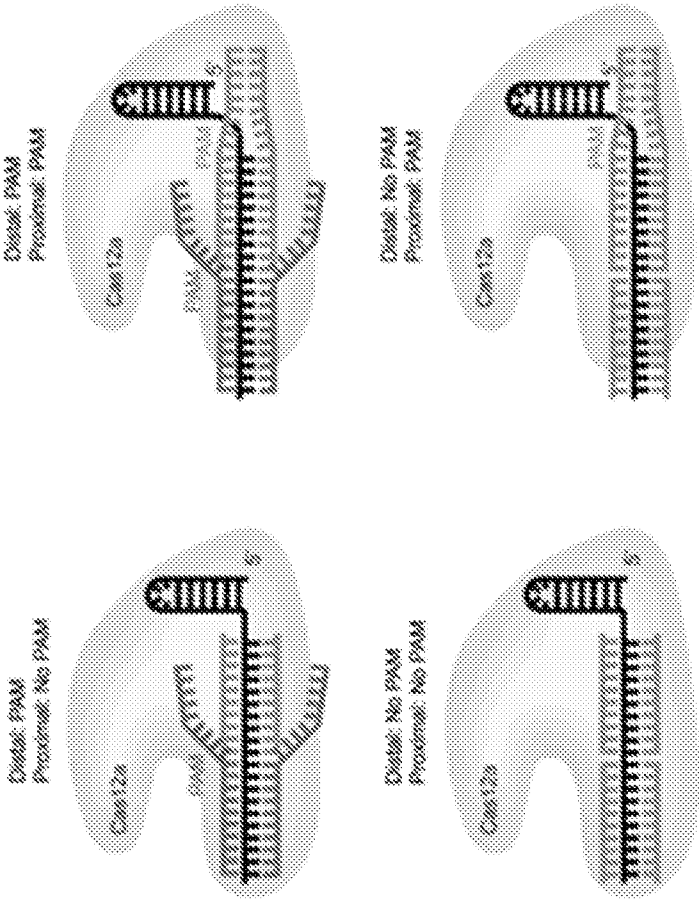
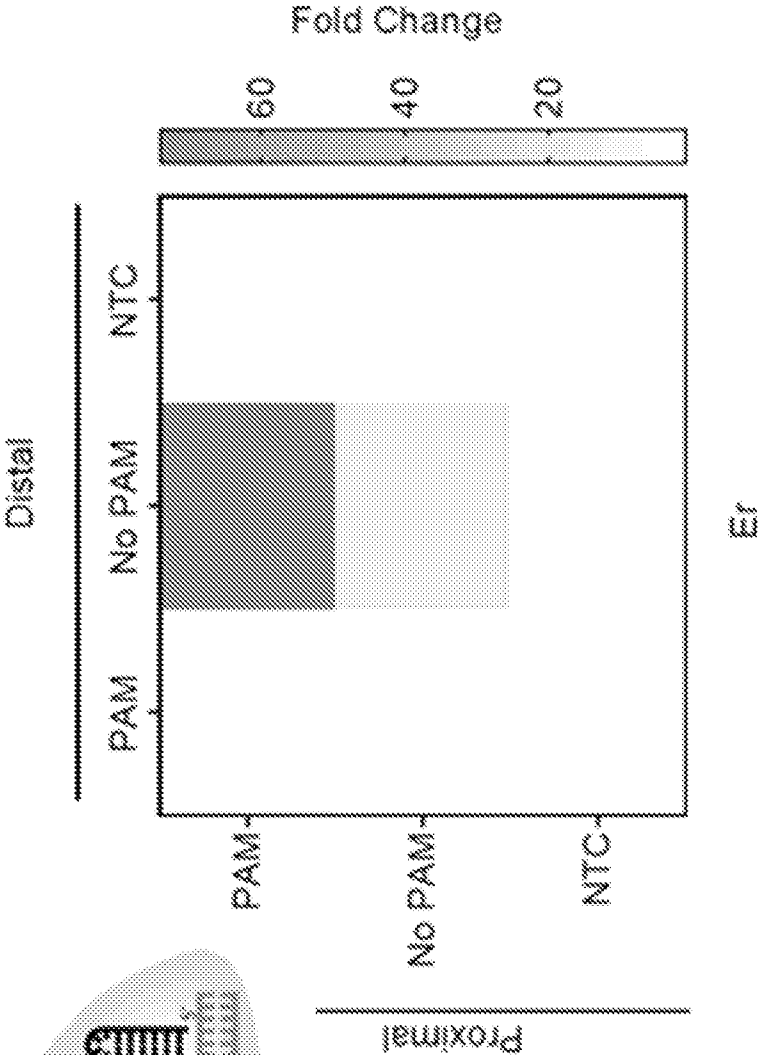
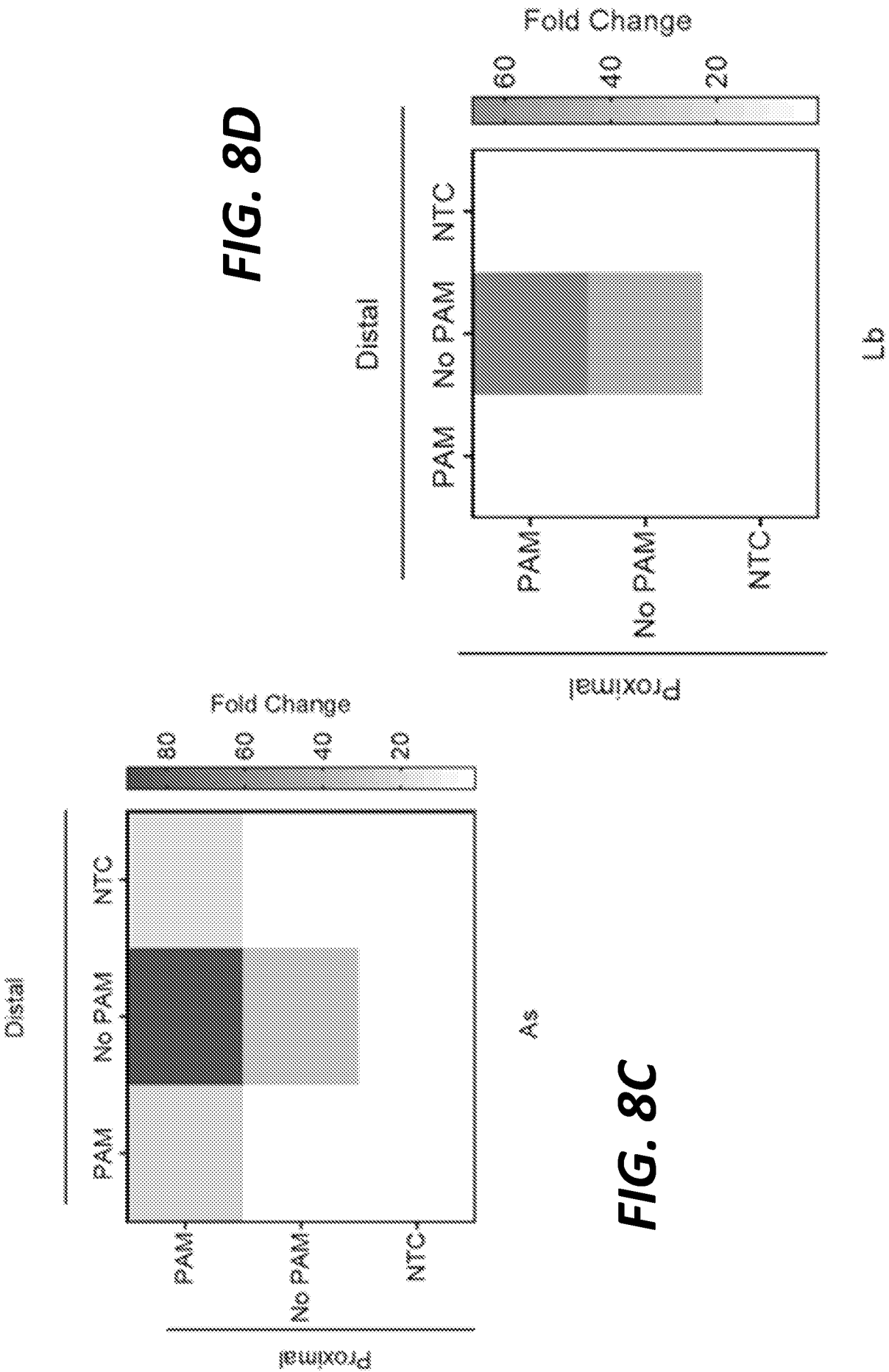


FIG. 8A

FIG. 8B





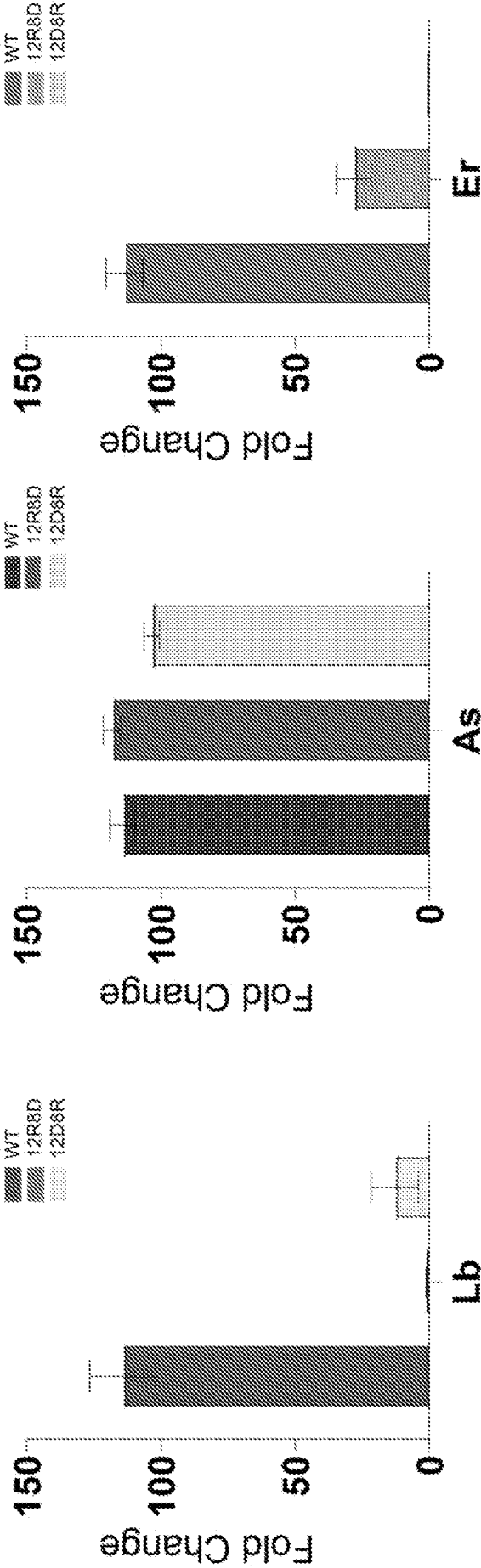


FIG. 9E

FIG. 9F

FIG. 9G

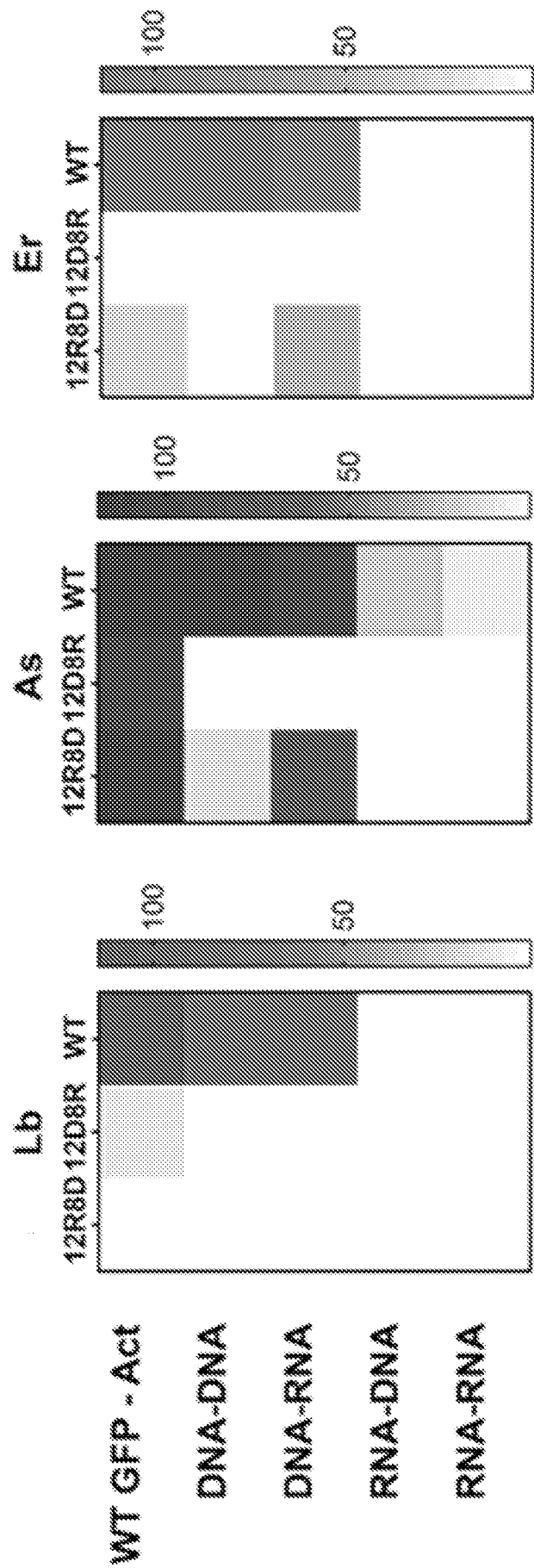


FIG. 10A

FIG. 10B

FIG. 10C

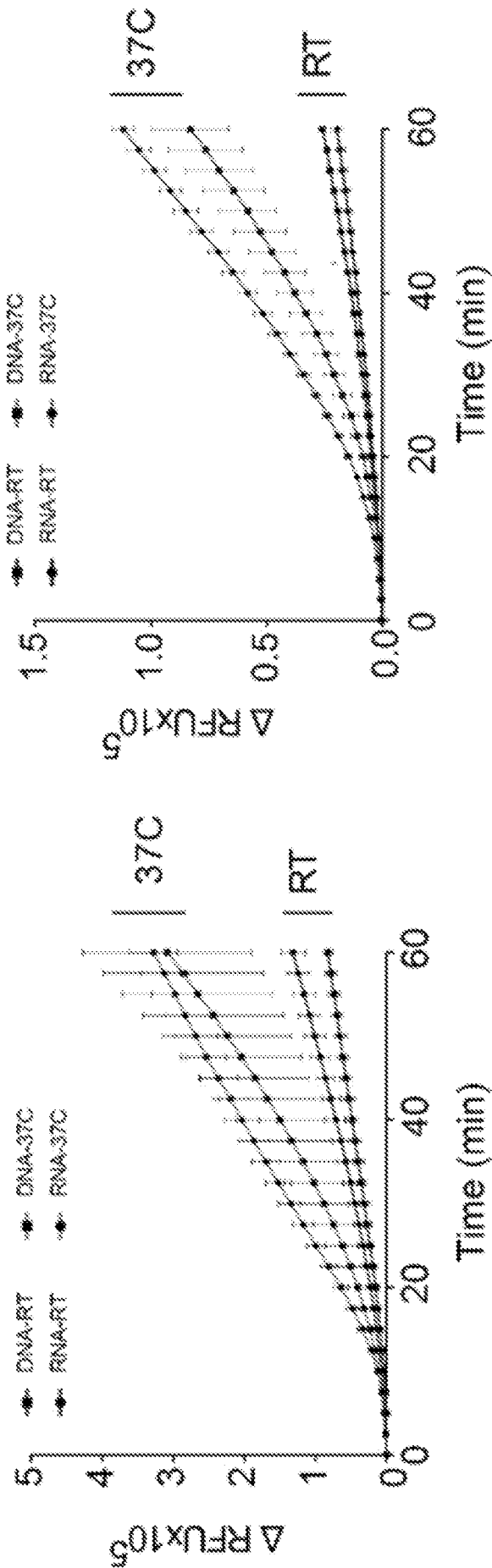


FIG. 11A

FIG. 11B

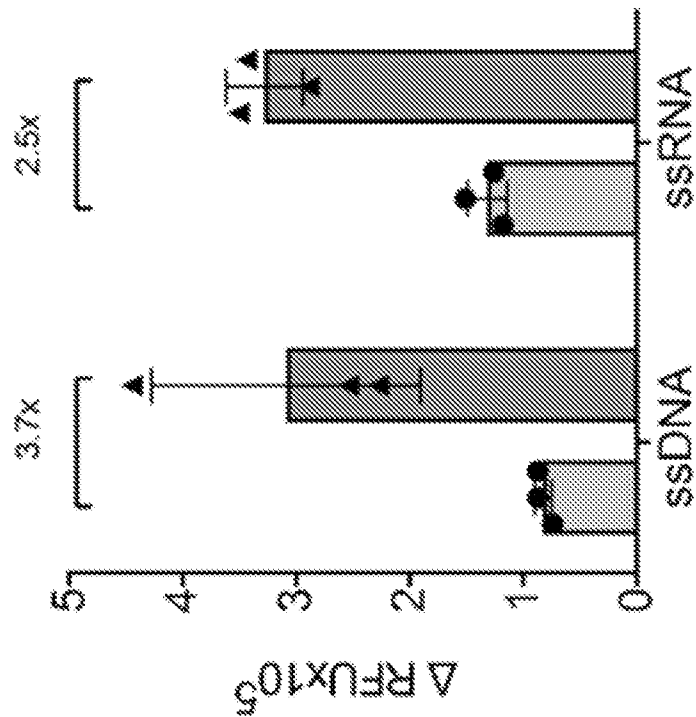


FIG. 11C

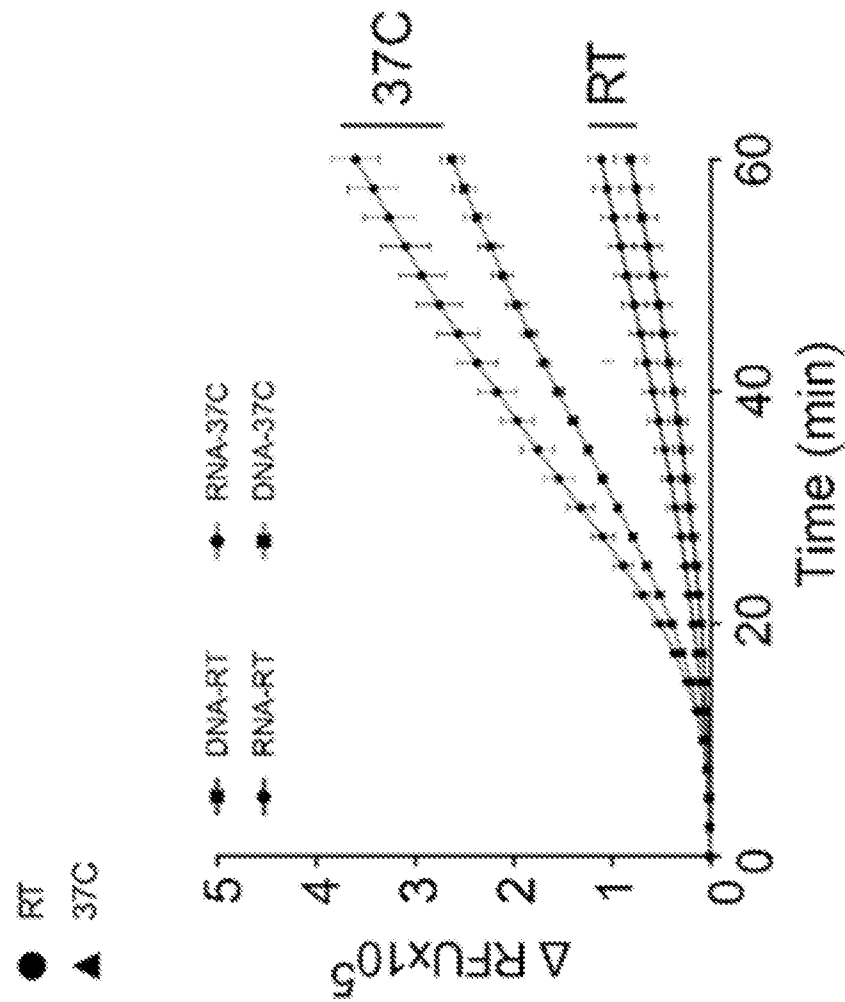


FIG. 11D

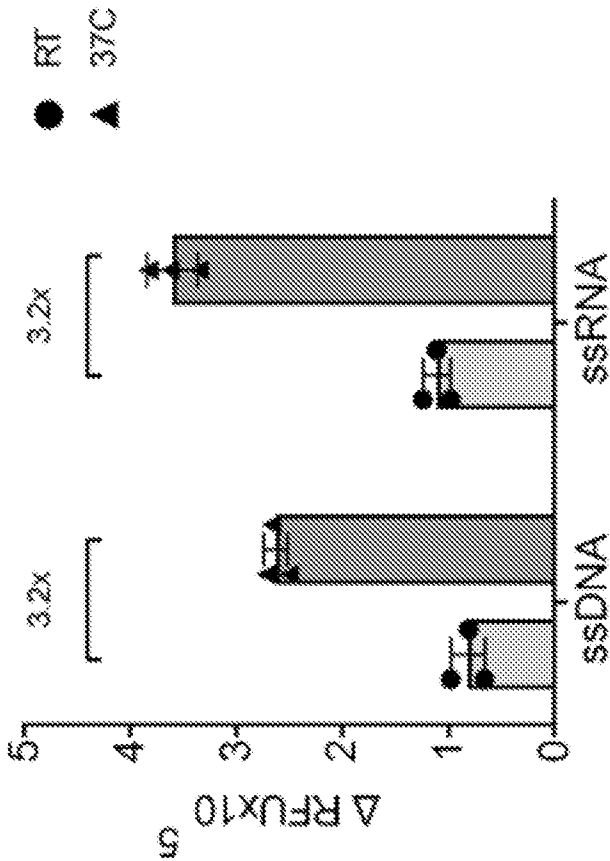


FIG. 11F

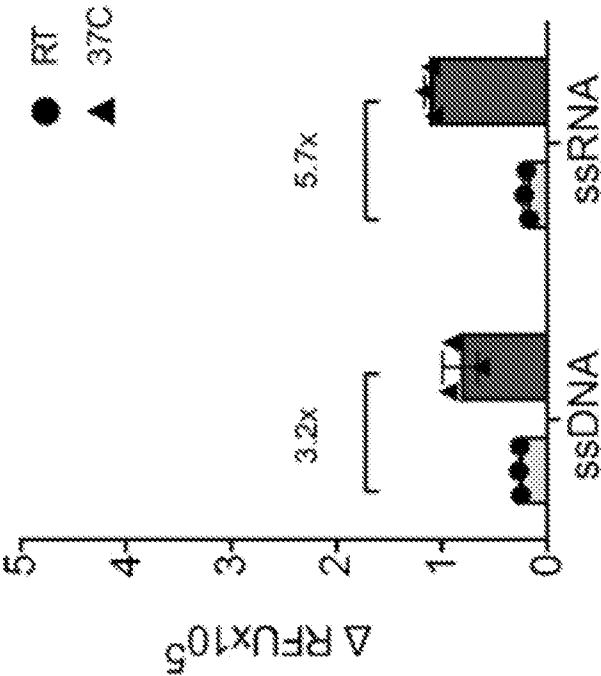


FIG. 11E

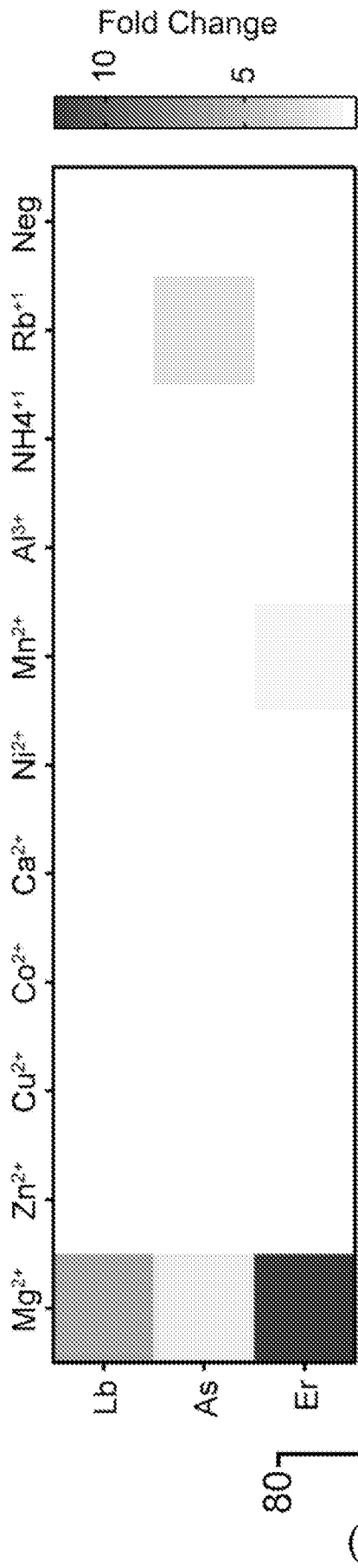


FIG. 12A

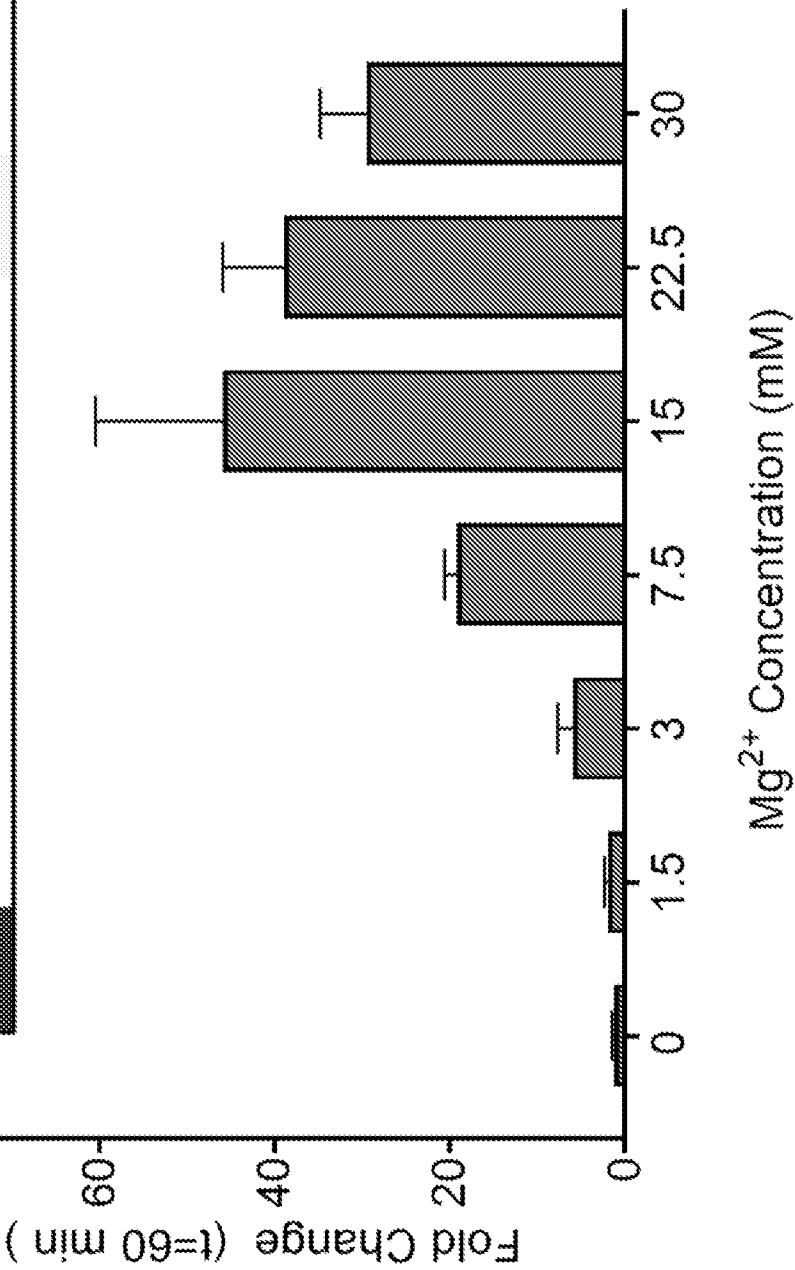


FIG. 12B

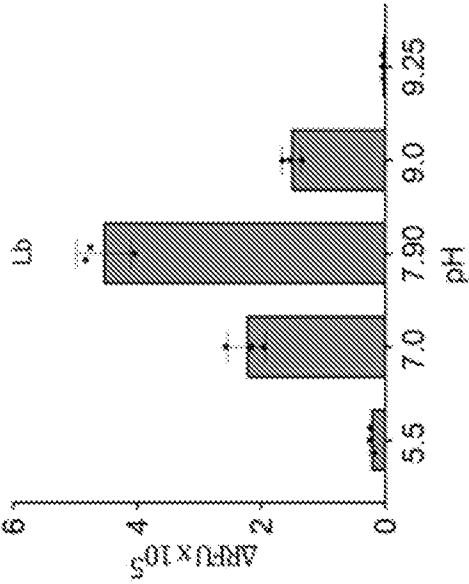


FIG. 13A

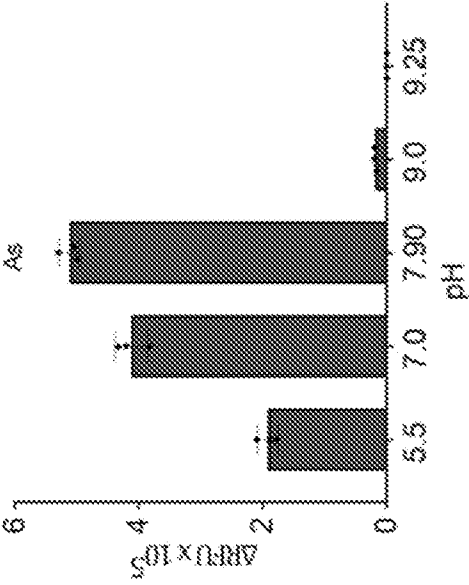


FIG. 13B

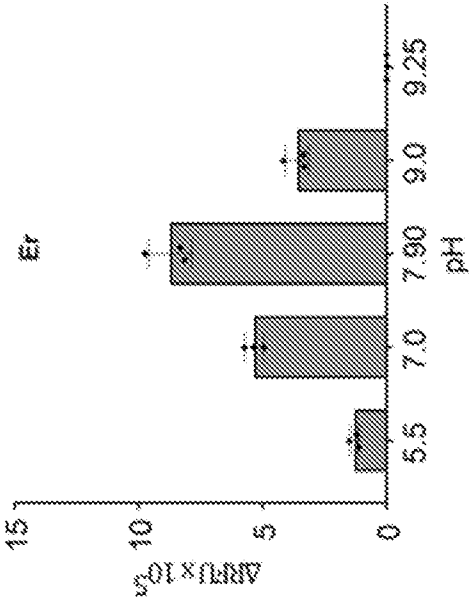


FIG. 13C

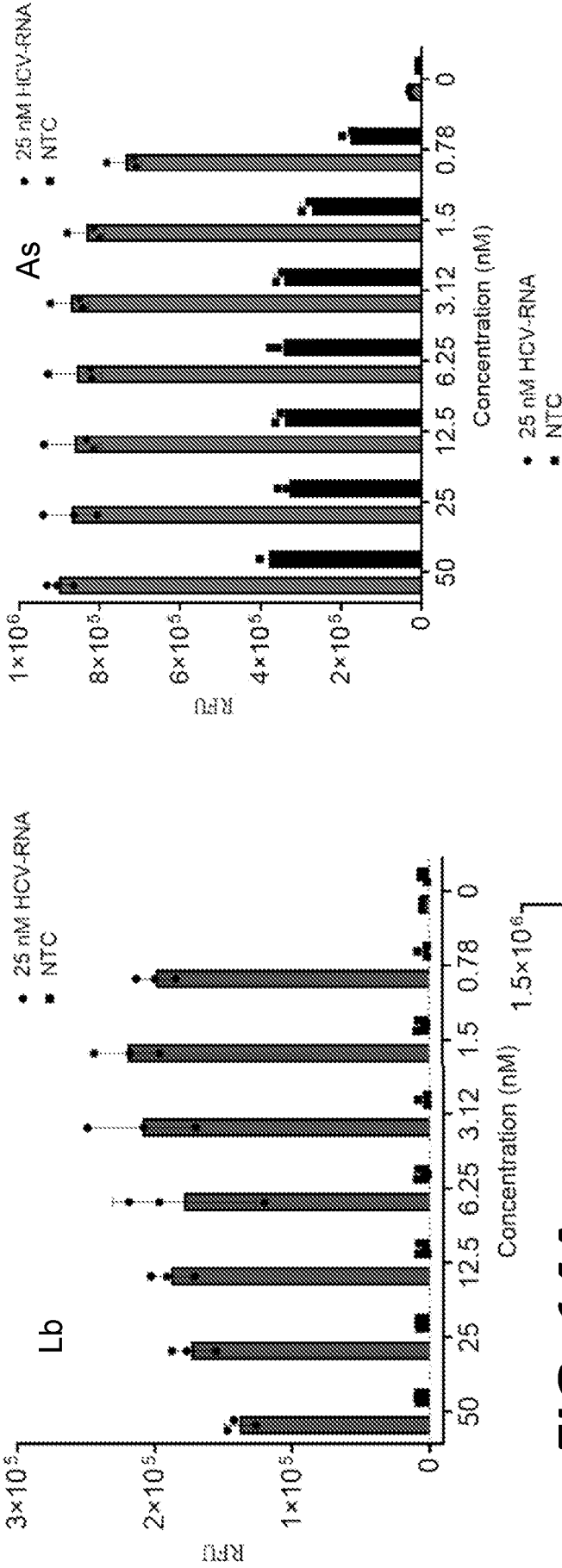


FIG. 14B

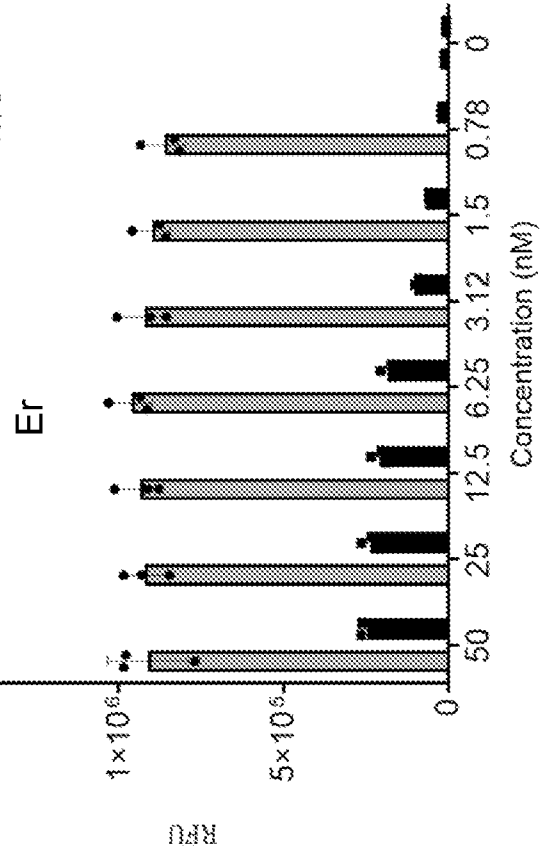


FIG. 14C

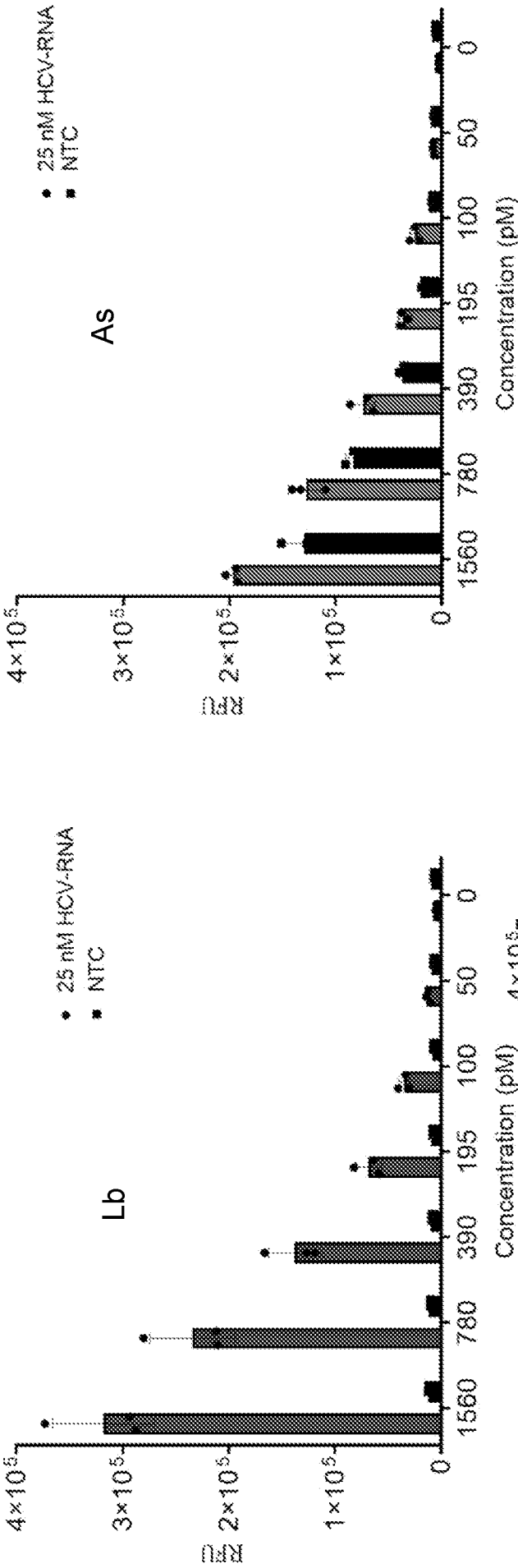


FIG. 15A

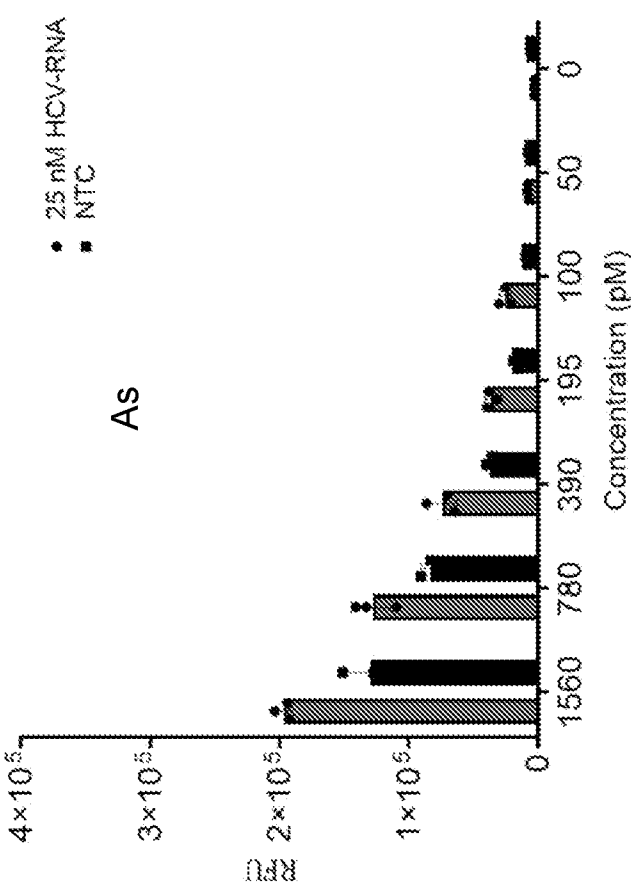
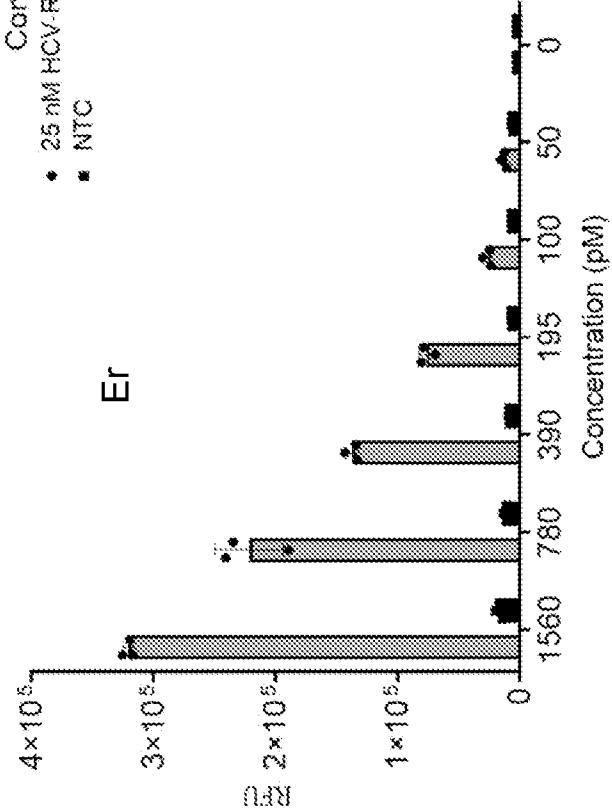


FIG. 15B



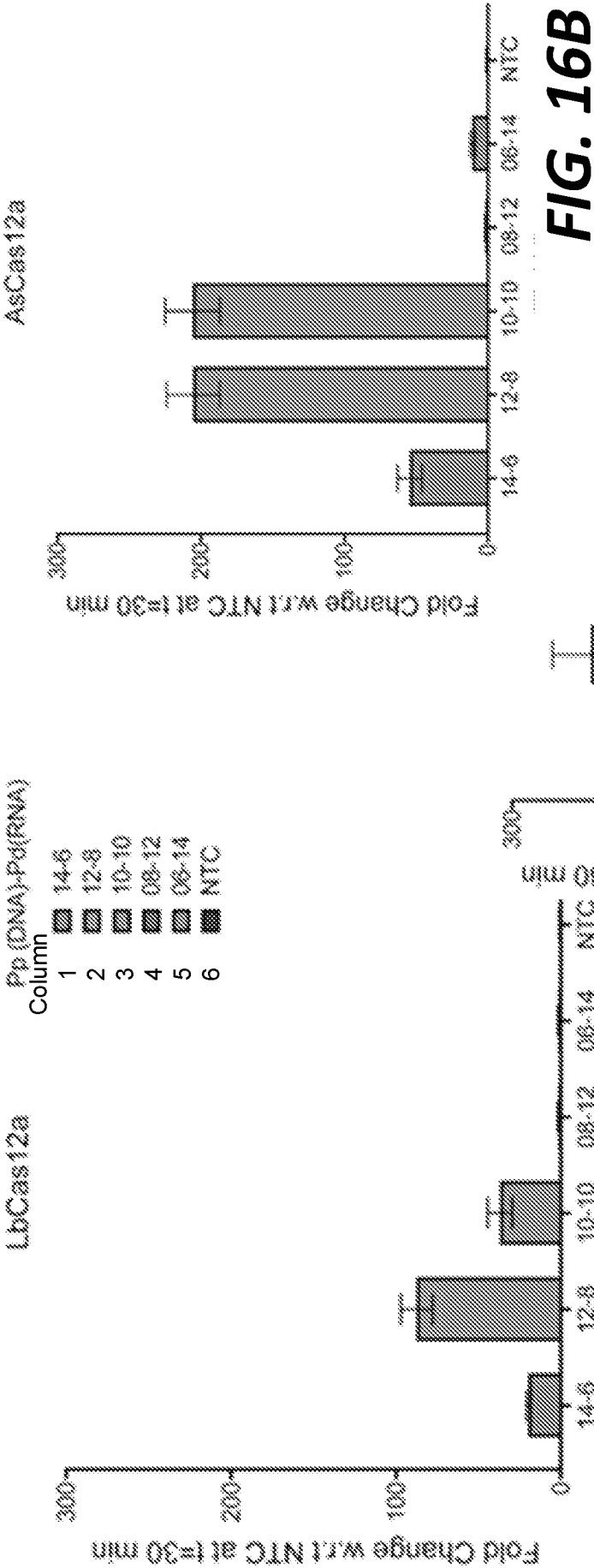


FIG. 16C

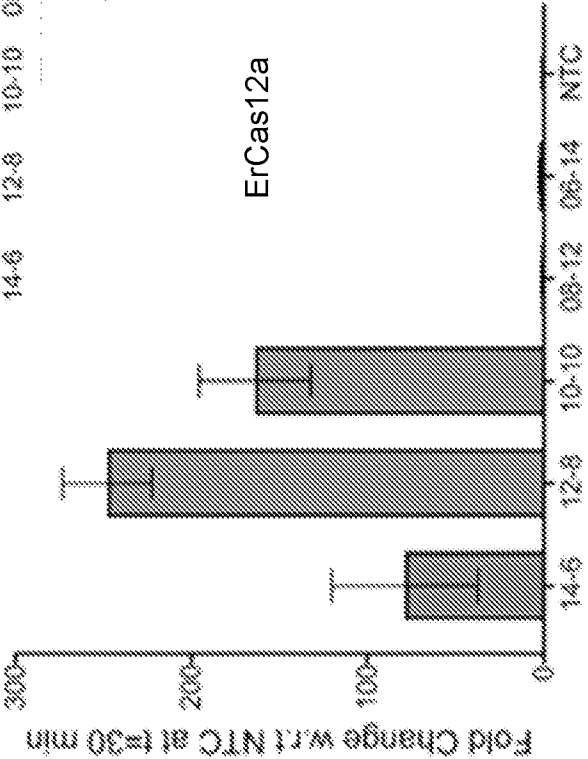


FIG. 16B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/79420

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C12Q 1/70, C12Q 1/6844 (2023.01)

ADD. C12N 15/63, C12Q 1/68 (2023.01)

CPC - INV. C12Q 1/6844

ADD. C12N 2310/20, C12Q 2563/107

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 9,850,525 B2 (AGILENT TECHNOLOGIES, INC) 26 DECEMBER 2017 (26.12.2017); col 2, ln 29-42; col 2, ln 43-46; col 2, ln 59- col 3, ln 9; col 3, ln 58-64	1-4, 14-21, 24
Y	XIONG et al. Functional DNA Regulated CRISPR-Cas12a Sensors for Point-of-Care Diagnostics of Non-Nucleic-Acid Targets. Journal of the American Chemical Society, 4 December 2019, Vol. 142, pages 207-213; Abstract, Figure 1, Figure 2A, p 209, col 1, para 1	1-4, 14-21, 24
Y	WO 2021/092519 A1 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION) 14 May 2021 (14.05.2021); Abstract; p 10, ln 6-14; p 21, ln 9-20; p 21, ln 23-32; p 44, ln 1-14	3-4, 21/(17-20)
Y	Li et al., CRISPR-Cas12a-assisted nucleic acid detection. Cell Discovery, 24 April 2018, Vol. 4, No. 20, pages 1-4; p 1, col 2, para 3	15
Y	US 2020/0131488 A1 (THE BROAD INSTITUTE, INC. et al.) 30 April 2020 (30.04.2020); Abstract	16
Y	TENG et al. CDetection: CRISPR-Cas12b-based DNA detection with sub-attomolar sensitivity and single-base specificity. Genome Biology 2019, Vol. 20, No. 132, pages 1-7; p 2, col 1, para 3	19-20, 21/(19-20)
Y	US 2019/0264186 A1 (THE BROAD INSTITUTE INC. et al.) 29 August 2019 (29.08.2019); para [0041]	20, 21/20

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

10 January 2023 (10.01.2023)

Date of mailing of the international search report

FEB 16 2023

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Kari Rodriguez

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/79420

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed.

b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/79420

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-13, 22-23, 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.