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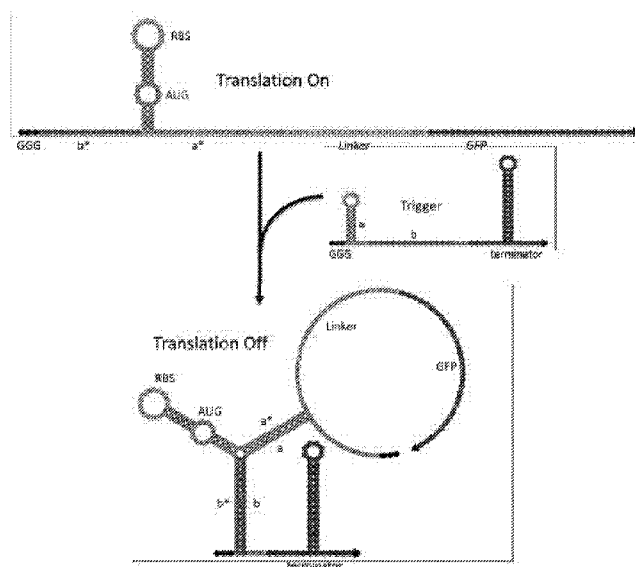
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(54) Title: SYNTHETIC NEAR-THRESHOLD TRANSLATIONAL REPRESSORS

FIG. 1



(57) Abstract: Provided herein are synthetic nucleic acid molecules and methods of using such synthetic nucleic acid molecules for strong repression of target gene expression. In particular, provided herein are methods for altering expression of a protein in a cell, where the method comprises introducing into a cell a protein coding sequence operably linked to a near-threshold translational repressor having first and second trigger recognition sequences that are fully or partially complementary to a repressing trigger RNA; and introducing into a cell the repressing trigger RNA.

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Synthetic Near-Threshold Translational Repressors

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/369,521, filed August 1, 2016; which is incorporated herein by reference as if set forth in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] Not applicable.

BACKGROUND

[0003] Synthetic biology is an emerging discipline that has great potential to respond to global pandemics. The increasing ability of synthetic biologists to repurpose and engineer natural biological components for practical applications has led to new opportunities for molecular diagnostics. Although recent advances have demonstrated the advantages of *de novo* design for riboregulators that increase gene expression in response to a trigger RNA, systems that demonstrate the inverse behavior, a decrease in gene expression upon triggering, have yet to be reported. Accordingly, there remains a need in the art for improved compositions, methods, and systems for strong repression of target gene expression.

BRIEF SUMMARY

[0004] In a first aspect, provided herein is a synthetic nucleic acid molecule comprising an RNA-sensing switch RNA sequence, wherein the switch RNA sequence comprises a near-threshold hairpin structure comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and first and second trigger recognition sequences located 5' and 3' to the hairpin structure, respectively; wherein the hairpin structure is configured to alternatively adopt a translationally active ON state or adopt a translationally inactive OFF state in the presence of a repressing trigger RNA, and wherein the second trigger recognition sequence does not encode an in-frame stop codon. The start codon can be located in a region of non-complementarity. The first trigger recognition sequence can have a length of 15 nucleotides. The second trigger recognition sequence can have a length of 12

nucleotides. The first and second trigger recognition sequences can be fully or partially complementary to a repressing trigger RNA.

[0005] In another aspect, provided herein is a method for altering expression of a protein, the method comprising providing a protein coding sequence operably linked to a near-threshold translational repressor having first and second trigger recognition sequences that are fully or partially complementary to a repressing trigger RNA; and providing the repressing trigger RNA, whereby expression of the protein is altered according to the level of repressing trigger RNA provided. Expression of two or more proteins can be altered by providing two or more protein coding sequences, each operably linked to a near-threshold translational repressor comprising first and second trigger recognition sequences that are fully or partially complementary to a single repressing trigger RNA. The fold-change in expression of the protein can be at least 25 fold. The fold-change in expression of the protein can be at least 50 fold.

[0006] In another aspect, provided herein is a method for altering expression of a protein in a cell, the method comprising introducing into a cell a protein coding sequence operably linked to a near-threshold translational repressor having first and second trigger recognition sequences that are fully or partially complementary to a repressing trigger RNA; and introducing into a cell the repressing trigger RNA, whereby expression of the protein is altered according to the level of repressing trigger RNA introduced to the cell. Expression of two or more proteins can be altered by introducing into the cell two or more proteins coding sequences, each operably linked to a near-threshold translational repressor comprising first and second trigger recognition sequences that are fully or partially complementary to a single repressing trigger RNA. The fold-change in expression of the protein can be at least 25 fold. The fold-change in expression of the protein can be at least 50 fold.

[0007] In another aspect, provided herein is a synthetic nucleic acid NAND logic circuit, wherein the NAND logic circuit comprises two or more near-threshold translational repressor (NeaTTR) hairpin modules comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and a trigger recognition sequence located 5' and 3' to the hairpin structure, wherein the NeaTTR hairpin modules are separated by a linker domain. The two or more NeaTTR hairpin modules can be operably linked to a downstream reporter element. The reporter element can be GFP or lacZ. The linker domain

can be 11 or 17 nucleotides. The NAND logic circuit can comprise three NeaTTR hairpin modules. The NAND logic circuit can comprise four or more NeaTTR hairpin modules.

[0008] In another aspect, provided herein is a synthetic nucleic acid NOR logic circuit, wherein the NOR logic circuit comprises at least one input RNA sensing hairpin module comprising an input RNA binding domain and a loop domain comprising first and second trigger RNA sequences; and a near-threshold translational repressor (NeaTTR) hairpin module comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and first and second trigger recognition sequences located 5' and 3' to the hairpin structure, respectively; wherein the first and second trigger recognition sequences are complementary to the first and second trigger RNA sequences, respectively, of the input RNA sensing hairpin module. The NeaTTR hairpin module can be operably linked to a reporter element. The reporter element can be GFP or lacZ. The NOR logic circuit can comprise two RNA sensing hairpin modules. The NOR logic circuit can comprise three RNA sensing hairpin modules. The NOR logic circuit can comprise four or more RNA sensing hairpin modules.

[0009] The foregoing and other advantages of the invention will appear from the following description. In the description, reference is made to the accompanying drawings, which form a part hereof, and in which there is shown by way of illustration a preferred embodiment of the invention. Such embodiment does not necessarily represent the full scope of the invention, however, and reference is made therefore to the claims and herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF DRAWINGS

[0010] The present invention will be better understood and features, aspects, and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

[0011] FIG. 1 is a schematic illustrating design of exemplary synthetic near-threshold translational repressors (NeaTTRs). The switch RNA (top) contains a weak RBS-bearing hairpin with sequence recognition sites on both sides to respond to the trigger or target RNA. Downstream gene expression is inactivated by the robust three-way junction structure formed

between the sensor and its cognate input trigger. The bottom of the switch RNA hairpin features a weak 5-bp stem engineered to form transiently and breathe open at temperatures near 37°C.

This unstable structure enables the switch RNA to allow translation of the downstream gene in the absence of the trigger RNA and to facilitate trigger RNA binding to deactivate expression.

[0012] FIG. 2 is a graph depicting ON/OFF GFP fluorescence levels obtained 5 hours (hr) after induction with IPTG for 48 NeaTTRs. Relative errors for the switch ON/OFF ratios were obtained by adding the relative errors of the switch ON and OFF state fluorescence measurements from three biological replicates in quadrature. Inset: ON/OFF GFP fluorescence measured for repressors of varying performance levels at different time points (3 hr, 4 hr, 5 hr) following induction.

[0013] FIG. 3 presents representative flow cytometry histograms of GFP fluorescence for three NeaTTRs with varying performance levels. Flow cytometry measurements were taken 5 hours after induction. OFF state (repressor with cognate trigger) is displayed on top of ON state (repressor with silent trigger, a control RNA with high secondary structure). Flow cytometry GFP fluorescence histograms for the three repressors showed an ON/OFF ratio of 126, 280, and 783, respectively.

[0014] FIGS. 4A-4D present data of an assessment of NeaTTR orthogonality. (a) Fold change for all trigger-switch combinations. Fold change was determined by taking the GFP output for a trigger-switch combination and dividing it by the GFP output from the switch with its cognate trigger. (b) Percent repression levels for all trigger-switch combinations. Percent repression was calculated by subtracting fluorescence output from the average non-cognate fluorescence of this switch and then dividing by the average value. (c) Library dynamic range in fold change and orthogonal library size. (d) Library dynamic range in cross-repression and orthogonal library size. The overall dynamic range corresponds to the minimum ON/OFF ratio (c) or maximum cross-repression (d) to expect in a network employing an orthogonal sub-library of switches of the specified size.

[0015] FIGS. 5A-5C are graphs demonstrating *in vitro* validation of NeaTTRs by plate reader. (a) GFP synthesis kinetics for NeaTTR N08 in presence of its cognate or silent trigger. (b) GFP synthesis kinetics for NeaTTR N09 in presence of its cognate or silent trigger. (c) ON/OFF GFP ratio at four time points (1hr, 2hr, 3hr, 4hr).

[0016] FIGS. 6A-6B are schematics illustrating NOT-AND (“NAND”) gates based on NeaTTRs. (a) Type I NAND gate design. Multiple repressor switches are aligned in upstream of the regulated gene in their active state. Complementary triggers serve as input RNAs that are required to block all active switches to turn off the gene expression. (b) Type II NAND gate design. It requires two partially complementary triggers to hybridize and give a functional trigger to switch off the gene expression.

[0017] FIG. 7 is a schematic illustrating a *N*-Input NOT-OR (“NOR”) Gate RNA. A single NeaTTR module is used to detect a cognate trigger RNA of sequence **a-b**. An array of input RNA sensing hairpins upstream of the NeaTTR module contain trigger RNA sequences sequestered within their loops. Upon binding of an input RNA, the hairpin sensing domain is unwound to free the trigger sequence. The trigger sequence then binds to the NeaTTR module through an intramolecular refolding mechanism and in turn deactivates gene expression.

[0018] FIGS. 8A-8C present *in vivo* measurements of multiple-input Type I NAND gates constructed from concatenated NeaTTR hairpin modules. (A) Truth tables obtained for six different two-input NAND gates constructed from different pairs of NeaTTR modules regulating GFP. (B) Truth tables obtained for four different three-input NAND gates constructed from different sets of NeaTTR modules regulating GFP. (C) Truth tables obtained for three different four-input NAND gates constructed from different sets of NeaTTR modules regulating GFP. Fold reduction of GFP fluorescence represents the fold decrease in GFP fluorescence obtained for the different input conditions compared to the NULL input case where no cognate inputs are expressed. All circuits show the desired NAND logic behavior with large reductions in GFP output only occurring in the logical FALSE case with all input RNAs expressed in the cell. GFP expression remains relatively high for the logical TRUE cases where at least one input RNA is not expressed. The geometric mean of GFP fluorescence was calculated via flow cytometry 6 hours after induction of RNA expression with IPTG.

[0019] FIG. 9 presents *in vivo* measurements of 29 one-input (aka 1-bit) NOR constructs. Fold-change GFP from geometric mean is measured after 5 hours of induction of input and switch RNA expression in the cell. The one-input (aka 1-bit) NOR constructs can be used to construct multi-bit NOR gates.

[0020] FIG. 10 presents *in vivo* measurements of a two-input (aka 2-bit) NOR gate. Fold reduction in GFP from the geometric mean is measured after induction of input and switch RNA

expression in the cell. The two-input NOR gate RNA was constructed by combining sensor domains from two validated one-input (1-bit) NOR constructs.

[0021] FIG. 11 presents *in vivo* measurements of NeaTTRs designed to detect full length mRNAs conferring antibiotic resistance and the *E. coli* small RNA *ryhB*. The mRNAs *cat* and *kanR* confer chloramphenicol and kanamycin resistance, respectively. GFP fluorescence was measured 5 hours after induction of RNA expression using IPTG.

[0022] While the present invention is susceptible to various modifications and alternative forms, exemplary embodiments thereof are shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the description of exemplary embodiments is not intended to limit the invention to the particular forms disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the spirit and scope of the invention as defined by the appended claims.

DETAILED DESCRIPTION

[0023] All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though set forth in their entirety in the present application.

[0024] The compositions, methods, and systems provided herein are based at least in part on the inventors' discovery of near-threshold translational repressors (NeaTTRs), a class of synthetic translational repressors comprising an RNA-sensing switch RNA and a repressing trigger RNA. The switch RNA possesses an unstable hairpin structure that can adopt a translationally active ON state or can enter a less active closed state to facilitate binding of the trigger RNA. We have designed and tested a library of 48 different NeaTTRs in *E. coli*. A subset of 20 NeaTTRs provide ON/OFF expression modulation of at least 50-fold, which corresponds to at least 98% repression. In contrast, previously reported translational repressors have been limited to at most 90%. Through comprehensive investigations of a library of 16 high performance devices, the inventors identified a set of 15 NeaTTRs that provide at least 40-fold repression and exhibit limited crosstalk interactions. NeaTTRs are also active in cell-free systems, signaling their potential use in paper-based or *in vitro* diagnostic devices. Also described herein are new methods of integrating NeaTTR elements into extended information-processing RNAs and trigger RNA complexes to achieve complex, multi-input logic expressions

that can be deployed *in vivo*, *in vitro*, or on paper. NeaTTRs can also be used for the detection of nearly arbitrary RNAs, including endogenous RNAs expressed by the cell or those associated with pathogens.

[0025] For purposes of convenience in the description, references to nucleic acid elements such as start codons, ribosomal binding site, 5' UTR, stem-loop, etc., may refer to either the RNA form or to the DNA form (i.e., to a DNA molecule that provides a template for transcription of the RNA). Similarly, when reference is made to modifying an RNA (e.g., by inserting an element such as a cis-repressive sequence) into the RNA, it is to be understood that the modification is generally accomplished by engineering the appropriate modification to a DNA molecule that provides a template for transcription of the RNA.

[0026] Accordingly, in a first aspect, provided herein is a synthetic RNA regulator that exploits a very unstable RNA secondary structure to enable signal output in the device ON state by facilitating ribosomal access and low OFF state expression by facilitating binding of a cognate trigger RNA with nearly arbitrary sequence. Such synthetic RNA regulators are referred to herein as “near-threshold translational repressors” (NeaTTRs) or “near-threshold repressor riboregulators.” As used herein, the term “near-threshold” refers to a switch RNA that has a secondary structure that is very close to being either in the translationally active or translationally repressed state, which enables the switch RNA to respond strongly to trigger RNA interactions that perturb its original secondary structure by a small number of base pairs.

[0027] As used herein, the terms “synthetic” and “engineered” are used interchangeably and refer to a non-naturally occurring nucleic acid molecule that has been created or modified by the hand of man (e.g., using recombinant DNA technology) or is derived from such a molecule (e.g., by transcription, translation, etc.). A nucleic acid molecule may be similar in sequence to a naturally occurring nucleic acid but typically contains at least one artificially created insertion, deletion, inversion, or substitution relative to the sequence found in its naturally occurring counterpart. A cell that contains a synthetic or engineered nucleic acid is considered to be an engineered cell.

[0028] In preferred embodiments, a near-threshold translational repressor is a synthetic nucleic acid molecule comprising an RNA-sensing switch RNA sequence, where the switch RNA sequence comprises an unstable hairpin structure comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising an initiation

codon, and first and second trigger recognition sequences located 5' and 3' to the hairpin structure, respectively; wherein the hairpin structure is configured to alternatively adopt a translationally active ON state or adopt a translationally inactive OFF state in the presence of a repressing trigger RNA, and wherein the second trigger recognition sequence does not encode an in-frame stop codon. A "hairpin" or "hairpin structure" refers to an intramolecular stem-loop structure of a nucleic acid sequence at the chosen assay temperature mediated by hybridization of partially or fully complementary sequences at the 5'- and the 3'-end of the nucleic acid sequence. As used herein, the term "unstable hairpin" refers to an RNA stem-loop structure that is not thermodynamically or kinetically favored (or is only weakly thermodynamically or kinetically favored). Such structures can adopt other secondary structures, either transiently or for longer periods of time, which can include additional unpaired bases. In some cases, unstable structures are characterized in terms of the fraction of time that they spend in the hairpin stem-loop form versus an unpaired structure where translation can occur. For example, the stem-loop structure may be more favorable (and be present for ~51% of the time), but the stem-loop can open transiently and, thus, transiently adopt a more unstable structure, at which point the ribosome can bind and begin translation. The fraction of time spent in the different states depends on the sequence and the temperature. As used herein, the terms "trigger" and "trigger recognition sequence" refer to a RNA molecule capable of binding to single-stranded regions immediately upstream and downstream of the switch RNA hairpin in order to form a structure that represses translational output.

[0029] As described in the following paragraphs and the Example provided below, near-threshold translational repressors of the invention comprise generally switch and trigger RNAs designed to fully or partially hybridize with one another. The switch RNA contains the coding sequence of the target gene being regulated. Referring to FIG. 1, near-threshold translational repressors comprise a hairpin structure containing an RBS sequence (AGAGGAGA) within its loop is located upstream of the target gene's open reading frame. The hairpin structure is followed by stem-forming region comprising a start codon (AUG), situated 6 nucleotides (nts) downstream. Preferably, the hairpin structure comprises a stem having a total of 11 base pairs and a 3-nt region of non-complementarity (i.e., a bulge) formed by the start (initiation) codon. The upper part of this stem can comprise 6 base pairs and is sufficiently weak to enable access to the RBS by the ribosome. The bottom part of this stem is a weak 5-bp region with the sequence

AACAA immediately downstream of the start codon. Importantly, this 5-bp region is sufficiently weak to make base pairing at the bottom of the hairpin transient. This unstable structure thus allows the ribosome to begin translation when the trigger RNA is absent. Upstream and downstream of the unstable hairpin are single-stranded trigger recognition sites that are 15 nts and 12 nts long, respectively. Between the 12-nt sensing sequence and the output gene is a linker sequence coding for a small additional peptides on the N terminus of the gene of interest. The loop of the switch can comprise 10 nts to 27 nts. The upper part of the stem can comprise 2 nts to 8 nts. The bottom part of the stem can comprise 2 nts to 8 nts. The trigger RNA comprises a 27-nt target sequence, where 10 nucleotides on its 5' end are hidden within a small hairpin, leaving only 17 nts exposed to minimize unintended interactions between the switch and non-cognate triggers.

[0030] In some cases, the near-threshold translational repressors (NeaTTRs) described herein comprise trigger RNAs having toehold lengths of shorter than 17 nts. Without being bound by any particular theory or mechanism, it is expected that trigger RNAs can have shorter RNA toehold lengths, thus providing more substantial secondary structure, and retain the high repression levels of the riboregulators while having improved orthogonality. Accordingly, the NeaTTRs described herein include those having trigger types featuring fewer exposed single-stranded domains. In some cases, the toehold domain length of the trigger is decreased while the size of the stem in the trigger is increased for improved orthogonality. In other cases, the triggers do not feature any toehold but have a stem with a loop of 6 nts or more. Such loop-mediated triggers will first bind to the linear sensing site of the switch RNA and unwind their stems to bind through the full 27-nt recognition site.

[0031] In some cases, a NeaTTR as provided herein comprises a shortened trigger sequence that is predicted to provide strong repression. For example, triggers shorter than 10 nucleotides nucleotides (e.g., 10, 9, 8, 7, 6 nucleotides, inclusive) can be used to provide substantial repression and improved orthogonality. In addition, triggers that have regions shorter than 10 nucleotides (e.g., 10, 9, 8, 7, 6 nucleotides, inclusive) that bind to the switch RNA be used to provide substantial repression and improved orthogonality. In some cases, the shorter trigger sequences are used in addition to the 5-bp unstable stem. Without being bound to any particular mechanism or mode of action, it is understood that shorter triggers that reduce the post-start codon duplex length and bind to locations further to the 3' end of the switch RNA will still

provide substantial repression and offer improved orthogonality. Coupling these shortened triggers with new secondary structures yield further enhancement.

[0032] When the switch and cognate trigger RNAs are both present, the two recognition sequences flanking the RBS hairpin bind to the trigger RNA through a kinetically favorable linear-linear interaction between **b** and **b*** domains (FIG. 1). This linear-linear interaction is independent of the current folding state of the near-threshold switch hairpin. When the 5-bp bottom stem of the hairpin stem forms transiently, the two exposed bases of the trigger **a** domain are able to bind to the corresponding bases downstream of the hairpin. The stem of the trigger RNA then completes a branch migration process to unwind and enable complete binding of the trigger RNA to the switch. This interaction forms the final inactivated three-way junction structure. In both prokaryotic and eukaryotic systems, the ribosome must be able to gain access to the start codon. Since the inactivated three-way junction structure robustly sequesters the RBS and start codon, downstream protein translation is strongly repressed under such conditions.

[0033] It is important to note that the trigger RNA can also adopt a single-stranded structure and still repress the switch RNA. The hairpin structure in the trigger RNA depicted in FIG. 1 is utilized purely to reduce the number of exposed single-stranded bases in the trigger to decrease the likelihood of crosstalk between repressors when deployed in the same cell or reaction volume. In addition, other methods can be employed to reduce the number of exposed bases in the trigger, for instance by enclosing much of the binding sequence within the loop of a stem-loop structured trigger as described herein. Such a trigger RNA would exploit a loop-linear interaction in order to bind to and inactivate the switch RNA. The NeaTTR design places few constraints on the sequence of the trigger RNA. The only major constraint is the requirement that the recognition sequence downstream of the hairpin (domain **a***) not code for an in-frame stop codon. This constraint can be avoided by moving the trigger RNA binding site by one or two nucleotides or by adding regions of non-complementarity (i.e., bulges) between the trigger RNA and the switch RNA to remove the stop codon.

[0034] As used herein, the term “non-complementarity” refers to an entity in a double stranded region of an RNA composition (wherein the double strand nature of the RNA composition may arise from intramolecular hybridization within one RNA molecule and/or arise from intermolecular hybridization between two RNA molecules) that comprises non-complementary nucleotides between the two strands of the double stranded region. Thus, the

region may be defined as a region of non-complementary nucleotides flanked by regions of double stranded RNA. In specific embodiments, the length of non-complementation is at least about 5 nucleotides. In other specific embodiments, the junction between the bubble and double stranded region comprises at least two T's. The terms "bubble" or "bulge" may also be used for the term "region of non-complementarity." It will be understood that the terms "bubble" and "bulge" imply no specific shape of said region, although in some embodiments it is shaped as a bubble. Complementarity of two sequences is generally determined by dividing the total number of nucleotides that participate in complementary base pairs (GC, AU, AT) when the sequences are aligned to produce the maximum number of complementary base pairs, counting all nucleotides in the two sequences (including those in bulges, mismatches, or inner loops) by the total number of nucleotides contained in both sequences. For example, consider two sequences of 19 and 20 nucleotides in length in which alignment to produce the maximum number of complementary base pairs results in 16 base pairs, 1 inner loop of 2 nucleotides, 1 mismatch, and 1 bulge (in the sequence with 20 nucleotides). The percent complementarity of the two sequences is $[(16+17)/39]100$. It will be appreciated that complementarity may be determined with respect to the entire length of the two sequences or with respect to portions of the sequences. As used herein, two sequences are considered "substantially complementary" herein if their complementarity is at least 50%.

[0035] As shown in FIG. 1, near-threshold translational repressors are in some cases operably linked to a reporter element (e.g., an *E. coli* lacZ reporter element encoding β -galactosidase) that is 3' to the hairpin structure. As used herein, the term "operably linked" refers to a relationship between two nucleic acid sequences wherein the production or expression of one of the nucleic acid sequences is controlled by, regulated by, modulated by, etc., the other nucleic acid sequence. Reporter proteins appropriate for the methods provided herein include, without limitation, enzymatic reporters (e.g., β -galactosidase, alkaline phosphatase, DHFR, CAT), fluorescent or chemiluminescent reporters (e.g., GFP, GFP variants, mCherry, luciferase, e.g., luciferase derived from the firefly (*Photinus pyralis*) or the sea pansy (*Renilla reniformis*) and mutants thereof), etc.

[0036] In another aspect, provided herein are methods of altering gene expression using the synthetic nucleic acid molecules described herein. In some cases, the method alters expression of an enzyme (or other protein) in a cell and comprises introducing into a cell an enzyme coding

sequence operably linked to a near-threshold translational repressor having first and second trigger recognition sequences that are fully or partially complementary to a repressing trigger RNA; and introducing into a cell the repressing trigger RNA, whereby expression of the enzyme is altered according to the level of repressing trigger RNA introduced to the cell.

[0037] In some cases, near-threshold translational repressors can be used to regulate expression of one or more polypeptides, including polypeptides representing entire metabolic pathways. In such cases, the method includes placing one or more polypeptides (e.g., an enzyme in a metabolic pathway) under control of NeaTTRs that respond to the same trigger RNA. The inventors have demonstrated that the near-threshold translational repressors provided herein yield unexpectedly high fold-change values, often in the 100-fold range. As used herein, the terms “expressing,” “expression,” or “express” refer to the production of a gene product (e.g., an mRNA transcript from a nucleic acid sequence encoding thereof). As used herein, the terms “gene product” and “expression product” generally refer to an RNA transcribed from the gene or a polypeptide encoded by an RNA transcribed from the gene. Thus a regulatory element, environmental condition, stimulus, etc., that alters the level of transcription or the stability of an RNA transcribed from a gene or alters its ability to serve as a template for translation will be said to alter expression of the gene. Similarly, a regulatory element, environmental condition, stimulus, etc., that alters the level of translation or stability of a polypeptide translated from an RNA transcribed from the gene will be said to alter expression of the gene.

[0038] Near-threshold translational repressors have multiple potential applications *in vitro* for diagnostic purposes. Integration of these near-threshold translational repressors into a low-cost, portable, and stable cell-free platform, such as one described by Pardee et al., *Cell* 159:940-954 (2014) and Pardee et al., *Cell* 165:1255-1266 (2016), could enable the applications below to be realized at under \$1 per test and deployed either in the field, in remote clinics, at the point of care, or even in the home.

[0039] In some cases, near-threshold translational repressors are incorporated into complex, multi-input logic circuits. For diagnostic purposes, multi-input logic circuits can be used to increase assay specificity or sensitivity. For instance, a NOT-AND (“NAND”) expression can reduce false positives by ensuring that more than one pathogen-associated RNA is present in a sample. A NOT-OR (“NOR”) expression can reduce false negatives by sensing more than one RNA in the same sample. *In vivo*, a multi-input logic circuit can be used to sense the set of

RNAs produced by a prokaryote in response to environment stresses. This RNA signature can be used to modulate production of enzymes or identify cells that are susceptible to antibiotics or screen for new antibiotic compounds. In addition, the circuits that sense a set of RNAs expressed by the host could be used to generate whole-cell biosensors that detect toxic chemicals in the environment.

[0040] A first synthetic nucleic acid logic circuit is the NOT-AND ("NAND") gate RNA. As described herein, one type of NAND gate RNAs has a Type 1 geometry and comprise two or more NeaTTR hairpin modules. The two or more NeaTTR hairpin modules are separated by a linker sequence. In some embodiments, the linker sequence is 11-nt or 17-nt in length such that the NeaTTR hairpins remain in the same reading frame and to ensure that they are single stranded when expressed as part of the gate RNA. In some embodiments the two or more NeaTTR hairpin modules are upstream of an operably linked reporter element. In some embodiments, the reporter element is GFP or lacZ. In some embodiments, the NAND gate comprise two NeaTTR hairpin modules. In some embodiments, the NAND gate comprises three NeaTTR hairpin sequences. In some embodiments the NAND gate comprises four NeaTTR hairpin sequences. In some embodiments, the NAND gate comprises more than four NeaTTR hairpin sequences. It is envisioned that each NeaTTR module in a single NAND gate RNA maybe be specific to a separate input RNA trigger sequences.

[0041] As described herein, a second type of NAND gate RNA has a Type 2 geometry and comprises a NeaTTR hairpin module, wherein the NeaTTR hairpin module comprises two input RNA recognition sites that are partially complementary to a first and a second input RNA sequence. The two input RNA recognition sites are located on the 5' and 3' regions upstream and downstream of the hairpin and each domain is designed to complement a separate input RNA sequence. For example, the 5' upstream input RNA recognition sequence is complementary to a first input RNA sequence and the 3' downstream input RNA recognition sequence is complementary to a second input RNA sequence. The two input RNAs are also partially complementary to one another and form a two-RNA complex that efficiently binds to the two input RNA recognition sites to repress translation from the NeaTTR hairpin module. In some embodiments, the input RNA complex comprises three partially complementary input RNAs that binds to the NeaTTR hairpin module. In some embodiments, the input RNA complex comprises four partially complementary input RNAs that binds to the NeaTTR hairpin module. In some

embodiments, the input RNA complex comprises more than four partially complementary input RNAs that binds to the NeaTTR hairpin module.

[0042] As described in the Example that follows, multiple NAND gate RNAs have been successfully implemented using validated NeaTRRs in Type I NAND geometries. For example, NAND gate RNAs were constructed by concatenating multiple NeaTTR hairpin modules upstream of the GFP open reading frame. The hairpin modules were separated by 11-nt or 17-nt spacer RNA sequences. These spacers ensured that the NeaTTR hairpins remained in the same reading frame and were designed to ensure that they were single stranded when expressed as part of the gate RNA. Referring now to FIG. 8, flow cytometry can be used to measure the geometric mean GFP fluorescence of cells expressing NAND gates and different combinations of input RNAs. The fold reduction in GFP was calculated by taking the GFP fluorescence measured for cells not expressing any cognate input RNAs (i.e., the NULL input case) and dividing it by the GFP fluorescence measured for cells expressing a specific combination of input RNAs. Measurements of six different two-input NAND gate circuits are shown in FIG. 8A. The NAND gate RNAs were constructed from pairs of NeaTTR hairpins upstream of GFP. For all devices, high fold reductions in GFP were obtained only when both input RNAs are expressed, which corresponds to the sole logical FALSE condition. In the other input combinations, GFP levels were not substantially reduced, as expected for these logical TRUE conditions. All six two-input NAND circuits thus show the desired NAND logical behavior. FIG. 8B presents measurements from a set of four different three-input NAND circuits. These circuits employed three NeaTTR hairpin modules concatenated upstream of GFP. Strong reductions in GFP output were only observed for the logical FALSE condition with all input RNAs expressed, while much smaller changes are observed for the logical TRUE states. FIG. 8C shows measurements from three exemplary four-input NAND circuits constructed from combinations of four NeaTTR hairpin modules arrayed upstream of GFP. These circuits also show the desired GFP output behavior in response to different combinations of input RNAs. The four-input devices showed lower fold reductions in general compared to the two-input and three-input circuits; however, the observed reductions were still substantially higher for the logical FALSE state compared to the logical TRUE states.

[0043] A second synthetic nucleic acid logic circuit is the NOT-OR (“NOR”) gate RNA. As described herein, the NOR gate RNA comprises one or more RNA sensing hairpin modules

comprising trigger RNA sequences within a loop domain and an input RNA binding domain, wherein the one or more RNA sensing hairpin modules are upstream of a NeaTTR module comprising an unstable hairpin structure comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising an initiation codon, and first and second trigger recognition sequences located 5' and 3' to the hairpin structure, respectively. The trigger sequences within the loop domain of the RNA sensing hairpin modules are complementary to trigger recognition sequences within the NeaTTR module. Binding of an input RNA to the input binding domain of the RNA sensing hairpin module initiates an intramolecular refolding reaction in which the trigger sequences of the RNA sensing hairpin bind the complementary trigger recognition sequences of the NeaTTR. In some embodiments, the NeaTTR is operably linked to a downstream reporter sequence. In some embodiments, the reporter sequence is a sequence encoding GFP or lacZ. In some embodiments, one input RNA sensing hairpin module is upstream of the NeaTTR module. In some embodiments, two input RNA sensing hairpin modules are upstream of the NeaTTR module. In some embodiments, three or more RNA sensing hairpin modules are upstream of the NeaTTR module. It is envisioned that each RNA sensing hairpin module in a single NOR gate RNA maybe be specific to a separate input RNA sequence.

[0044] With respect to "NOR" gates, FIG. 9 illustrates the basic molecular mechanism of NOR gate RNAs in a simplified 1-bit system in which a single sensor hairpin is positioned upstream of a NeaTTR module. In such cases, a trigger RNA A can bind to the A* binding site in the sensor hairpin and unwind its stem. This event then exposes an intramolecular trigger containing the a and b sequences that can then bind to the downstream NeaTTR module at sites a* and b* to repress translation of the output gene. As described in the Example that follows, flow cytometry can be used to measure gate RNAs several hours after induction of RNA expression using IPTG. The gate RNAs displayed wide variations in function, with some displaying very little change in GFP output upon expression of the cognate input RNA. However, 7 out of the 29 devices (24%) provided at least 50-fold reductions in GFP expression upon detection of the input RNA and two provided very large reductions of over 200-fold.

[0045] In some cases, near-threshold translational repressors are useful in diagnostic methods. Devices that repress translation can be used for turning off expression of an enzyme that is otherwise fed forward in the circuit (e.g. a transcription factor or an RNA polymerase).

This type of control can halt all downstream reactions based on the downstream gene. In some cases, near-threshold translational repressors are useful for the detection of natural RNAs. Since the a* and b* domains within NeaTTR hairpins (referring to FIG. 1) can adopt virtually any sequence, they can be used to detect natural RNAs associated with drug resistance, viruses, hereditary diseases, and microorganisms. Referring now to FIG. 11, NeaTTRs can be responsive to natural RNAs. For example, systems can be configured to turn off expression of GFP in response to detection of their target RNAs and bind to the target sequence 5'-b-a-3'. In some cases, reductions of GFP fluorescence are 6-fold or more. Referring now to FIG. 11, an exemplary sensor, which was configured to detect the catalase mRNA (*cat*) responsible for chloramphenicol resistance, provided a fold reduction of 15 upon *cat* binding. Riboregulators that turn off protein expression in response to a target pathogen-, resistance- or disease-related RNA could be used, for instance, as kill switches that trigger cell death when a bacterium picks up a drug resistance mutation or a resistance-conferring plasmid (e.g., a toxin-antitoxin system can be disrupted when the riboregulator ceases production of the antitoxin). These riboregulators can also be deployed using cell-free systems for RNA detection in a diagnostic device.

[0046] NeaTTRs can also be used to implement time-dependent reactions and/or control the amount of a protein produced. For example, a switch RNA can be transcribed at the outset of the diagnostic reaction to begin production of its output protein. At a later time, a trigger RNA can be added or expressed that turns off production of the output protein, thus creating a time-dependent expression profile. Repressors can also be used in complementary reactions with activating riboregulators. In this context, a repressor would be used to detect the same target RNA as an activator. Interaction of the target RNA with both riboregulators would result in changes in the production of two enzymes, and yield clearer diagnostic results. Repressor systems can also be used in diagnostic systems for control reactions to determine if a technician is carrying out an assay properly. For instance, the repressor can be used to detect a control RNA or DNA that is amplified along with a sample from a patient. If repressor output is low, it means that the amplification reaction was successful (provided the cell-free reaction is active). If repressor output is high, it means that the amplification reaction failed but that the cell-free diagnostic stage was successful. An activating riboregulator to detect a control RNA would also be used for such a set of validation assays to determine if the cell-free reaction is functional when amplification is successful.

[0047] 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 the invention pertains. All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

[0048] All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

[0049] The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

[0050] The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, i.e., “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[0051] As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

[0052] As used herein, the terms “approximately” or “about” in reference to a number are generally taken to include numbers that fall within a range of 5% in either direction (greater than or less than) the number unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value). Where ranges are stated, the endpoints are included within the range unless otherwise stated or otherwise evident from the context.

[0053] The present invention has been described in terms of one or more preferred embodiments, and it should be appreciated that many equivalents, alternatives, variations, and modifications, aside from those expressly stated, are possible and within the scope of the invention. The invention will be more fully understood upon consideration of the following non-limiting Examples.

EXAMPLE

[0054] This example demonstrates near-threshold translational repressors (NeaTTRs) and uses thereof.

[0055] *In Vivo NeaTTR Validation:* A total of 48 NeaTTRs were designed and constructed (Table 1), and the 48 pairwise interactions between the cognate trigger RNA and a non-cognate trigger RNA with high secondary structure (the “silent” trigger) were tested in *E. coli* BL21 star DE3. Green fluorescent protein (GFP) was used as the output protein for characterizing switch output through flow cytometry. ON state expression levels were obtained from cells expressing the switch and a non-cognate trigger. OFF state expression levels were obtained from cells expressing the switch and its cognate trigger. Flow cytometry histograms were then used to obtain the median GFP expression from both ON and OFF strains and then used to compute the ON/OFF ratio. Considerable variations in ON/OFF ratios among all repressors were observed (FIG. 2), but overall the cognate pairs showed significant repression with an average of 50-fold change in GFP output. Out of the 48 devices, 12 provided ON/OFF levels exceeding 100-fold and 5 provided ON/OFF exceeding 250-fold. Thus a substantial fraction of these NeaTTRs provided high dynamic range without any additional parameter tuning from empirical measurements. We expect that further improvements can be gained through forward engineering approaches we have previously described⁶.

[0056] Time-course measurements revealed increasing levels of repression over time as ON state cells produced increasing amounts of GFP. A set of four different NeaTTRs with four different levels of dynamic range are shown in the inset of FIG. 2. Differences in the ON/OFF levels of the four devices increased over time to a final value of approximately 780-fold for the highest performance NeaTTR (NeaTTR No. 1) after 5 hours of induction.

[0057] Representative flow cytometry histograms of three different repressors are provided in FIG. 3. In general, the OFF state of the repressors display wider fluorescence distributions. In particular, NeaTTR 13 possessed a nearly bimodal OFF state distribution with the dominant population at the higher fluorescence level. This distribution lead to the overall lower ON/OFF ratio obtained for NeaTTR 13 compared to devices 1 and 5. The ON state expression of the devices exhibited much tighter distributions overall and provide very high absolute levels of translation.

[0058] *Evaluation of NeaTTR orthogonality:* To measure the orthogonality of the devices, we isolated a subset of 16 repressors that displayed high ON/OFF GFP levels at 5 hours and assayed in *E. coli* all 256 pairwise combinations of switch and trigger plasmids. The silent trigger with high secondary structure was also tested as the 17th trigger for all switches. We used flow cytometry to quantify GFP output from all interactions. FIGS. 4A-4B provide orthogonality matrices depicting the degree of crosstalk between devices. FIG. 4a shows the ON/OFF levels obtained from all pairwise interactions. These values were obtained along each column by taking the fluorescence level for each interaction with the switch RNA and dividing it by the fluorescence level obtained with cognate trigger RNA. Thus, all values along the diagonal of FIG. 4a are equal to 1. Superimposed on most of the elements in the orthogonality matrix are numbers giving the ON/OFF level for each pairwise combination having a fold change of at least 40. Almost all noncognate pairs showed over 50-fold GFP fluorescence output compared with its cognate pair. FIG. 4b provides an orthogonality matrix obtained from percent repression levels. These percent repression levels were determined by first calculating the average GFP expression level of the switch RNA when expressed with each of the 15 non-cognate triggers in the library and the silent trigger. This mean ON state expression level was then used to determine the percent repression levels for all 17 different triggers expressed against a given switch. Based on this definition using the mean ON state, roughly half of the switch-trigger combinations will actually provide enhanced expression levels and negative percent repression. Although this

metric may sound counter-intuitive, it does provide a good measure of the typical percent repression to expect for a switch RNA when the cognate trigger is expressed. Furthermore, it removes the effects of outlier expression levels obtained for specific non-cognate triggers that can exaggerate the degree of crosstalk between devices.

[0059] Using the orthogonality matrices shown in FIGS. 4a-4b, we then determined subsets of the 16 member high performance NeaTTR library that provided a desired threshold level of crosstalk. In terms of fold-change measurements, 15 riboregulators formed a subset expected to provide at least 40-fold ON/OFF levels if used in combination (FIG. 4c). This 40-fold mark means that you would expect to have a 40-fold difference between each repressor in the orthogonal set when its corresponding trigger RNA is expressed compared to the ON-state expression level when any of the other non-cognate triggers is expressed. For more stringent crosstalk requirements, a subset of 8 devices will provide at least 100-fold dynamic range and a subset of 6 devices 200-fold dynamic range. FIG. 4d depicts the size of orthogonal subsets obtained when applying the same considerations to the percent repression experimental data. An orthogonal subset of 8 devices, for instance, will provide cross-repression levels of below 15% if deployed at the same time. A subset of 9 devices will provide cross-repression levels of at most 20%. Previously reported repressing riboregulators have demonstrated lower degrees of orthogonality compared to the ones demonstrate here. Mutalik *et al.* showed that they could generate a set of six translational riboregulators with under 20% crosstalk⁴, while Takahashi described a library of seven transcriptional attenuators with under 20% crosstalk in percent repression. Using shortened triggers or those with reduced trigger sequence accessibility, we expect that the orthogonality of our repressors can be substantially improved. Furthermore, our repressors demonstrate markedly higher fold-change values often in the 100-fold range while those of Mutalik *et al.* and Takahashi *et al.* are generally below 20-fold.

[0060] *In vitro validation by plate reader:* Cell-free tests were conducted to validate NeaTTR performance *in vitro*. Plasmids for transcribing two representative switches were mixed with their cognate trigger or silent trigger in 1:4 ratio in cell free reaction system, and GFP synthesis kinetics was monitored by plate reader as shown in FIGS. 5a-5b. A cell-free reaction system without any plasmids was used as background which was later subtracted from GFP output for calculating GFP ON/OFF ratio in FIG. 5c. Switches with cognate triggers clearly showed significant repression in GFP synthesis, and displayed 5 to 6 fold ON/OFF GFP ratio

after 1 hour of reaction. The successful use of NeaTTRs in cell-free systems demonstrates that these riboregulators could be used for regulating synthetic gene networks in vitro and could be deployed as part of cell-free diagnostics used either in liquid form or in paper-based (or, more generally, porous substrate) reaction systems^{10,11}.

[0061] *In vivo evaluation of NeaTTR Type I NAND logic gates:* Tests of three Type I NAND gates (FIG. 6A) were conducted in *E. coli*. Each of these NAND gates consists of two NeaTTR unstable switches upstream of a GFP reading frame and thus evaluates the expression NAND(A,B). Input A corresponds to the trigger RNA that binds to the 5' NeaTTR switch module and input B corresponds to the trigger RNA that binds to the 3' NeaTTR switch module.

[0062] FIGS. 8A-8C show the performance of multiple NAND gate RNAs hours after induction of RNA transcription. We have successfully implemented multiple NAND gate RNAs using validated NeaTTRs in Type I NAND geometries. NAND gate RNAs were constructed by concatenating multiple NeaTTR hairpin modules upstream of the GFP open reading frame. The hairpin modules were separated by 11-nt or 17-nt spacer RNA sequences. These spacers ensured that the NeaTTR hairpins remained in the same reading frame and were designed to ensure that they were single stranded when expressed as part of the gate RNA. Data were obtained by using flow cytometry to measure the geometric mean GFP fluorescence of cells expressing the NAND gates and different combinations of input RNAs. The fold reduction in GFP was calculated by taking the GFP fluorescence measured for cells not expressing any cognate input RNAs (i.e., the NULL input case) and dividing it by the GFP fluorescence measured for cells expressing a specific combination of input RNAs. Measurements of six different two-input NAND gate circuits are shown in FIG. 8A. See Table 4. The NAND gate RNAs were constructed from pairs of NeaTTR hairpins upstream of GFP. For all devices, high fold reductions in GFP are obtained only when both input RNAs are expressed, which corresponds to the sole logical FALSE condition. In the other input combinations, GFP levels are not substantially reduced, as expected for these logical TRUE conditions. All six two-input NAND circuits thus show the desired NAND logical behavior.

[0063] FIG. 8B presents measurements from a set of four different three-input NAND circuits (Table 5). These circuits employed three NeaTTR hairpin modules concatenated upstream of GFP. Strong reductions in GFP output were only observed for the logical FALSE condition with all input RNAs expressed, while much smaller changes were observed for the

logical TRUE states. FIG. 8C shows measurements from three different four-input NAND circuits (Table 6) constructed from combinations of four NeaTTR hairpin modules arrayed upstream of GFP. These circuits also showed the desired GFP output behavior in response to different combinations of input RNAs. The four-input devices showed lower fold reductions in general compared to the two-input and three-input circuits; however, the observed reductions were still substantially higher for the logical FALSE state compared to the logical TRUE states.

[0064] Validation of “1-Bit” NOR Gates

[0065] We have confirmed the basic molecular mechanism of the NOR gate RNAs using a simplified 1-bit system in which a single sensor hairpin is positioned upstream of a NeaTTR module (FIG. 9, inset). In this gate RNA, a trigger RNA A can bind to the A* binding site in the sensor hairpin and unwind its stem. This event then exposes an intramolecular trigger containing the a and b sequences that can then bind to the downstream NeaTTR module at sites a* and b* to repress translation of the output gene.

[0066] A library of 29 different 1-bit NOR gates (Table 2) was constructed based on the same NeaTTR module and tested for regulation of GFP as the output protein. The gate RNAs were measured via flow cytometry five hours after induction of RNA expression using IPTG. The gate RNAs displayed wide variations in function, with some displaying very little change in GFP output upon expression of the cognate input RNA. However, 7 out of the 29 devices (24%) provided at least 50-fold reductions in GFP expression upon detection of the input RNA and two provided very large reductions of over 200-fold.

[0067] Validation of a Two-Input NOR Gate

[0068] We have successfully implemented a two-input NOR gate based on the general design depicted in FIG. 7. The system features a NeaTTR domain downstream of two hairpins that detect different input RNAs (see Table 2) and harbor intramolecular triggers within their loops. The two-input NOR gate was constructed using the previously validated 1-bit NOR constructs, specifically the 2nd and 4th ranked devices shown in FIG. 9. The NOR gate was measured five hours after induction of circuit RNA expression using IPTG. FIG. 10 shows the fold reduction in GFP expression for the four possible combinations of input RNAs 1 and 2. The highest GFP expression levels are obtained when neither of the inputs is expressed. In the presence of any of the input RNAs, GFP fold reductions of at least 10 are observed, as expected for a functional NOR gate.

[0069] Detection of Natural RNAs Using NeaTTRs

[0070] Since the a* and b* domains within NeaTTR hairpins (FIG. 1) can adopt virtually any sequence, they can be used to detect natural RNAs associated with drug resistance, viruses, hereditary diseases, and microorganisms. FIG. 11 displays results obtained from measurements in *E. coli* of three different NeaTTRs responsive to natural RNAs. These systems turn off expression of GFP in response to detection of their target RNAs and bind to the target sequence 5'-b-a-3'. We observed reductions of GFP fluorescence of 6-fold or more. The best performing sensor, which detects the catalase mRNA (*cat*) responsible for chloramphenicol resistance, provided a fold reduction of 15 upon *cat* binding. Riboregulators that turn off protein expression in response to a target pathogen-, resistance- or disease-related RNA could be used, for instance, as kill switches that trigger cell death when a bacterium picks up a drug resistance mutation or a resistance-conferring plasmid (e.g., a toxin-antitoxin system can be disrupted when the riboregulator ceases production of the antitoxin). These riboregulators can also be deployed using cell-free systems for RNA detection in a diagnostic device.

[0071] All three gates show at least 10-fold increases in GFP expression within 3 hours of induction compared to the sole logical FALSE case with inputs A and B present. Fold-change values increase with induction time for all three NAND gates. The circuit shown in FIG. 8B provides the most even GFP expression levels for the three logical TRUE cases, with fold changes of between 40 and 50 at the 5-hour time point. The circuit shown in FIG. 8C yields the highest fold-change levels overall with between 60- to 152-fold for the 5-hour time point. The success of these three NeaTTR-based gate RNAs demonstrates the robustness of the Type I design for constructing multi-input NAND logic.

Table 1. NeaTTR Devices

Device #	Device name	Switch RNA Sequence	Trigger RNA sequence
1	STUR_NN_gen1_N020	GGAUGAAUGAUUACACUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAU AACAAGCACGAAUUGACUACACUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:1)	GGGACGAAUUGAUU UGUCAAUUCGUGCG UGUAUAUCAUUCAU CAU (SEQ ID NO:101)
2	STUR_NN_gen1_N010	GGGUAGUAAAGAUUAGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAU AACAAGUACAAGAACCGAAACGAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:2)	GGGACAAGAACA ACGGUUCUUGUACU AUCUAAUCUUAACU AAC (SEQ ID NO:102)

3	STUR_WW_gen1_N008	GGGUACUCAAGAAUCCAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCACAUACUAAAUUACUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:3)	GGGACAUACUAUAU CGUUAGUAUGUGAU GGAAUUCUUGAGUA AUG (SEQ ID NO:103)
4	STUR_NN_gen1_N021	GGGACUACUAUUCACUUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCCGACACUACAAUUUCGAAAC CUGGCGGCAGCGCAA (SEQ ID NO:4)	GGGCGACACUAAUA GUGUAGUGUCGGAU AAGUGAAUAGUAGU AGA (SEQ ID NO:104)
5	STUR_NN_gen1_N013	GGGACAAUCAAAUACAAAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCACAUACAAAGCAAACGAAA CCUGGCGGCAGCGCAA (SEQ ID NO:5)	GGGACAUACAAUGA ACUUUGUAUGUGAU UUGUAUUUGAUUGU AGC (SEQ ID NO:105)
6	STUR_NN_gen1_N002	GGGAUGAUUUGAAUUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCACACUACACUAAACAUAAAC CUGGCGGCAGCGCAA (SEQ ID NO:6)	GGGACACUACAACU CAGUGUAGUGUGCG UAAUUCAAUAUCAU ACU (SEQ ID NO:106)
7	STUR_NN_gen1_N015	GGGUCCAAAUCUUAUUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCACUACCUCGAAUACUUAAC CUGGCGGCAGCGCAA (SEQ ID NO:7)	GGGACUACCUCACA CUCGAGGUAGUGAU AAGUAAAGAUUUGGA AGU (SEQ ID NO:107)
8	STUR_NN_gen1_N023	GGGCAAAUACUCCAUAUCUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCACAUAAACUCACUGAUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:8)	GGGACAUAAACCUA UGAGUUUAUGUGCG AUAUGGAGUAUUUG AAA (SEQ ID NO:108)
9	STUR_NN_gen1_N024	GGGCUCCUAUCACUUUACUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCACACUAAUACAAAUUC AAC CUGGCGGCAGCGCAA (SEQ ID NO:9)	GGGACACUAACCAU AUAGUUAGUGUGCG UAAAGUGAUAGGAG UAA (SEQ ID NO:109)
10	STUR_NN_gen1_N003	GGGAGUAUAAGAUAGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGUACGAAGCAACGAACAUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:10)	GGGACGAAGCAUAA AGUUGCUUCGUACU AUCUAUCUUAUACU AAC (SEQ ID NO:110)
11	STUR_NN_gen1_N019	GGGACUAAUCAGAUUCUACUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCACCUAAACAAGACUAAU AAC CUGGCGGCAGCGCAA (SEQ ID NO:11)	GGGACCUAACAUA ACUUGUUAGGUGCG UAGAUCUGAUUAGU GUG (SEQ ID NO:111)
12	STUR_NN_gen1_N012	GGGUGAUUGAAUAAGAAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCCGAUAAUGACACUAA CUAA CCUGGCGGCAGCGCAA (SEQ ID NO:12)	GGGCGAUAAUGUUC CGUCAUUAUCGGCA UUCUUAUUCAAUCA UAG (SEQ ID NO:112)
13	STUR_WW_gen1_N010	GGGCAAAGUAUCCAUCAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCACAUACUAGAAUCUAAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:13)	GGGACAUACUAACA AUCUAGUAUGUGAA UGAUGGAUACUUUG AAA (SEQ ID NO:113)
14	STUR_NN_gen1_N007	GGGCAAGAUUUAGUAGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCCGAACGACCGAAAUUGAUAA CCUGGCGGCAGCGCAA (SEQ ID NO:14)	GGGCGAACGACGAA ACGGUCGUUCGGAA UCUACUAAAUCUUG AAA (SEQ ID NO:114)

15	STUR_NN_gen1_N001	GGGCAAUUAGUACUAUCCUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAGUCACAUAAACGCAAACUAAAC CUGGCGGCAGCGCAA (SEQ ID NO:15)	GGGCACAUAAACAUA ACGGUUAUGUGACG GAUAGUACUAAUUG AAA (SEQ ID NO:115)
16	STUR_NN_gen1_N009	GGGCGAAGAUGAUACAAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAGCCACCUCACACUCAACAUAAC CUGGCGGCAGCGCAA (SEQ ID NO:16)	GGGCACCUCACCUA CGUGUGAGGUGGCA UUGUAUCAUCUUCG UAU (SEQ ID NO:116)
17	STUR_WW_gen1_N016	GGGAUACUUUCAAACUUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCACAUAAUCGACUGAAUAA CCUGGCGGCAGCGCAA (SEQ ID NO:17)	GGGACACUAAUACU ACGAUUAGUGUGAU AAGUUUGAAAGUAU AAG (SEQ ID NO:117)
18	STUR_WW_gen1_N020	GGGUACCUUCAAUUCUUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCACCUCACUAACAUAUCAAC CUGGCGGCAGCGCAA (SEQ ID NO:18)	GGGACCUCACUUAC AUUAGUGAGGUGAU GGAAUUUGAAGGUA AUG (SEQ ID NO:118)
19	STUR_NN_gen1_N011	GGGAUCAAUCAAUUCUACUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCACAUAAACCUAAGAACUAA CCUGGCGGCAGCGCAA (SEQ ID NO:19)	GGGACAUAAACAUA GAGGUUUAUGUGAG UAGAAUUGAUUGAU AAG (SEQ ID NO:119)
20	STUR_WW_gen1_N018	GGGAUUCACUUAACAAGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCACAUAGACCAUAGAACGAA CCUGGCGGCAGCGCAA (SEQ ID NO:20)	GGGACAUAGACAAU UUGGUCUUAUGUGAA UCUUGUAAGUGAAU AUG (SEQ ID NO:120)
21	STUR_WW_gen1_N012	GGGAUGUGAUUACUAGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCCGAACAAGCAAUACAAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:21)	GGGCGAACAAGAAC AUGCUUGUUCGGAA UCUAGUAAUCACAU CUA (SEQ ID NO:121)
22	STUR_WW_gen1_N009	GGGUCCAUAUACUUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUACCUAAUUCUGAGAUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:22)	GGGAACCUAUUGUA CGAAUUAGGUUGAA UUGAAGUAUAUGGA AUC (SEQ ID NO:122)
23	STUR_WW_gen1_N023	GGGCAAUUCUAAUCCUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCCAAUAGAGCAAAGCAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:23)	GGGCAAUAGAUAG AGCUCUAUUUGGAU AGGAUUAGGAAUUG AAA (SEQ ID NO:123)
24	STUR_NN_gen1_N008	GGGACAAAUCAGAUAAACUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAGCCAAUAGAUAGAACACGAAA CCUGGCGGCAGCGCAA (SEQ ID NO:24)	GGGCAAUAGAUCAA ACUAUCUAUUGGCG UUUAUCUGAUUUGU AUG (SEQ ID NO:124)
25	STUR_WW_gen1_N011	GGGAUCCAAGUUAUCAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAUCACAUAAAGACCUCAUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:25)	GGGCACAUAAACUA AUCUUUAUGUGUAA UGAUAAUUGGAU GAA (SEQ ID NO:125)
26	STUR_WW_gen1_N014	GGGAUGAUCGAAACGAAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAUAACAAGAAUAGACAAUAGAAA CCUGGCGGCAGCGCAA (SEQ ID NO:26)	GGGCAAGAAUAACA UUCUAUUCUUGUAA UUCGUUUCGAUCAU AUG (SEQ ID NO:126)

27	STUR_WW_gen1_N003	GGGAAUGAAGAUAGUGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCCGAAUAAGCGAACUAAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:27)	GGGCGAAUAAGAAU GCGCUUAUUCGGAA UCACUAUCUUCAU AAC (SEQ ID NO:127)
28	STUR_WW_gen1_N017	GGGAUCUCACCGUGUCUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCAACAUACAUCUAACAAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:28)	GGGAACAUACAAU UGAUGUAUGUUGAU AGACACGGUGAGAU AGA (SEQ ID NO:128)
29	STUR_WW_gen1_N005	GGGACUCCAAUUGACGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUACAUAUAGACUAAACAUA CCUGGCGGCAGCGCAA (SEQ ID NO:29)	GGGCAUAUAGAAUA GAGUCUAUAUGUAA UCGUCAAUUGGAGU AGA (SEQ ID NO:129)
30	STUR_NN_gen1_N004	GGGUAAUGAAGUGAAUACUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCCAAUUGAUACUAAACCGAA CCUGGCGGCAGCGCAA (SEQ ID NO:30)	GGGCAAUGAUCAU CGUAUCAUUUGGCG UAUUCACUUCAUUA ACA (SEQ ID NO:130)
31	STUR_NN_gen1_N016	GGGUCCCAUUAUCUUAACCUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCCUACACUACCAUUCCTGAAC CUGGCGGCAGCGCAA (SEQ ID NO:31)	GGGCUACACUAAAC AGGUAGUGUAGGCG GUAAGAUAAUGGGA GUA (SEQ ID NO:131)
32	STUR_WW_gen1_N006	GGGCGAUUAGAGUAAAGAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUGCAAUAGAACGAUACAAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:32)	GGGCAAUAGAAUA CCGUUCUAUUGCAU CUUACUCAUAUCG AUU (SEQ ID NO:132)
33	STUR_WW_gen1_N024	GGGACUACUCCAUUCUAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCCAUACCUCAUACAACGAAAC CUGGCGGCAGCGCAA (SEQ ID NO:33)	GGGCAUACCUCUAC AAUGAGGUUAGGAA UAGAAUGGAGUAGU AAG (SEQ ID NO:133)
34	STUR_WW_gen1_N021	GGGCGACUUAACUACUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCACUCCACGGAAACUACUAAAC CUGGCGGCAGCGCAA (SEQ ID NO:34)	GGGACUCCACGAAU AUCCGUGGAGUGAU AGUAGUUUAAGUCG UAG (SEQ ID NO:134)
35	STUR_WW_gen1_N013	GGGAUGUGAACUUAAGAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUGCGAAAUACGGAAAUAGAAA CCUGGCGGCAGCGCAA (SEQ ID NO:35)	GGGCGAAUACAAU ACCGUAUUUCGCAU CUUUAAGUUCACAU CGA (SEQ ID NO:135)
36	STUR_NN_gen1_N018	GGGCAAUCCUAUUCUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUUCUACAACUCAAUCACUCAAC CUGGCGGCAGCGCAA (SEQ ID NO:36)	GGGCUACAUAAC AUGAUGUUGAGAAU GAUGAAUAGGAUUG AGA (SEQ ID NO:136)
37	STUR_WW_gen1_N001	GGGUAAAGAAUUAAGAAUCAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCAGCGAACACGAAACAUA CCUGGCGGCAGCGCAA (SEQ ID NO:37)	GGGAGCGAACAAUA ACGUGUUCGCUGAU GAUUCUAAUUCUUA ACA (SEQ ID NO:137)
38	STUR_NN_gen1_N022	GGGUCAAUCCGAUAAUCUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUUCUCCAAGGCAAUGAUAAA CCUGGCGGCAGCGCAA (SEQ ID NO:38)	GGGACUCCAAGAU AGCCUUGGAGUAAA GAUUAUCGGAUUGA AUG (SEQ ID NO:138)

39	STUR_WW_gen1_N007	GGGAAUGAAUGAUGAGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUGAGCGAACGAGCAAUGGAAA CCUGGCGGCAGCGCAA (SEQ ID NO:39)	GGGAGCGAACGCGA ACUCGUUCGCUCAA UCUCAUCAUUCAUU AAG (SEQ ID NO:139)
40	STUR_NN_gen1_N006	GGGAUUUAGAAGUAAGUAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUUCGAAGAUUUGGACGAUAA CCUGGCGGCAGCGCAA (SEQ ID NO:40)	GGGCGAAGAUAGAU ACAUAUCUUCGAAU ACUUACUUCUAAA CUA (SEQ ID NO:140)
41	STUR_WW_gen1_N004	GGGAUUUACUUAGAUACUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUACAUAUACAUACGAACGAA CCUGGCGGCAGCGCAA (SEQ ID NO:41)	GGGACAUAUACUUU CAUGUAUAUGUAAA GUAUCUAAGUAAU GAA (SEQ ID NO:141)
42	STUR_WW_gen1_N015	GGGCGAAUUGAAAUGAAAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUGACGGAAUAGAAACUAAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:42)	GGGACGGAAUACA CUCUAUUCGUCAU UUCAUUCAAUUCG UAA (SEQ ID NO:142)
43	STUR_WW_gen1_N019	GGGCAUUUACCCUACUAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUACACUCCAAAUCAAAGAUAA CCUGGCGGCAGCGCAA (SEQ ID NO:43)	GGGCACUCCAAUAG GAUUUGGAGUGUAA UAGUAGGGUAAAUG AGA (SEQ ID NO:43)
44	STUR_WW_gen1_N002	GGGUAAUACUUACCGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCACUCCAACGGAACAAUCAAC CUGGCGGCAGCGCAA (SEQ ID NO:44)	GGGACUCCAACUAA ACCGUUGGAGUGAU AUCGGUAAGUAAU GAA (SEQ ID NO:144)
45	STUR_WW_gen1_N022	GGGCUCAUACUUCACUAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUACACCUAACAUAAUCACUAAAC CUGGCGGCAGCGCAA (SEQ ID NO:45)	GGGCACCUAACUAA CAUGUUAGGUGUAA UAGUGAAGUAUGAG AUG (SEQ ID NO:145)
46	STUR_NN_gen1_N005	GGGAUCUCCAUCUAUCCAUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUCCAUAUAGUAACAAGCAGAA CCUGGCGGCAGCGCAA (SEQ ID NO:46)	GGGCAUUAAGUUAA GUUACUUAUUGGAU GGAUAGAUGGAGAU GAU (SEQ ID NO:146)
47	STUR_NN_gen1_N014	GGGUAGAUUAAGAGUGAUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAUGACCUAAACGCACGAACUAAAC CUGGCGGCAGCGCAA (SEQ ID NO:47)	GGGACCUAAACUAA CGCGUUUAGGUCAA UCACUCUUAUUCUA CUA (SEQ ID NO:147)
48	STUR_NN_gen1_N017	GGGCAAUCUCCAUAACGUUUGUUAUA GUUAUGAACAGAGGAGACAUAAACAUG AACAAAGCACUUAACCGAAUAACUAA CCUGGCGGCAGCGCAA (SEQ ID NO:48)	GGGACUUAACGAA ACGGUUUAAGUGCA CGUAUUGGAGAUUG GGA (SEQ ID NO:148)

Table 2. 1-Input ("1-bit") NOR Devices

Device Number	Device Name	5'-3' Switch RNA Sequence	Trigger RNA Sequence
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1	STUR_NOR_ Gen3_Hpin5	GGGAUCAAAGUAUACAAGGGUAGGGUAGGGUUAU CGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCGAUAACCCUACCCUACCGCCUCAAUUC GAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGUU AUGAACAGAGGAGACAUAAACAUGAACAAUCCGAC ACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:49)	GGGCUGCAGGUGA CAGAUUACACCUG CAGCGAUAAACCCU ACCCUACCCUUGU AUACUUUGAU (SEQ ID NO:149)
2	STUR_NOR_ Gen2_Hpin1	GGGUCUUAUGUGUAUGUUUCUGUCUUGUCUUUAU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGAUAAGACAAGACAGAGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:50)	GGGACAAGGACUC UGAAGUGAGUCCU UGUAGAUAAAGAC AAGACAGAAACAU ACACAUAGA (SEQ ID NO:150)
3	STUR_NOR_ Gen3_Hpin7_ mut	GGGAGUAAAGUAUAGAACGCUUGAUGUUGAUGUU AGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAUAGUCUAAACAUCAACAAGCGCCUCAAUUC GAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGUU AUGAACAGAGGAGACAUAAACAUGAACAAUCCGAC ACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:51)	GGGUGACAUAUCC AAGAGCGGAUAUG UCACUAAACAUCAA CAUCAAGCGUUCU AUACUUUACU (SEQ ID NO:151)
4	STUR_NOR_ Gen2_Hpin9_ mutRepstem	GGGUCGUAAAGUUUAAGUCUGUUUCUGUCUGUU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGAACAGACAGAAACAGAGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAAGUU AUGAACAGAGGAGACAUAAACAUGAACAAUCCGAC ACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:52)	GGGACCUCAAUUC AUCUGGGAAUUGA GGUAGAACAGACA GAAACAGACUAA ACUUUAACGA (SEQ ID NO:152)
5	STUR_NOR_ Gen3_Hpin11	GGGACUAAGACUAAAUGAUGUAUAGAUUGAAGAU UGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCAAUUCUCAAUCUUAACAGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:53)	GGGACGUGCGCAG CUCCUACUGCGCA CGUCAAUUCUCAA UCUAUACAUAUU UAGUCUUAGU (SEQ ID NO:153)
6	STUR_NOR_ Gen2_Hpin13	GGGUUCUUAAGUUUAUGUAGGUCUGUUUAUCUUGUU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGAACAAGAUAAACAGACCGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:54)	GGGUGACCUGAAC CGAACUGUUCAGG UCAAGAACAAGAU AACAGACCUACAU AAACUAAGAA (SEQ ID NO:154)
7	STUR_NOR_ Gen3_Hpin6_ mut	GGGACGACUUAAGAUUACGGUAUGAGUUUAGAUAU UGCGACACUAAUAGUGUAGUGUCGGAUAAGUAAU AGUAGUCAAUUCUAAACUUAUACCGCCUCAAUUC GAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGUU AUGAACAGAGGAGACAUAAACAUGAACAAUCCGAC ACUACAAUUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:55)	GGGAGUGCCACAG GCUCGACUGUGGC ACUCAAUUCUAAA CUCAUACCGUAAU CUUAAGUCGU (SEQ ID NO:155)

8	STUR_NOR_ Gen2_Hpin12	GGGUUAGGUAUGUGUAUUGAUUGAUUUGAUGCUU GUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUACAAGCAUCAAAUCAAUCGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:56)	GGGACCCUACUCC AGCACAGGAGUAG GGUACAAGCAUCA AAUCAAUCAAUAC ACAUACCUAA (SEQ ID NO:156)
9	STUR_NOR_ Gen2_Hpin16	GGGAGUAAGGUAAGGUGAGUGCGUUGUCUUUAGU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGACUAAAAGACAACGCACGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:57)	GGGUCACUACUGG GAUUAUACCAGUAG UGAAGACUAAAGA CAACGCACUCACC UUACCUUACU (SEQ ID NO:157)
10	STUR_NOR_ Gen2_Hpin3	GGGUCUGUUUUAUGUGUAUGUGUCUGUCUUGUCUG CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGCAGACAAGACAGACACGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:58)	GGGACGUUAGUCG AUGUCGCGACUAA CGUAGCAGACAAG ACAGACACAUACA CAUAAACAGA (SEQ ID NO:158)
11	STUR_NOR_ Gen3_Hpin3_ mut	GGGAGCUUAAUGAUAGAAUGCGUUGAUGUUAUGUA AGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCUUAACAUAACAACGCAGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:59)	GGGUCGGUGCGU AACGGCAACGCAC CGACUUAACA UCAACGAUUCUA UCAUUAAGCU (SEQ ID NO:159)
12	STUR_NOR_ Gen3_Hpin7	GGGAGUAAAGUAUAGAACGCUUGAUGUUGAUGUU AGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCUAAACAUAACAUAAGCGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:60)	GGGUGACAUAUCC AAGAGCGGAUAUG UCACUAAACAUA CAUCAAGCGUUCU AUACUUUACU (SEQ ID NO:160)
13	STUR_NOR_ Gen2_Hpin2	GGGAGUUGUAUGUGUAUGGUCUGUCUUGUUCUUAU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGAUAGAACAAGACAGACGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:61)	GGGACGCGCUAGC GCAUCGGCUAGCG CGUAGAUAGAACA AGACAGACCAUAC ACAUACAACU (SEQ ID NO:161)
14	STUR_NOR_ Gen3_Hpin4_ mut	GGGAUGAUAAAGAUUGAUGCUGUUAUGUUGAUUU AGCGACACUAAUAGUGUAGUGUCGGAUAAGGAAU AGUAGUCUAAAUCAACAUAACAGCGCCUCAAUUC GAGGCAAGACUACUAAUUCACUUAUUGUUAUAGUU AUGAACAGAGGAGACAUAACAUGAACAAUCCGAC ACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:62)	GGGAGCCAUGAUC GCCCAGGAUCAUG GCUCUAAAUCAAC AUAACAGCAUCAA UCUUUAUCAU (SEQ ID NO:162)

15	STUR_NOR_ Gen3_Hpin4_ mut	GGGAUGAUAAAGAUUGAUGCUGUUAUGUUGAUUU AGCGACACUAAUAGUGUAGUGUCGGAUAAUGAAU AGUAGUCUAAAUCAACAUAAACAGCGCCUCAAUUC GAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGUU AUGAACAGAGGAGACAUAAACAUGAACAAUCCGAC ACUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:63)	GGGAGCCAUGAUC GCCCAGGAUCAUG GCUCUAAAUCAAC AUAACAGCAUCAA UCUUUAUCAU (SEQ ID NO:163)
16	STUR_NOR_ Gen2_Hpin6	GGGAGUAAAGUAAAGGUGUCGUUGUAUGUUCGUU CUCGACACUAAUAGUGUAGUGUCGGAUAAAGUGAA UAGUAGUAGAACGAACAUACAACGAGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:64)	GGGACACCGGAGG CGUGCACCUCCGG UGUAGAACGAACA UACAACGACACCU UUACUUUACU (SEQ ID NO:164)
17	STUR_NOR_ Gen2_Hpin15	GGGUCUAGUGUCGUCGUUGUCUUGUAUGGUUCUU GUCGACACUAAUAGUGUAGUGUCGGAUAAAGUGAA UAGUAGUACAAGAACCAUACAAGACGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:65)	GGGACGAUAUACG GGUCUACGUUAU CGUACAAGAACCA UACAAGACAACGA CGACACUAGA (SEQ ID NO:165)
18	STUR_NOR_ Gen2_Hpin18	GGGUUAGUAUUGUAGUUGUCUGUAUUUGUCUCUG CUCGACACUAAUAGUGUAGUGUCGGAUAAAGUGAA UAGUAGUAGCAGAGACAAAUACAGAGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:66)	GGGCAGGGUACUU GUACUGAAGUACC CUGAGCAGAGACA AAUACAGACAACU ACAAUACUAA (SEQ ID NO:166)
19	STUR_NOR_ Gen3_Hpin5_ mutA	GGGAUCAAAAGUAUACAAGGGUAGGGUAGGGUUAU CGCGACACUAAUAGUGUAGUGUCGGAUAAAGUGAU AGUAGUCGAUAACCCUACCCUACCGCCUCAAUUCG AGGCAAGACUACUUAUUCACUUAUUGUUUAUAGUUA UGAACAGAGGAGACAUAAACAUGAACAAUCCGACA CUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:67)	GGGCUGCAGGUGA CAGAUUACACCUG CAGCGAUAAACCCU ACCCUACCCUUGU AUACUUUGAU (SEQ ID NO:167)
20	STUR_NOR_ Gen3_Hpin10_ _mut	GGGACUGGAUACUUAUACGGUUCUGUAGGUUCUU AGCGACACUAAUAGUGUAGUGUCGGAUAAAGUAAA UAGUAGUCUAAAGAACCUACAGAACCGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:68)	GGGCCAUACCGUG CAACUACCAGGUA UGGCUAAGAACCU ACAGAACCGUAUA AGUAUCCAGU (SEQ ID NO:168)
21	STUR_NOR_ Gen2_Hpin4	GGGUUAGUGUAUGGUUAGUCGUUCUUGUUCUUAU CUCGACACUAAUAGUGUAGUGUCGGAUAAAGUGAA UAGUAGUAGAUAAAGAACAAGAACGAGCCUCAAUU CGAGGCAAGACUACUUAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUUCGAAACCUGGCGGCAGCGCAA (SEQ ID NO:69)	GGGAGCCGUGCAG CGUGCACUGCACG GCUAGAUAAAGAAC AAGAACGACAUAC CAUACACUAA (SEQ ID NO:169)

22	STUR_NOR_ Gen3_Hpin8	GGGACGUACUAAUUGAUUUCGUCUUGUCUUGUUAU UGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCAAUACAAGACAAGACGAGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:70)	GGGUUCCCGUCAC UGGUACGUGACGG GAACAAUACAAGA CAAGACGAAAUCA AUUAGUACGU (SEQ ID NO:170)
23	STUR_NOR_ Gen2_Hpin5	GGGAGUGUAUUGUAUGUGUCUUGUUCUUGUUCGU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGACGAACAAGAACAAGAGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:71)	GGGACCUGCUCAC CUUCAUGUGAGCA GGUAGACGAACAA GAACAAGACACAU ACAAUACACU (SEQ ID NO:171)
24	STUR_NOR_ Gen2_Hpin14	GGGUCCUGUGUCUUUAUGUCUUGUUCUGUUUAUC UUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAAGAUAAACAGAACAAAGAGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:72)	GGGACCGUAACGA AAUCCAUCGUUAC GGUAAGAUAAACA GAACAAGACAUAA AGACACAGGA (SEQ ID NO:172)
25	STUR_NOR_ Gen2_Hpin17 _mut	GGGUCUGUGUAUUUCUGUUCUUAUGUCUUGUCUU AUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA AGUAGUAUAAGACAAGACAUAAAGAGCCUCAAUUC GAGGCAAGACUACUAAUUCACUUAUUGUUAUAGUU AUGAACAGAGGAGACAUAAACAUGAACAAUCCGAC ACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:73)	GGGACAUCAGAGA CUAGUAUCUCUGA UGUAUAAGACAAG ACAUAAGAACAGA AAUACACAGA (SEQ ID NO:173)
26	STUR_NOR_ Gen2_Hpin10	GGGAUGUAUGUGUAAUGGUCUGUUUAUGUAUGUU CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGAACAUAACAUAACAGAGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:74)	GGGAGUCACGCGC UACGUUGCGCGUG ACUAGAACAUAACA UAAACAGACCAUU ACACAUACAU (SEQ ID NO:174)
27	STUR_NOR_ Gen3_Hpin2	GGGAAUCUUAAUCGUUAUAGGUCUUUGUCUUGUUC UGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCAGAACAAAGACAAAGACCGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:75)	GGGAGUCCUUGAG GUGCGACUCAAGG ACUCAGAACAAAGA CAAAGACCUAUAC GAUUAAGAUU (SEQ ID NO:175)
28	STUR_NOR_ Gen3_Hpin9	GGGAGUAGUAGUAAAUGUGUCUUGUAUCUUGUUC UGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUCAGAACAAAGAUACAAGACGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:76)	GGGCCACUUCUGA CUGCGAUCAGAAG UGGCAGAACAAAGA UACAAGACACAUU UACUACUACU (SEQ ID NO:176)

29	STUR_NOR_Gen2_Hpin1I	GGGAGUAAGUUGAAAGUAGGUUAUGUUUAUGUUG CUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAA UAGUAGUAGCAACAUAAACAUAAACCGCCUCAAUU CGAGGCAAGACUACUAAUUCACUUAUUGUUUAUAGU UAUGAACAGAGGAGACAUAAACAUGAACAAUCCGA CACUACAAUUUCGAAACCUGGCGGCAGCGCAAA (SEQ ID NO:77)	GGGACCCUCAUUG CUCUACCAAUGAG GGUAGCAACAUAA ACAUAACCUACUU UCAACUUACU (SEQ IDNO:177)
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Table 3. 2-Input NOR Devices

Device Name	2-input NOR Hairpin BA
Switch RNA Sequence	GGGUCGUUAAGUUUAAGUCUGUUUCUGUCUGUUCUCGACACUAAUA GUGUAGUGUCGGAUAAGUGAAUAGUAGUAGAACAGACAGAAACAGAG CCUCAAUUCGAGGCAAGUCUUAUGUGUAUGUUUCUGUCUUGUCUUUA UCUCGACACUAAUAGUGUAGUGUCGGAUAAGUGAAUAGUAGUAGUA AAGACAAGACAGAGCCUCAAUUCGAGGCAAGACUACUAAUUCACUUAUU GUUAUAGUUUAUGAACAGAGGAGACAUAAACAUGAACAAUCCGACACUA CAAUUUCGA (SEQ ID NO:78)
Input A (Binds at 5' site on switch RNA)	GGGACCUCAAUUAUCUGGGAAUUGAGGUAGAACAGACAGAAACAGA CUUAAACUUAUACGA (SEQ ID NO:79)
Input B (Binds at 3' site on switch RNA)	GGGACAAGGACUCUGAAGUGAGUCCUUGUAGAUAAAGACAAGACAGA AACAUACACAUAGA (SEQ ID NO:80)

Table 4. 2-Input NAND Constructs

Device Number	Device Name	Input RNAs Used (Listed by binding location 5' to 3' on switch RNA)	Switch RNA Sequence
1	STUR_NAND_L11_N008_H pFD	F, D	GGGCUCCUAUCACUUUACUUGUUUAUGUUAUGAA CAGAGGAGACAUAAACAUGAACAAAGCACACUAAUCU ACAAAUUCCAAAGCCACAUACUAAUCAGAUUCUAC UUGUUUAUGUUUAUGAACAGAGGAGACAUAAACAU GAACAAGCACCUAACAAGACUAAUC (SEQ ID NO:81)
2	STUR_NAND_L17_N006_H pFD	F, D	GGGCUCCUAUCACUUUACUUGUUUAUGUUAUGAA CAGAGGAGACAUAAACAUGAACAAAGCACACUAAUCU ACAAAUUCACCGCAAAUACAAUAAACACUAAUCAG AUCUACUUGUUUAUGUUAUGAACAGAGGAGACAU AACAUGAACAAGCACCUAACAAGACUAAUC (SEQ ID NO:82)
3	STUR_NAND_L17_N008_H pDB	D, B	GGGACUAAUCAGAUUCUACUUGUUUAUGUUAUGAA CAGAGGAGACAUAAACAUGAACAAAGCACCUAACAA GACUAAUCAUAUACAAUACCUCCAAAUCAUCAA UUCUACUUGUUUAUGUUAUGAACAGAGGAGACAU AACAUGAACAUCACAUAAACCUAAGAACU (SEQ ID NO:83)

4	STUR_NAND _L11_N007_H pDC	D, C	GGGACUAAUCAGAUACUUGUUUAUAGUUAUGAA CAGAGGAGACAUAAACAUGAACAAAGCACCUAACAA GACUAAUCCAAAUCAAAUCACAAUCAAUACAAA UUGUUUAUAGUUAUGAACAGAGGAGACAUAAACAU GAACAAUCACAUACAAAGCAAACGA (SEQ ID NO:84)
5	STUR_NAND _L11_N002_H pCF	C, F	GGGACAAUCAAAUACAAAUUGUUUAUAGUUAUGA ACAGAGGAGACAUAAACAUGAACAAUCACAUACAA AGCAAACGAACAAUACCCGACUCCUAUCACUUUA CUUGUUUAUAGUUAUGAACAGAGGAGACAUAAACAU GAACAAGCACACUAAACUACAAAUUC (SEQ ID NO:85)
6	STUR_NAND _L17_N001_H pCD	C, D	GGGACAAUCAAAUACAAAUUGUUUAUAGUUAUGA ACAGAGGAGACAUAAACAUGAACAAUCACAUACAA AGCAAACGAAACAUAAAAGCCAACUAAACUAAUCA GAUCUACUUGUUUAUAGUUAUGAACAGAGGAGACA UAAACAUGAACAAAGCACCUAACAAGACUAAUC (SEQ ID NO:86)

Table 5. 3-Input NAND Constructs

Device #	Device Name	Input RNAs Used (Listed by binding location 5' to 3' on switch RNA)	Switch RNA Sequence
1	STUR_NAN D_link17_N0 3_003_HpCB D	C, B, D	GGGACAAUCAAAUACAAAUUGUUUAUAGUUAUGAACAGAG GAGACAUAAACAUGAACAAUCACAUACAAAGCAAACGAAA UACCGCAAAUACCAUUAUCAAUCAAUUCUACUUGUUUAUAG UUAUGAACAGAGGAGACAUAAACAUGAACAAUCACAUAAA CCUAAGAACUAAGCCGACAAUACUAAACACUAAUCAGAUUC UACUUGUUUAUAGUUAUGAACAGAGGAGACAUAAACAUGAA CAAGCACCUAACAAGACUAAUC (SEQ ID NO:87)
2	STUR_NAN D_link11_N0 3_004_HpBC D	B, C, D	GGGAUCAAAUUCUACUUGUUUAUAGUUAUGAACAGAG GAGACAUAAACAUGAACAAUCACAUAAACCUAAGAACUCA AUUUCGCAUACAAUCAAUACAAAUUGUUUAUAGUUAUGA ACAGAGGAGACAUAAACAUGAACAAUCACAUACAAAGCAA ACGACAAAUACAAAUACUAAUCAGAUUCUACUUGUUUAUAG UUAUGAACAGAGGAGACAUAAACAUGAACAAAGCACCUAAC AAGACUAAUC (SEQ ID NO:88)
3	STUR_NAN D_link11_N0 3_006_HpFC D	F, C, D	GGGCUCCUAUCACUUUACUUGUUUAUAGUUAUGAACAGAG GAGACAUAAACAUGAACAAAGCACACUAAACUACAAAUUCGC AAAUACAUCACAAUCAAUACAAAUUGUUUAUAGUUAUGA ACAGAGGAGACAUAAACAUGAACAAUCACAUACAAAGCAA ACGACAAAUACAAAUACUAAUCAGAUUCUACUUGUUUAUAG UUAUGAACAGAGGAGACAUAAACAUGAACAAAGCACCUAAC AAGACUAAUC (SEQ ID NO:89)
4	STUR_NAN D_link11_N0 3_001_HpFD C	F, D, C	GGGCUCCUAUCACUUUACUUGUUUAUAGUUAUGAACAGAG GAGACAUAAACAUGAACAAAGCACACUAAACUACAAAUCCA AAGCCACAUACUAAUCAGAUUCUACUUGUUUAUAGUUAUGA ACAGAGGAGACAUAAACAUGAACAAAGCACCUAACAAGACU AAUCCAAAUCAAAUCACAAUCAAUACAAAUUGUUUAUAG UUAUGAACAGAGGAGACAUAAACAUGAACAAUCACAUACA AAGCAAACGA (SEQ ID NO:90)

NAND Inputs	
Input RNA Letter	Input RNA Sequence
A	GGGACAAGAACAAUACGGUUCUUGUACUAUCUAAUCUUACUAAAC (SEQ ID NO:94)
B	GGGACAUAAACAUAGAGGUUUAUGUGAGUAGAAUUGAUUGAUAAAG (SEQ ID NO:95)
C	GGGACAUACAAUGAACUUUGUAUGUGAUUUGUAUUUGAUUGUAGC (SEQ ID NO:96)
D	GGGACCUAACAUAACUUGUUAGGUGCGUAGAUCUGAUUAGUGUG (SEQ ID NO:97)
E	GGGCGACACUAAUAGUGUAGUGUCGGAUAAGUGAAUAGUAGUAGA (SEQ ID NO:98)
F	GGGACACUAACCAUAUAGUUAGUGUGCGUAAAGUGAUAGGAGUAA (SEQ ID NO:99)
Noncognate trigger	GGGCGAUAAUGUCCGUCAUUAUCGGCAUUCUUAUUCAAUCAUAG (SEQ ID NO:100)

Table 6. 4-Input NAND Constructs

Device Number	Device Name	Input RNAs Used (Listed by binding location 5' to 3' on switch RNA)	Switch RNA Sequence
1	STUR_NA ND_link11_ N04_010_F DCB	F, D, C, B	GGGCUCCUAUCACUUUACUUGUUUAGUUUUGAACAGAGGAGACA CAUAAACAUGAACAAAGCACACUAAUACAAAUCCAAAGCCACA UACUAAUCAGAUUCUACUUGUUUAGUUUUGAACAGAGGAGACA UAACAUGAACAAAGCACCUAACAAGACUAAUCCAAAUCAAUACA CAAUCAAUACAAAUUGUUUAGUUUUGAACAGAGGAGAGACAU AAACAUGAACAAUCACAUACAAAGCAAACGACACAUACCUUCAU CAAUCAAUUCUACUUGUUUAGUUUUGAACAGAGGAGAGACAUAA CAUGAACAAUCACAUAAACCUAAGAACUAACCUGGCGGCAGCG CAA (SEQ ID NO:91)
2	STUR_NA ND_link17_ N04_011_F BDC	F, B, D, C	GGGCUCCUAUCACUUUACUUGUUUAGUUUUGAACAGAGGAGACA CAUAAACAUGAACAAAGCACACUAAUACAAAUCCUAAUUCU AUCCAGCAUCAAUCAAUUCUACUUGUUUAGUUUUGAACAGAG GAGACAUAAACAUGAACAAUCACAUAAACCUAAGAACUAAGCCG ACAAUACUAAACACUAAUCAGAUUCUUGUUUAGUUUUGAAC AGAGGAGACAUAAACAUGAACAAAGCACCUAACAAGACUAAUCAA CUCAAUUCGCAGAUACAAUCAAUACAAAUUGUUUAGUUUAG GAACAGAGGAGACAUAAACAUGAACAAUCACAUACAAAGCAAAC GAAACCUGGCGGCAGCGCAA (SEQ ID NO:92)

3	STUR_NA ND_link17_ N04_001_D FCB	D, F, C, B	GGGACUAAUCAGAUACUACUUGUUUAUAGUUAUGAACAGAGGAG ACAUAACAUGAACAAGCACCUAACAAGACUAAUCAACUACAAA UACAAUAACUCCUAUCACUUUACUUGUUUAUAGUUAUGAACAGA GGAGACAUAAACAUGAACAAGCACACUACUACAAAUUCACAAU UACCGACGCAAUACAAUCAAUACAAAUUGUUUAUAGUUAUGAA CAGAGGAGACAUAAACAUGAACAACUACAUACAAAGCAAACGAA AUACCGCAAUACCAUAUCAUCAAUUCUACUUGUUUAUAGUUA UGAACAGAGGAGACAUAAACAUGAACAACUACAUAAACCUAAGA ACUAACCUGGCGGCAGCGCAA (SEQ ID NO:93)
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[0072] Discussion

[0073] We have demonstrated a new type of *de novo*-designed translational repressor that exploits an unstable RNA secondary structure to enable signal output in the device ON state by facilitating ribosomal access and low OFF state expression by facilitating binding of a cognate trigger RNA with nearly arbitrary sequence. These NeaTTR systems provided remarkably high repression with ON/OFF ratios up to nearly 800-fold. They exhibited orthogonality that surpassed those reported for previous RNA-based repressors, forming an orthogonal library of 15 different devices expected to provide ON/OFF levels of at least 40-fold. We attribute the high repression levels of the devices to the favorable reaction kinetics and thermodynamics provided by precisely designed RNAs and the use of the near-threshold switch RNA structure to simultaneous enable translation and a strong binding site for interactions with the trigger RNA. A crucial part of the observed orthogonality of the NeaTTRs is the use of trigger RNAs in which a substantial portion of their interaction region with the switch RNA is sequestered within a hairpin. This design feature means that each trigger only provides 17 exposed nucleotides. Exposed, single-stranded trigger domains are a major source of device crosstalk in other repressors, and decrease output of noncognate switch RNAs by binding non-specifically and impairing RBS access. Since trigger RNAs are typically expressed at higher concentrations than the switch RNAs *in vivo*, these nonspecific damping effects can be substantial. Accordingly, reduction of the single-stranded region of the trigger RNA to only 17-nts substantially attenuates this effect. Lastly, our validation of the NeaTTRs in both *in vivo* contexts in *E. coli* and *in vitro* conditions in cell-free reactions means that they can be deployed in applications such as metabolic engineering, whole-cell biosensing, or for *in vitro* diagnostics.

[0074] Given the high dynamic range and orthogonality of these devices, it is expected that they can be deployed to regulate multiple enzymes within a metabolic pathway and enable fine control of enzyme levels through tuning of the levels of the corresponding trigger RNA.

Moreover, entire metabolic pathways can be regulated by placing each enzyme under control of NeaTTRs that respond to the same trigger RNA. The ability of NeaTTRs to detect nearly arbitrary RNAs also means that they could be used to respond to the expression of endogenous or pathogen associated RNAs within the cell to provide dynamic and response regulation of gene expression. Such capabilities could be useful not only for metabolic engineering, but also for developing whole-cell biosensors or potentially for rapid, high-throughput screening antimicrobial compounds. For diagnostic applications, riboregulators that turn off gene expression in response a target RNA could be used prevent a given reaction from occurring, which could be helpful given the limited energy bandwidth of cell-free reactions, or to provide dynamic expression conditions, for instance the expression of a protein for a limited amount of time at the outset of a diagnostic test.

[0075] RNA-only logic systems:

[0076] The high dynamic range and orthogonality of NeaTTRs enable them to be incorporated into biomolecular circuits for evaluating complex logic expressions. The NeaTTRs on their own act as inverters with expression of the trigger RNA or input RNA turning off protein expression. In addition, NeaTTRs can be used to evaluate both NAND and NOR logic expressions. We show in Figure 6 two different designs for carrying out NAND logic. Type I NAND logic can be achieved using an extended gate RNA that features multiple MeTR switch RNA modules concatenated upstream of an open reading frame (FIG. 6a). Each of the corresponding trigger RNAs for the switch modules thus functions as an input molecule. Expression of these input RNAs enables them to bind to the cognate sensor binding site and deactivate the RBS and start codon of the switch RNA. However, if one or more of the set of input RNAs is absent, the RBS and start codons of the corresponding switch RNAs will remain exposed and enable translation to still occur. On the other hand, if all the input RNAs are expressed, each of the switch RNAs will be repressed and translation will cease. This behavior corresponds to NAND logic. In order for such NAND gate RNAs to work effectively, care must be taken to ensure that none of the switch RNA modules nor the linker sequences that separate them encodes an in-frame stop codon. Furthermore, each of the switch RNAs must also be in the same reading frame as the downstream gene.

[0077] FIG. 6B shows the design of a Type II NAND circuit. This RNA circuit employs only a single switch RNA module but now divides the trigger RNA sequence between two different

input RNAs 1 and 2 that have complementary hybridization domains. Upon expression of both inputs, the two will hybridize to one another and form a complete trigger RNA to abolish translation from the complementary switch RNA. When only one of the input RNAs is expressed, the region complementary to the switch RNA is designed to be sufficiently short to either prevent input RNA binding or to provide insufficient base pairing to prevent translation from occurring. Thus, gene expression is only turned off when both input RNAs are present, which corresponds to 2-input NAND logic. Like the Type I devices, Type II NAND logic can be extended to greater than 2-input expressions. For an N -input NAND circuit, the trigger RNA sequence can be divided among N different input RNAs that must hybridize to one another to generate a fully assembled and functional trigger RNA for the corresponding switch RNA. Alternatively, the trigger RNA can be divided between input bits 1 and N and the intervening bits 2 through $N-1$ can be designed to hybridize directly with another and form an N -RNA complex that can repress the switch RNA. In this design, input 2 would bind through two hybridization domains to input 1 and input 3. Or in general, input m would bind to inputs $m-1$ and $m+1$ through hybridization domains. Finally, a combination of both the above input RNA design strategies could be employed and spacers between hybridization domains and/or trigger RNA portions could be used to increase the activity of the final assembled trigger.

[0078] NOR logic requires RNA circuit output to be active only if all input RNAs are not expressed. The design of such a NOR gate circuit is shown in FIG. 7. This gate RNA features a single switch RNA located just upstream of the open reading frame of the output gene. Upstream of the switch RNA are two or more hairpins each designed to detect the input RNA molecules of the circuit. Each of these hairpins contains the sequence of the cognate trigger for the switch RNA. However, these trigger sequences are sequestered within the stem loops of each of the hairpins. Consequently, in the absence of the corresponding input RNA, the trigger sequences are unable to hybridize to the sensor RNA recognition site and translation will continue to occur. When an input RNA is expressed, the input RNA will bind through a toehold-mediated linear-linear interaction and unwind most (or all depending on the final design) of the hairpin stem. The resulting weakened hairpin structure is no longer strongly sequestered and thus is available for binding to the switch RNA. Accordingly, the gate RNA will undergo an intramolecular refolding process and the newly exposed trigger RNA will bind to the switch RNA region. Following trigger binding, the RBS and start codon of the switch RNA will be strongly repressed and thus

prevent translation from occurring. Since all of the upstream hairpins are designed to bind to an input RNA and harbor trigger RNA sites, binding of any input RNA to the gate RNA will cause output gene expression to be turned off. In principle, any number of input RNA sensing hairpins can be arrayed upstream of the switch RNA meaning that the system is capable of N -input NOR expressions. Lastly, the trigger RNA sequence need not comprise part of the stem of the input-sensing hairpin. The trigger RNA can be contained entirely within the loop of the hairpin, provided it is not sufficiently exposed to enable hybridization with the switch RNA sensing domain.

[0079] Importantly, this NOR gate concept can also be applied to other riboregulators beyond NeaTTRs. The upstream hairpins that sense the input RNAs can be designed to expose arbitrary RNA sequences. Thus, they can be used to activate (or deactivate) any existing riboregulator, including toehold switches⁶, toehold repressors, beacon riboregulators, and STARs⁹, among others. For repressing riboregulators, these types of gate RNAs would carry out NOR logic. For activating riboregulators like the toehold switch, these types of gate RNAs would carry out OR logic. An important advantage of this approach compared to OR logic gates of concatenated activating riboregulators, which are equivalent to the Type I NAND gate RNAs, is that translation of the output gene always begins at the same location and thus the output of the gate RNA is the same regardless of the input RNA used. Furthermore, gate RNAs of this type do not need to be screened for in-frame stop codons upstream of the start codon site, and translation will not be affected by the procession of the ribosome through areas in the gate RNA with high secondary structure, which occurs for gate RNAs of the Type I NAND variety. It is possible to implement this OR and NOR logic functionality of the gate RNA using separate RNA hairpin species that are not covalently bound to one another. However, the use of a single RNA to encode all the sensing hairpins leads to enhanced reaction co-localization, since both switch and trigger are within the same molecule and need not find each other in the cytoplasm or solution through random diffusion. Consequently, these extended gate RNAs should provide much better reaction kinetics and improved performance overall compared to less novel nucleic acid networks comprised of multiple distinct RNA molecules in place of the single gate RNA molecule.

[0080] Materials and Experimental Procedures

[0081] *Materials and Bacterial Strains:* All DNA oligonucleotides were designed using NUPACK software package⁸ and purchased from Integrated DNA Technologies. *E. coli* strains DH5 α (endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17(rK- mK+) λ -) and BL21 star DE3 (F-ompT hsdSB (rB-mB-) gal dcm rne131 [DE3]; Invitrogen) were used in this study.

[0082] *Plasmid Construction:* Both insert and vector backbone DNA oligonucleotides were amplified via PCR and assembled using Gibson assembly with 30-bp overlap regions¹². Triggers were inserted into pET15b-derived vectors, whereas the switch system was inserted into a pCOLADuet-derived vector.

[0083] *Growth and Expression Conditions:* *E. coli* were grown in LB broth with appropriate antibiotics at 37°C. For flow cytometry measurements, overnight cultures of cells picked from individual colonies were diluted 100 fold with fresh media shaken at 37°C for 80 minutes before induction. 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) were used to induce the cells. Measurements were taken at least 3 hours after induction.

[0084] *Flow Cytometry Measurements and Analysis:* Flow cytometry was performed using a Stratadigm S1000EXi flow cytometer with an A600 HTAS (High Throughput Auto Sampler). Cells were diluted with phosphate buffered saline (PBS) before measurement. Forward scatter (FSC) was used for trigger, and ~40,000 individual cells were analyzed using custom Matlab scripts.

[0085] *Cell-free Test with Plate Reader:* PURExpress® In Vitro Protein Synthesis Kit (NEB) was used for cell free tests with 100 ng switch plasmid and 400 ng trigger plasmid in 20 μ l reaction system. Synergy H1 Microplate reader (Biotek) was used to monitor GFP synthesis kinetics in 4 hours.

CLAIMS

We claim:

1. A synthetic nucleic acid molecule comprising:
an RNA-sensing switch RNA sequence, wherein the switch RNA sequence comprises:
a near-threshold hairpin structure comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and
first and second trigger recognition sequences located 5' and 3' to the hairpin structure, respectively;
wherein the hairpin structure is configured to alternatively adopt a translationally active ON state or adopt a translationally inactive OFF state in the presence of a repressing trigger RNA, and wherein the second trigger recognition sequence does not encode an in-frame stop codon.
2. The synthetic nucleic acid molecule of claim 1, wherein the start codon is located in a region of non-complementarity.
3. The synthetic nucleic acid molecule of claim 1, wherein the first trigger recognition sequence has a length of 15 nucleotides.
4. The synthetic nucleic acid molecule of claim 1, wherein the second trigger recognition sequence has a length of 12 nucleotides.
5. The synthetic nucleic acid molecule of claim 1, wherein the first and second trigger recognition sequences are fully or partially complementary to a repressing trigger RNA.
6. A method for altering expression of a protein, the method comprising
providing a protein coding sequence operably linked to a near-threshold translational repressor having first and second trigger recognition sequences that are fully or partially complementary to a repressing trigger RNA; and
providing the repressing trigger RNA, whereby expression of the protein is altered according to the level of repressing trigger RNA provided.

7. The method of claim 6, wherein expression of two or more proteins are altered by providing two or more protein coding sequences, each operably linked to a near-threshold translational repressor comprising first and second trigger recognition sequences that are fully or partially complementary to a single repressing trigger RNA.
8. The method of claim 6, wherein the fold-change in expression of the protein is at least 25 fold.
9. The method of claim 6, wherein the fold-change in expression of the protein is at least 50 fold.
10. A synthetic nucleic acid NOT-AND (NAND) logic circuit, the NAND logic circuit comprising:
two or more near-threshold translational repressor (NeaTTR) hairpin modules comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and a trigger recognition sequence located 5' and 3' to the hairpin structure, wherein the NeaTTR hairpin modules are separated by a linker domain.
11. The NAND logic circuit of claim 10, wherein the two or more NeaTTR hairpin modules are operably linked to a downstream reporter element.
12. The NAND logic circuit of claim 11, wherein the reporter element is GFP or lacZ.
13. The NAND logic circuit of claim 10, wherein the linker domain is 11 or 17 nucleotides.
14. The NAND logic circuit of claim 10, wherein the NAND logic circuit comprises three NeaTTR hairpin modules.
15. The NAND logic circuit of claim 10, wherein the NAND logic circuit comprises four or more NeaTTR hairpin modules.

14. A synthetic nucleic acid NOT-OR (NOR) logic circuit, the NOR logic circuit comprising:
at least one input RNA sensing hairpin module comprising an input RNA binding domain and a loop domain comprising first and second trigger RNA sequences; and
a near-threshold translational repressor (NeaTTR) hairpin module comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and first and second trigger recognition sequences located 5' and 3' to the hairpin structure, respectively; wherein the first and second trigger recognition sequences are complementary to the first and second trigger RNA sequences, respectively, of the input RNA sensing hairpin module.
15. The NOR logic circuit of claim 14, wherein the NeaTTR hairpin module is operably linked to a reporter element.
16. The NOR logic circuit of claim 15, wherein the reporter element is GFP or lacZ.
17. The NOR logic circuit of claim 14, wherein the NOR logic circuit comprises two RNA sensing hairpin modules.
18. The NOR logic circuit of claim 14, wherein the NOR logic circuit comprises three RNA sensing hairpin modules.
19. The NOR logic circuit of claim 14, wherein the NOR logic circuit comprises four or more RNA sensing hairpin modules.
20. A method for altering expression of a protein in a cell, the method comprising:
introducing into a cell a protein coding sequence operably linked to a near-threshold translational repressor having first and second trigger recognition sequences that are fully or partially complementary to a repressing trigger RNA; and

introducing into a cell the repressing trigger RNA, whereby expression of the protein is altered according to the level of repressing trigger RNA introduced to the cell.

21. The method of claim 20, wherein expression of two or more proteins are altered by introducing into the cell two or more protein coding sequences, each operably linked to a near-threshold translational repressor comprising first and second trigger recognition sequences that are fully or partially complementary to a single repressing trigger RNA.

22. The method of claim 20, wherein the fold-change in expression of the protein is at least 25 fold.

23. The method of claim 20, wherein the fold-change in expression of the protein is at least 50 fold.

FIG. 1

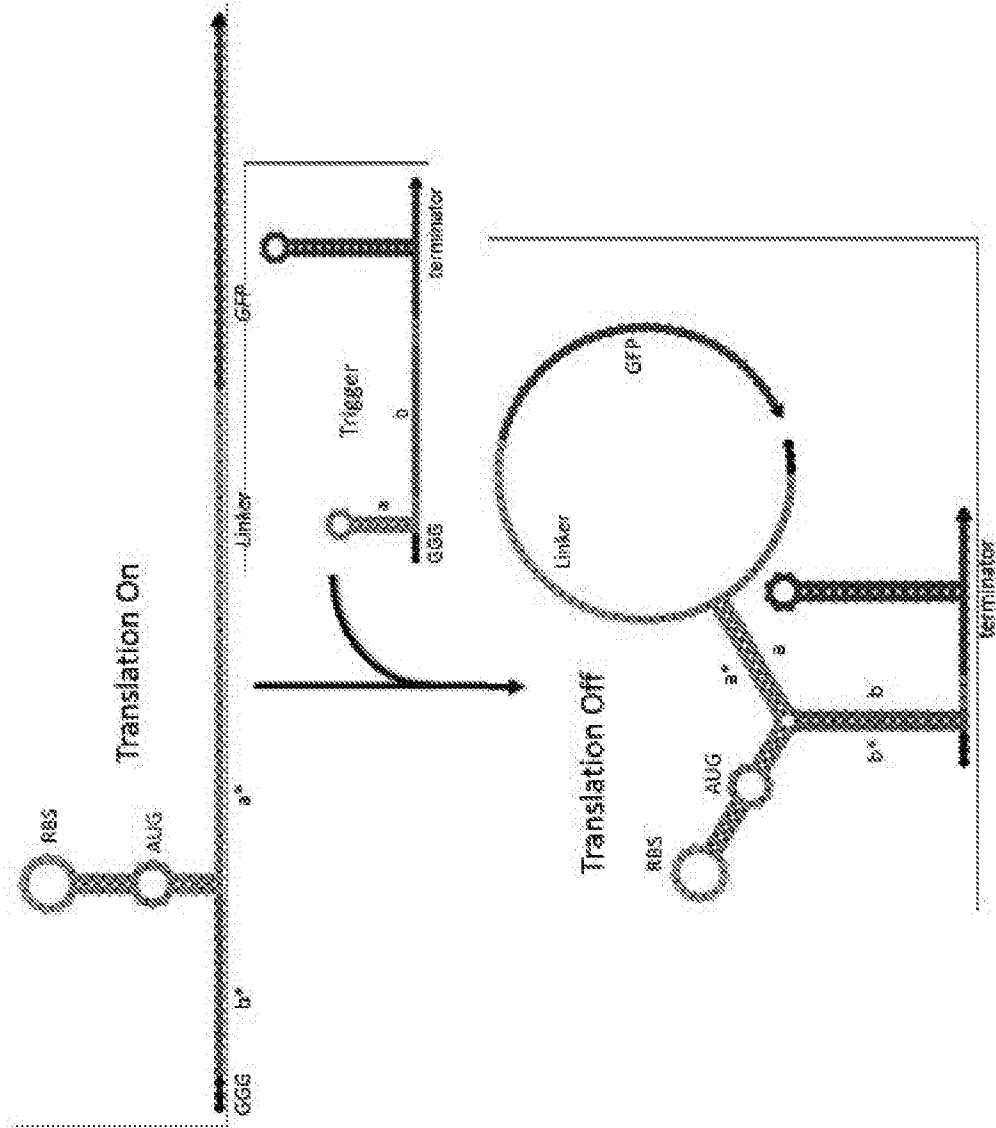


FIG. 2

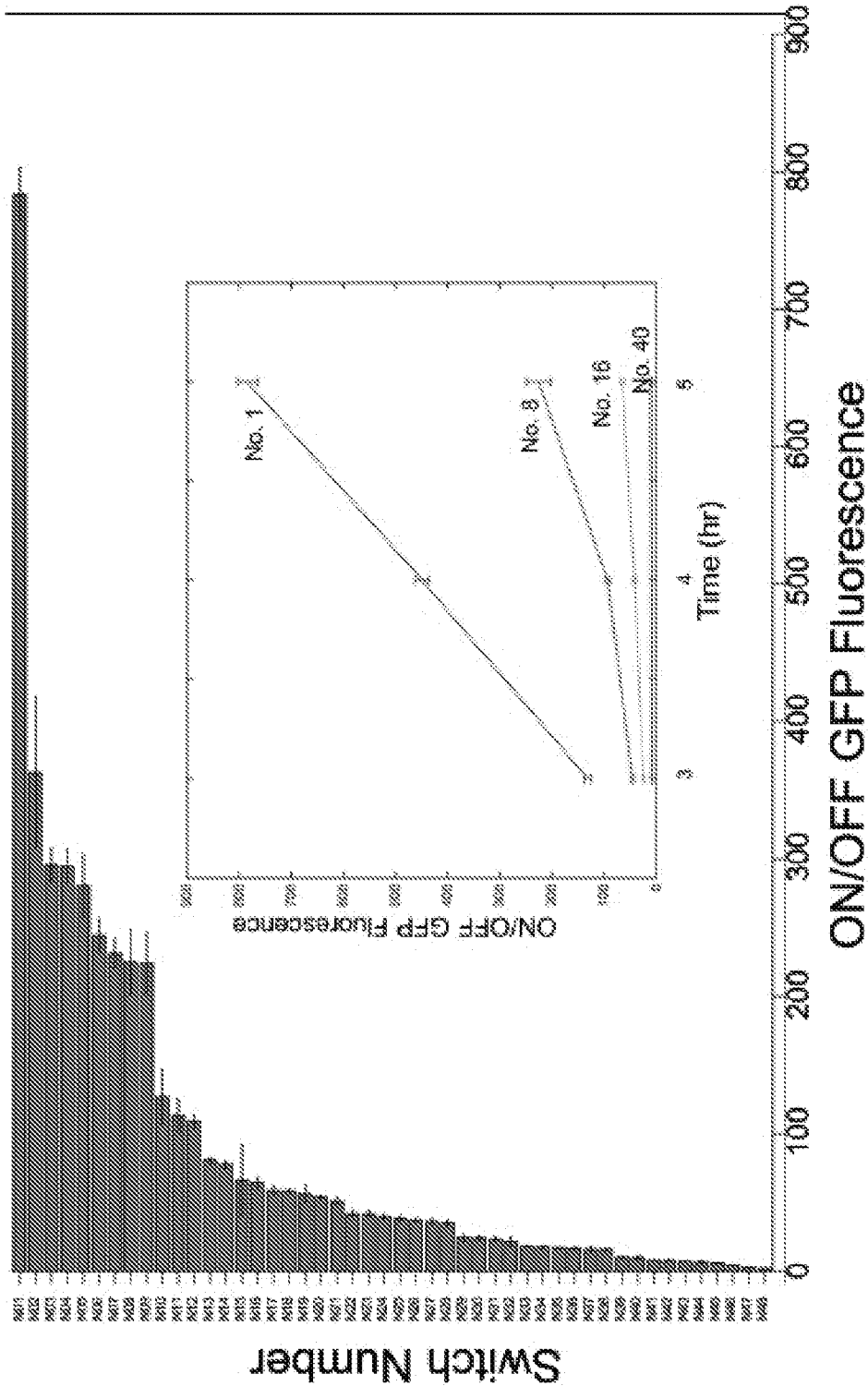
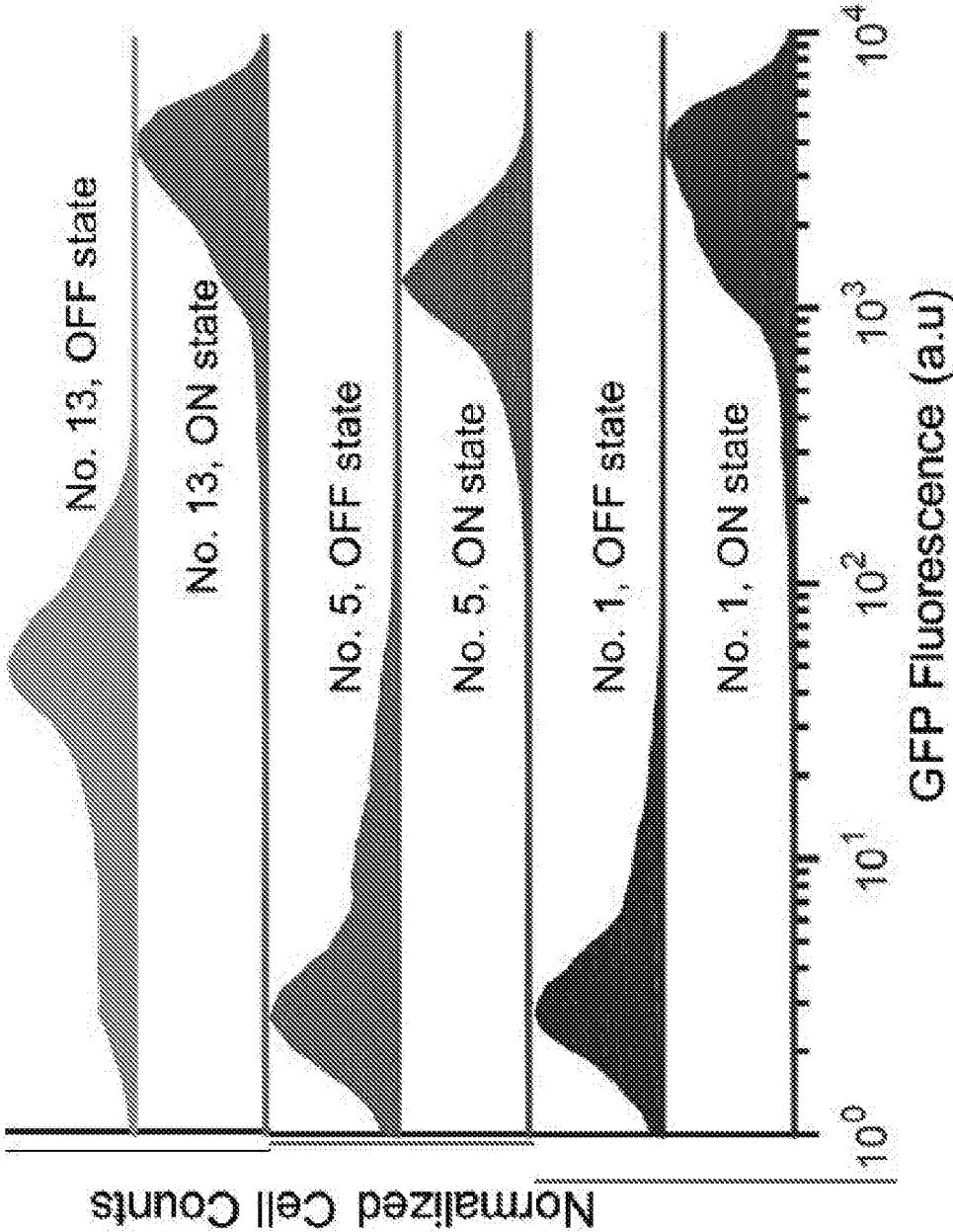
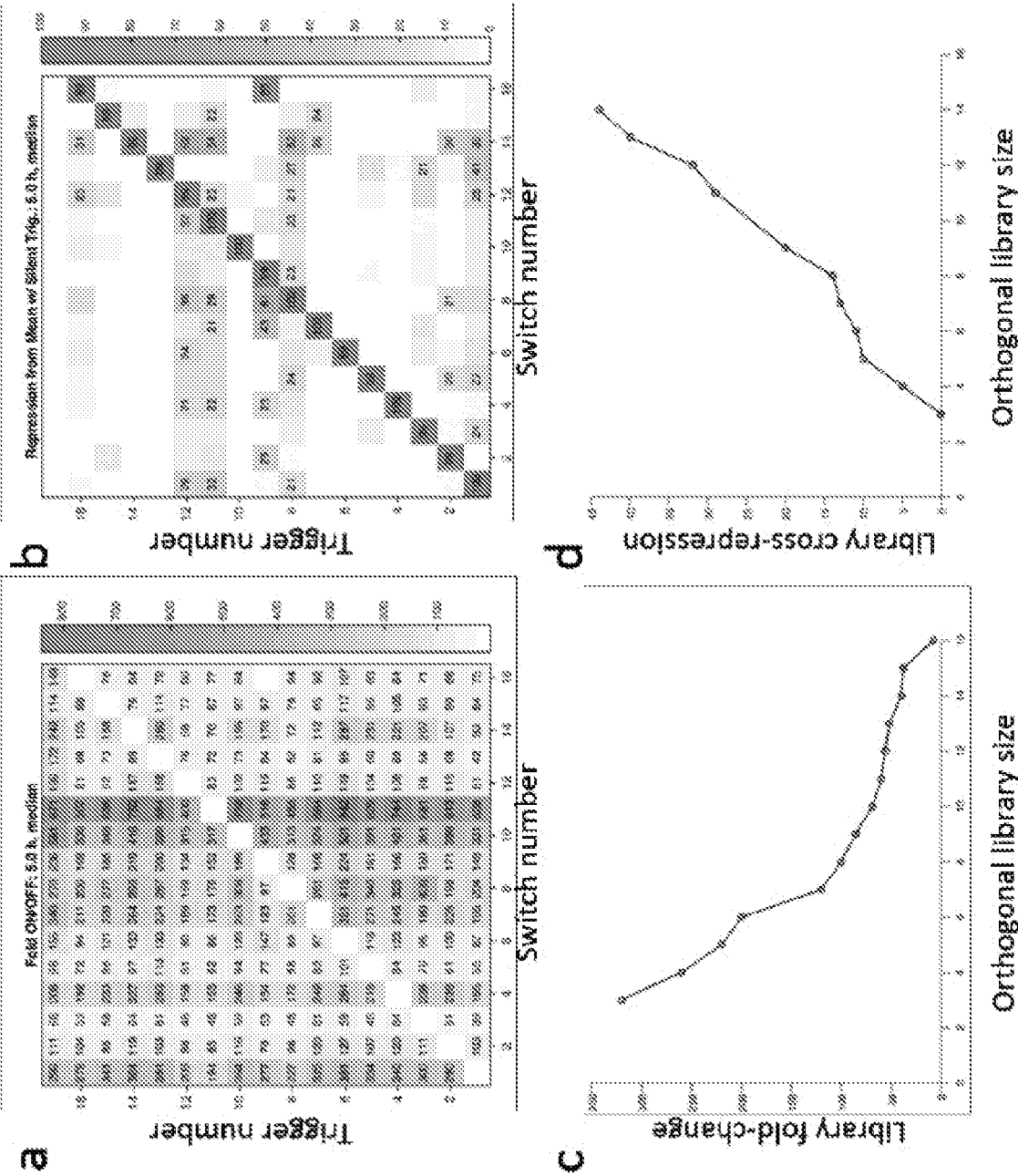


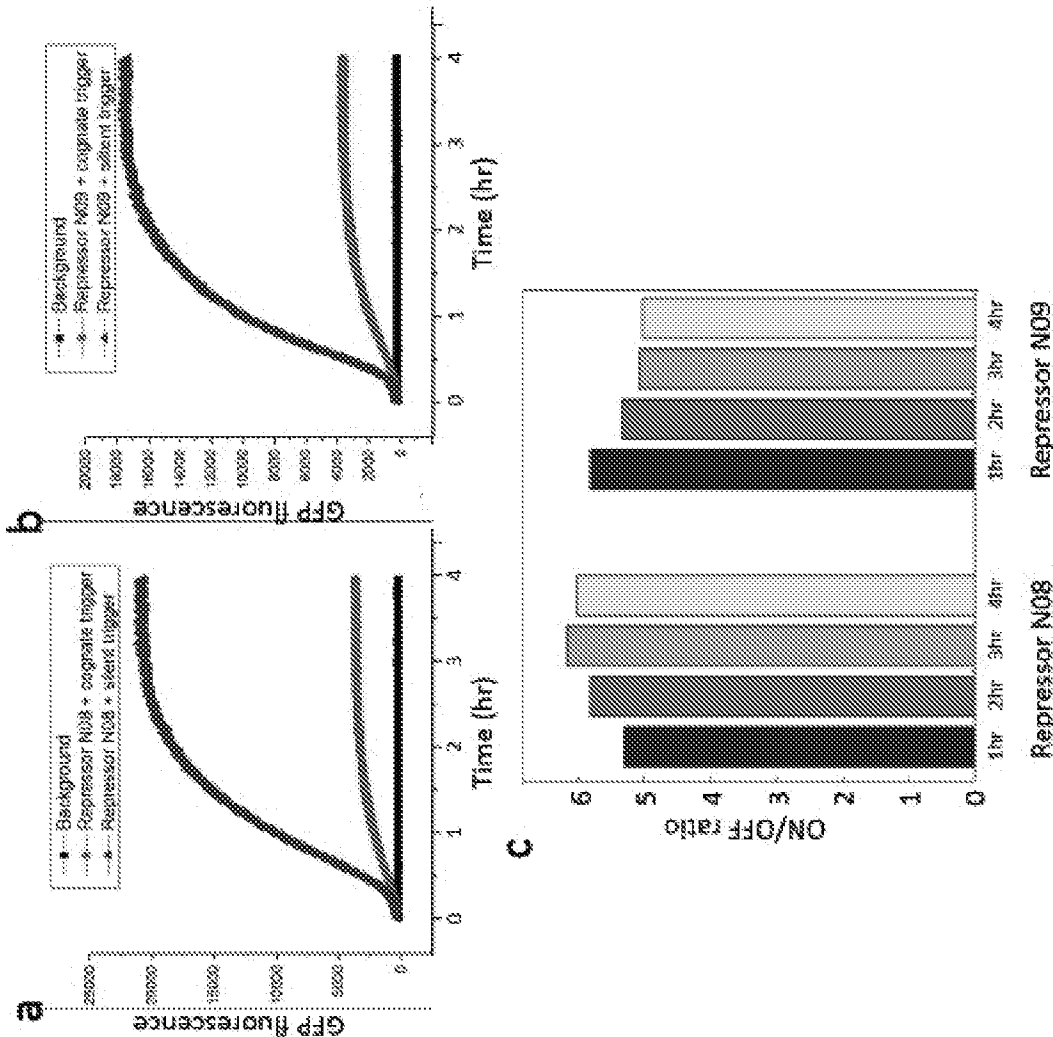
FIG. 3



FIGS. 4A-4D



FIGS. 5A-5C



FIGS. 6A-6B

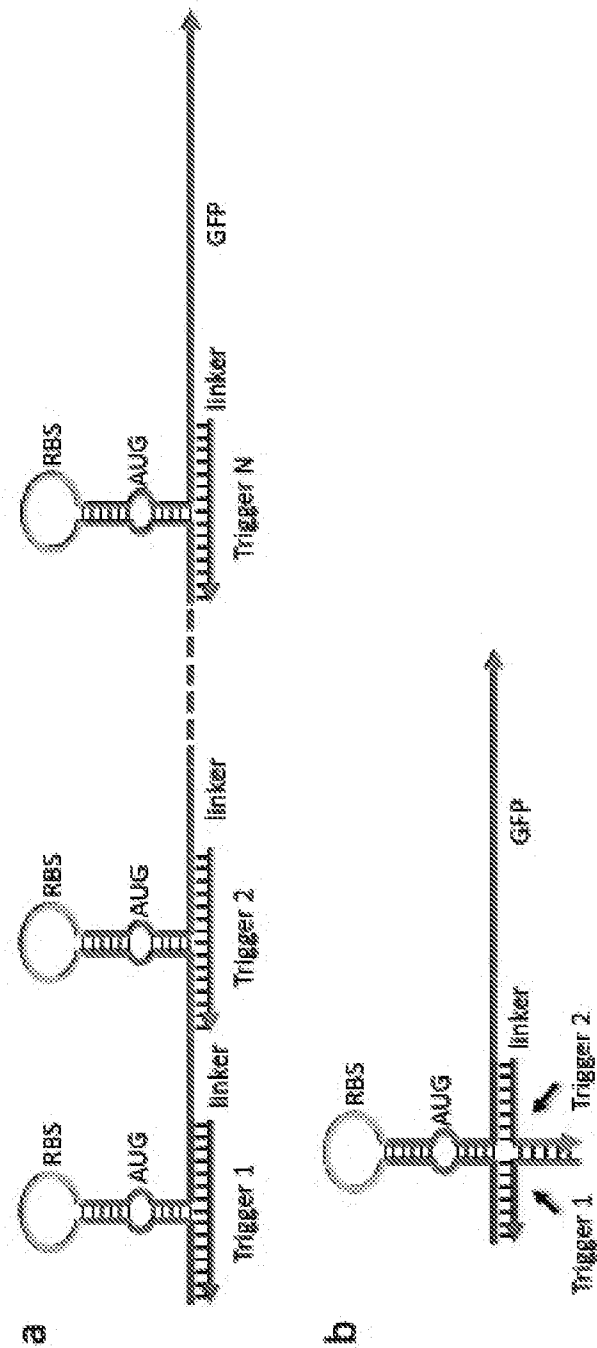
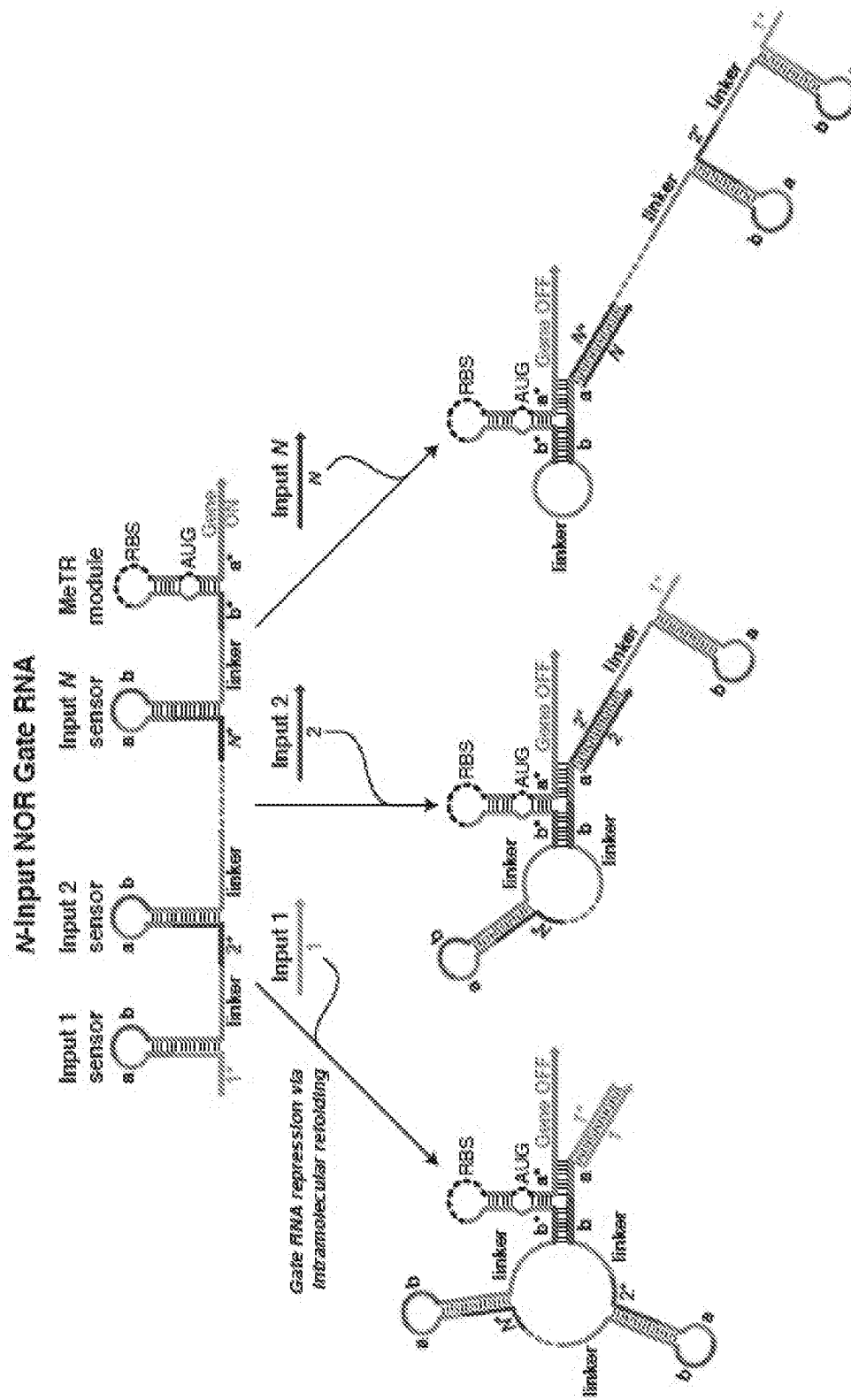


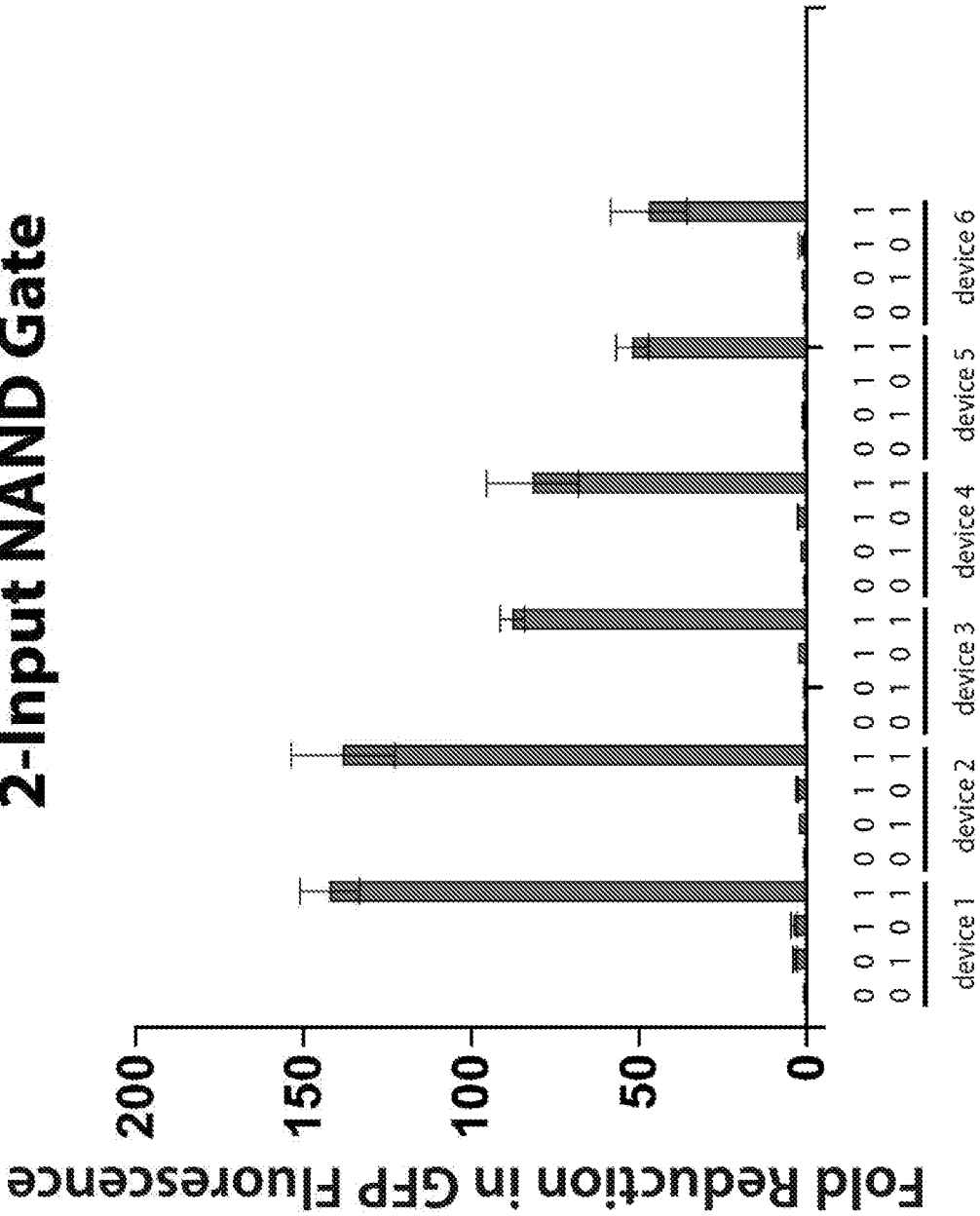
FIG. 7



FIGS. 8A-8C

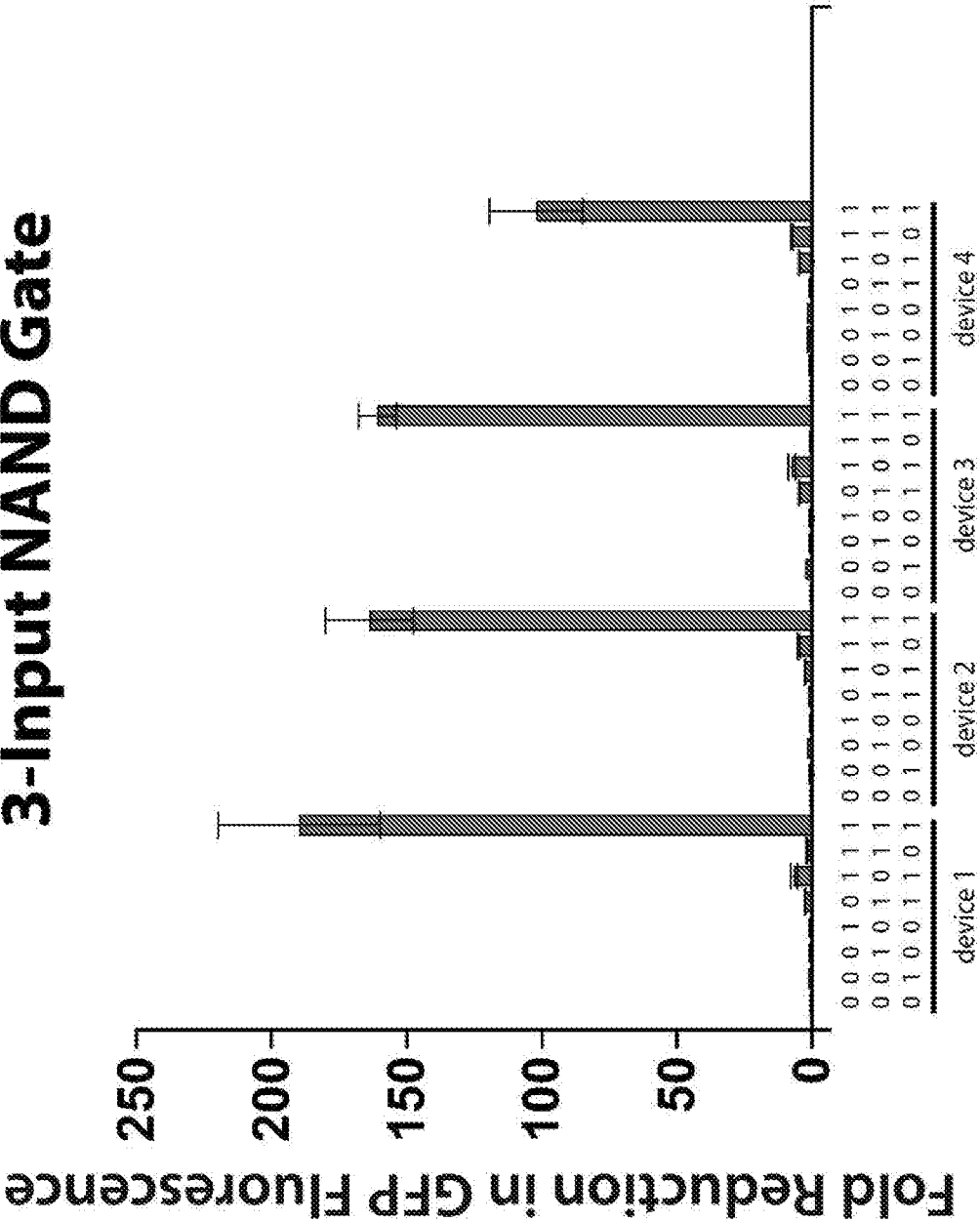
2-Input NAND Gate

A.



FIGS. 8A-8C, CONTINUED

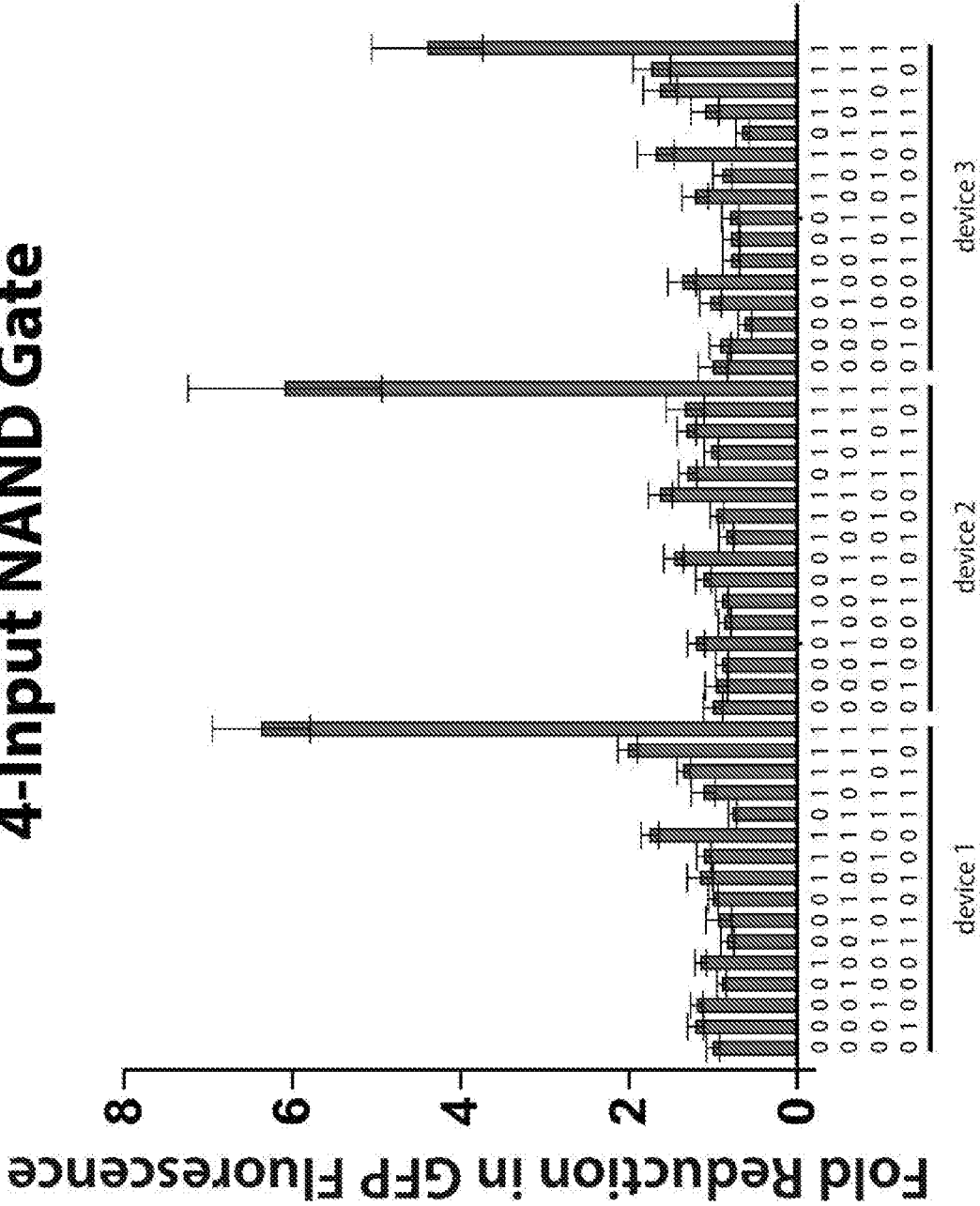
3-Input NAND Gate



B.

FIGS. 8A-8C, CONTINUED

4-Input NAND Gate



C.

FIG. 9

1-Bit NOR Gate

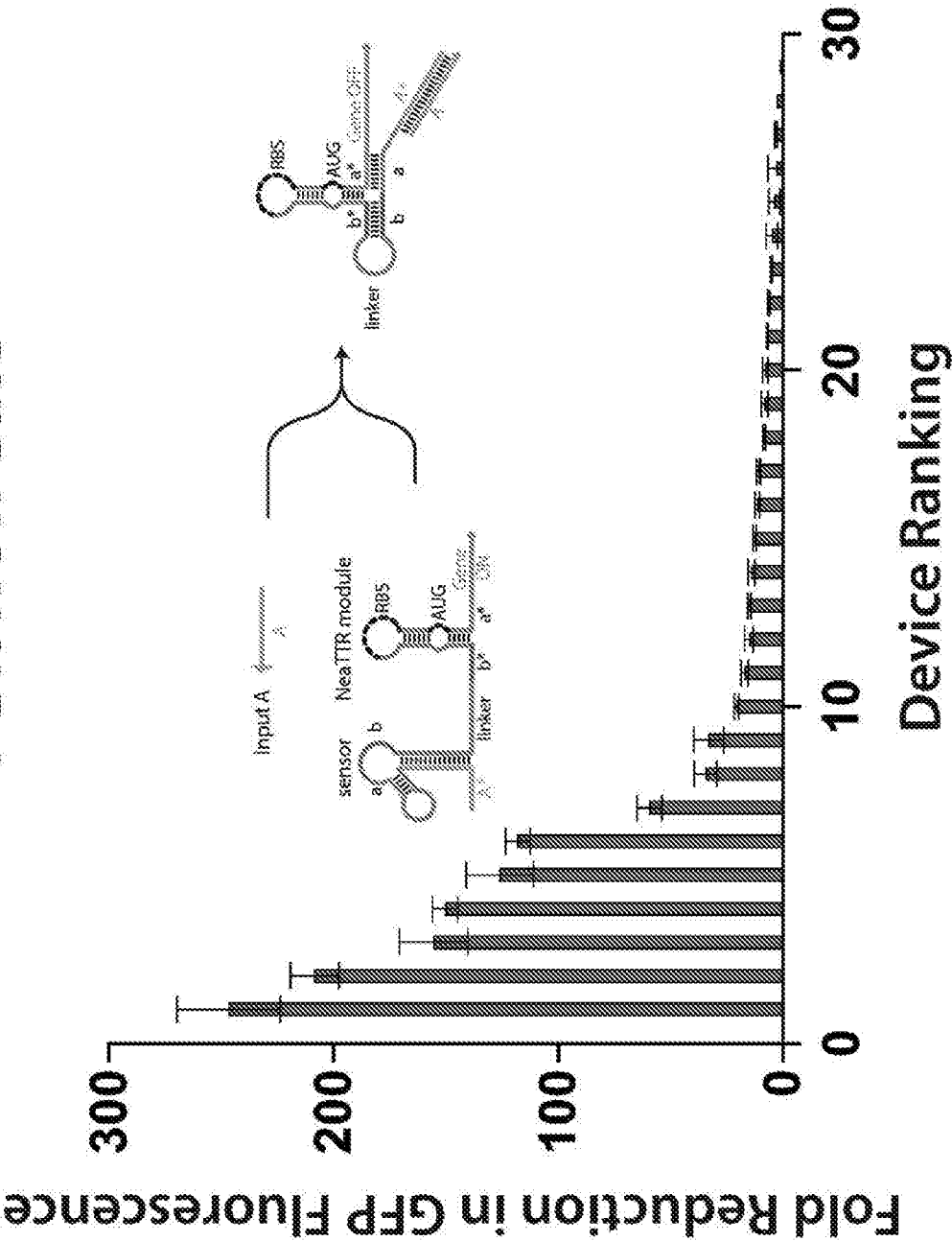


FIG. 10

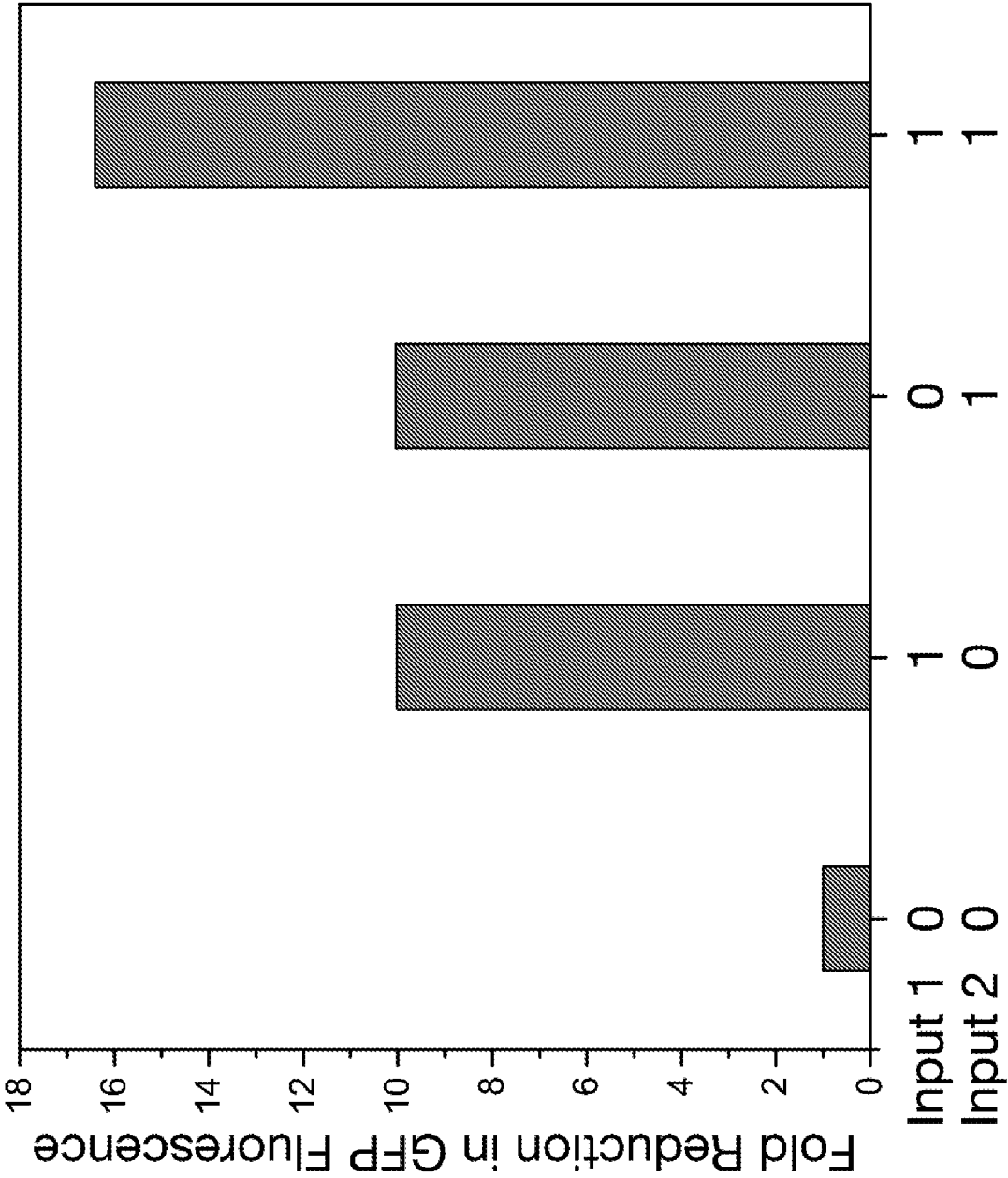
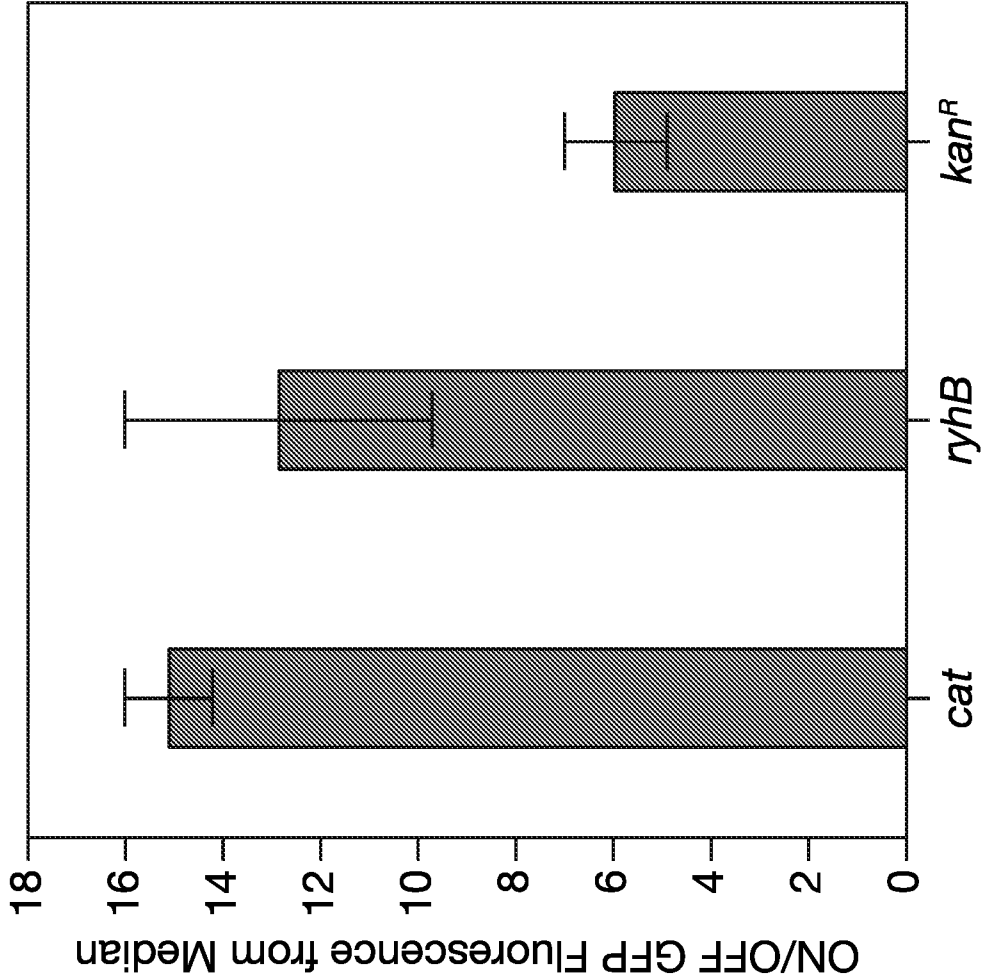


FIG. 11



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/44815

A. CLASSIFICATION OF SUBJECT MATTER
 IPC - C12N 15/113, 15/67, 5/10; C12Q 1/68 (2017.01)
 CPC - C12N 15/113, 15/67, 5/10, 15/8209; C12Q 1/6897

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.
A	WO 2016/011089 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 21 January, 2016; page 6, lines 21-22; page 18, lines 16-18; page 25, line 5; page 27, lines 15-16; page 31, lines 19- page 32, line 2; page 33, lines 7-17; page 39, lines 7-9; figures 11A, 12A	1-9, 14b, 15b, 16-23
A	US 2015/0275203 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE et al.) 01 October, 2015; paragraphs [0012], [0065], [0089], [0101], [0124], [0146]; figures 11A-11B, 12A, 13A	1-9, 14b, 15b, 16-23
A	(KRISHNAMURTHY, M et al.) Tunable Riboregulator Switches for Post-Transcriptional Control of Gene Expression. ACS Synthetic Biology. 27 July, 2015; Vol. 4, No. 12; pages 1-31; abstract; page 5, paragraph 2; page 8, paragraph 1; page 10, paragraph 4; page 22, paragraph 1; figures 1A-1B; DOI: 10.1021/acssynbio.5b00041	1-9, 14b, 15b, 16-23
P, Y	WO 2017/205668 A1 (ARIZONA BOARD OF REGENTS ON BEHALF OF ARIZONA STATE UNIVERSITY et al.) 30 November, 2017; whole document	1-9, 14b, 15b, 16-23

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

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

06 December 2017 (06.12.2017)

Date of mailing of the international search report

04 JAN 2018

Name and mailing address of the ISA/

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Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/44815

Box No. I 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:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☒ on paper or in the form of an image file.
 - b. ☒ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/44815

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.:
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:

- Please See the Next Supplemental Page-

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.:
Group I, Claims 1-9, 14b, 15b, 16-23

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.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US17/44815

-Continued from Box III: Observations where unity of invention is lacking -

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, Claims 1-9 and 14-23 are directed toward a synthetic nucleic acid molecule, comprising a synthetic nucleic acid NOT-OR (NOR) logic circuit; and methods for altering expression of a protein therewith.

Group II, Claims 10-13 are directed toward a synthetic nucleic acid NOT-AND (NAND) logic circuit

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a second trigger recognition sequence that does not encode an in-frame stop codon, not present in Group II; the special technical features of Group II include a NAND logic circuit, not present in Group I.

Groups I and II share the technical features including: a synthetic nucleic acid logic circuit, the logic circuit comprising: at least one near-threshold translational repressor (NeaTTR) hairpin module comprising a loop-forming region comprising a ribosomal binding site (RBS) and a stem-forming region comprising a start codon, and a trigger recognition sequence located 5' and 3' to the hairpin structure.

However, these shared technical features are previously disclosed by WO 2016/011089 A1 to President and Fellows of Harvard College et al. (hereinafter 'Harvard').

Harvard discloses a synthetic nucleic acid logic circuit (a synthetic nucleic acid logic circuit; page 2, line 24 - page 3, line 2; page 32, line 26 - page 33, line 17), the logic circuit comprising: at least one near-threshold translational repressor (NeaTTR) hairpin module (the logic circuit comprising: at least one toehold repressor (near-threshold translational repressor (NeaTTR) hairpin module); page 31, lines 7 - page 32, line 17) comprising a loop-forming region comprising a ribosomal binding site (RBS) (comprising a loop-forming region comprising a ribosomal binding site (RBS); page 31, lines 7 - page 32, line 17) and a stem-forming region comprising a start codon (a stem-forming region comprising a start codon; page 31, lines 7 - page 32, line 17), and a trigger recognition sequence located 5' and 3' to the hairpin structure (and trigger sequences located 5' and 3' to the hairpin structure; page 31, lines 7 - page 32, line 17).

Since none of the special technical features of the Groups I and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Harvard reference, unity of invention is lacking.