



- (51) **International Patent Classification:**
C12N 15/85 (2006.01) *C12N 1/21* (2006.01)
- (21) **International Application Number:**
PCT/US2016/054767
- (22) **International Filing Date:**
30 September 2016 (30.09.2016)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
62/235,186 30 September 2015 (30.09.2015) US
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- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

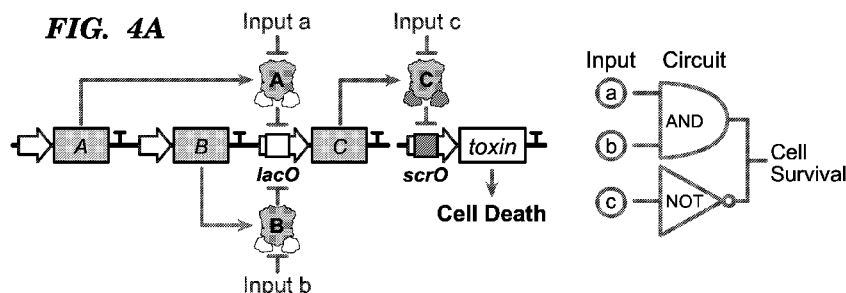
Declarations under Rule 4.17:

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

Published:

- without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) **Title:** DEADMAN AND PASSCODE MICROBIAL KILL SWITCHES



(57) **Abstract:** Provided herein are systems, methods and compositions for rendering cells or the expression of an effector protein sensitive to a predetermined condition. In one aspect, cells can be rendered dependent upon the presence of an environmental agent, *e.g.*, an exogenous agent, without which the cell will default to expression of a death protein and be killed. In another aspect, cells can be rendered sensitive to the presence of a set of predetermined conditions such that cells will only grow when two or more necessary exogenous agents are supplied, and without either of which, the cells are killed. In this aspect, hybrid transcription factors provide a vast array of possible predetermined conditions.

DEADMAN AND PASSCODE BIOLOGICAL KILL SWITCHES**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims benefit under 35 U.S.C. §119(e) of U.S. Provisional Application Serial No. 62/235,186 filed on September 30, 2015, the contents of which are herein incorporated by reference in their entirety

FIELD OF THE INVENTION

[0002] This invention relates to genetically engineered biological circuits and their uses.

GOVERNMENT SUPPORT

[0003] This invention was made with Government Support under Contract No. HDTRA1-14-1-0006 awarded by the Defense Threat Reduction Agency, Contract No. N00014-11-1-0725, awarded by the Office of Naval Research, and Contract No. FA9550-14-1-0060 awarded by the Air Force Office of Scientific Research. The Government has certain rights in the invention.

BACKGROUND

[0004] With the advent of synthetic biology, genetically modified microorganisms have been increasingly used for biomedical, industrial and environmental applications¹⁻⁶. Deployment of these engineered microbes in large scales and open environments calls for the development of safe and secure means to restrain their proliferation. Pioneering biocontainment systems used metabolic auxotrophy in which target cells could only grow in the presence of an exogenously supplied metabolite^{7,8}, and the recent creation of an *E. coli* strain with an altered genetic code enabled production of synthetic auxotrophy strains in which an exogenous supply of non-natural amino acids is required for cell survival^{9,10}. Traditional metabolic auxotrophy strains are hampered by the potential for inadvertent complementation by crossfeeding or by the presence of the metabolite in heterogenous environments, and synthetic auxotrophy systems rely on extensive genome-wide engineering that can be impractical for many industrial production and biotherapeutic microbes. Furthermore, they are intrinsically difficult to reprogram for different environmental conditions, limiting their application.

SUMMARY OF THE INVENTION

[0005] Described herein, in part, are synthetic biological biocontainment circuits. In some embodiments, a switch termed herein as a “Deadman kill switch” that uses, in part, a transcription-based monostable toggle design to provide rapid and robust target cell killing in the absence of an input survival signal or condition is used, and, in some embodiments, a synthetic biological circuit termed herein a “Passcode circuit” or “Passcode kill switch” that uses hybrid transcription factors

(TFs) to construct complex environmental requirements for cell survival, are provided. As described herein, a tripartite strategy of (i) TF protein engineering to detect diverse input signals, (ii) robust circuit design to provide signal processing, and (iii) redundant toxin-induced and protease-mediated cell killing mechanisms was used. The resulting engineered biocontainment systems described herein are modular, flexible and extensible, and are useful across many industrial and biotherapeutic applications.

[0006] Accordingly, in some aspects, provided herein are programmable synthetic biological circuits responsive to a selected combination of predetermined inputs, the circuits comprising:

- i) an effector expression module comprising a nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first modulator protein is present;
- ii) a first modulator protein expression module comprising a nucleic acid sequence encoding the first modulator protein, operably linked to a second nucleic acid regulatory element comprising an AND circuit, whereby expression of the first modulator protein requires the presence of each of a selected set of at least two predetermined input agents, and in the absence of any one of the at least two predetermined input agents, expression of the first modulator protein is altered, resulting in expression of the effector protein.

[0007] In some embodiments of these aspects and all such aspects described herein, the second nucleic acid regulatory element comprises a DNA binding site for a second modulator protein.

[0008] In some embodiments of these aspects and all such aspects described herein, the first modulator protein is a repressor protein, and where, in the absence of any one of the at least two predetermined input agents, expression of the repressor protein is inhibited, resulting in expression of the effector protein.

[0009] In some embodiments of these aspects and all such aspects described herein, the programmable synthetic biological circuit further comprises a set of at least two hybrid modulator protein expression modules, each of which comprises nucleic acid sequence encoding a hybrid modulator protein and operatively linked regulatory sequences that permit the expression thereof, each hybrid modulator protein comprising:

- a) an input agent-responsive domain of an input agent-sensitive transcription factor; and
- b) the DNA binding domain of the second modulator protein,

wherein the binding of each respective hybrid modulator protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is altered by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid modulator protein, and

wherein each respective hybrid modulator protein alters expression from the first modulator protein expression module in the absence of its respective input agent.

[0010] In some embodiments of these aspects and all such aspects described herein, the at least two hybrid modulator proteins are each hybrid repressor proteins, where the binding of each respective hybrid repressor protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is altered by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid modulator protein, and wherein each respective hybrid modulator protein alters expression from the first modulator protein expression module in the absence of its respective input agent.

[0011] In some aspects, provided herein are compositions comprising one or more nucleic acids that alone or together constitute any of the programmable synthetic biological circuits described herein.

[0012] In some embodiments of these aspects and all such aspects described herein, the expression modules are comprised by a single nucleic acid construct.

[0013] In some embodiments of these aspects and all such aspects described herein, the hybrid modulator expression modules and first modulator expression module are on the same nucleic acid construct.

[0014] In some embodiments of these aspects and all such aspects described herein, the hybrid repressor expression modules, first repressor expression module and effector expression module are on the same nucleic acid construct.

[0015] Provided herein, in some aspects, are host cells comprising one or more nucleic acids that alone or together constitute any of the programmable synthetic biological circuits described herein.

[0016] In some embodiments of these aspects and all such aspects described herein, the cell is a bacterial, fungal, plant or animal cell.

[0017] In some aspects, provided herein, are programmable synthetic biological circuits responsive to a selected combination of predetermined inputs, the circuit comprising:

- i) an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first repressor protein is present;
- ii) a first repressor protein expression module comprising nucleic acid sequence encoding the first repressor protein, operably linked to a second nucleic acid regulatory element comprising an AND circuit, whereby expression of the first repressor protein requires the presence of each of a selected set of at least two predetermined input agents, and in the absence of any one of the at least two predetermined input agents, expression of the first repressor protein is repressed, resulting in expression of the effector protein.

[0018] In some embodiments of these aspects and all such aspects described herein, the second nucleic acid regulatory element comprises a DNA binding site for a second repressor protein.

[0019] In some embodiments of these aspects and all such aspects described herein, the first nucleic acid regulatory element comprises a DNA binding site for the first repressor protein.

[0020] In some embodiments of these aspects and all such aspects described herein, the predetermined input agent comprises an environmental input agent.

[0021] In some embodiments of these aspects and all such aspects described herein, the programmable synthetic biological circuits further comprise a set of at least two hybrid repressor protein expression modules, each of which comprises nucleic acid sequence encoding a hybrid repressor protein and operatively linked regulatory sequences that permit the expression thereof, each hybrid repressor protein comprising:

- a) an input agent-responsive domain of an input agent-sensitive transcription factor; and
- b) the DNA binding domain of the second repressor protein,

wherein the binding of each respective hybrid repressor protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is inhibited by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid repressor protein, and

wherein each respective hybrid repressor protein represses expression from the first repressor protein expression module in the absence of its respective input agent.

[0022] In some embodiments of these aspects and all such aspects described herein, the binding and activity of the first repressor protein is inhibited by the binding of an input agent different from members of the selected set of at least two input agents, thereby establishing a NOT circuit, such that in the absence of the different input agent, the first repressor protein binds the first nucleic acid regulatory element and represses expression of the effector protein, but in the presence of the different input agent, the first repressor protein does not bind the first nucleic acid regulatory element and permits expression of the effector protein.

[0023] In some embodiments of these aspects and all such aspects described herein, the first repressor protein is a hybrid repressor protein comprising an input agent-responsive domain of an input agent-sensitive transcription factor and the DNA binding domain of the second repressor protein, wherein the input agent responsive domain and the DNA binding domain are heterologous to each other.

[0024] In some embodiments of these aspects and all such aspects described herein, the effector is a toxin or a reporter protein.

[0025] In some embodiments of these aspects and all such aspects described herein, the toxin is selected from the group consisting of a restriction endonuclease, a DNA gyrase inhibitor, a ribonuclease-type toxin.

[0026] In some embodiments of these aspects and all such aspects described herein, the first repressor protein is selected from a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR

polypeptide, a CelR polypeptide, MalI polypeptide, ScrR polypeptide, TetR polypeptide, or a hybrid comprising the DNA binding domain of one such polypeptide fused to the input agent-responsive domain of another such polypeptide.

[0027] In some embodiments of these aspects and all such aspects described herein, the second repressor protein is selected from a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CelR polypeptide, MalI polypeptide, ScrR polypeptide, TetR polypeptide, or a hybrid comprising the DNA binding domain of one such polypeptide fused to the input agent-responsive domain of another such polypeptide.

[0028] In some embodiments of these aspects and all such aspects described herein, the input agent-responsive domain of each hybrid repressor protein is selected from the environmental agent binding domains of a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CelR polypeptide, MalI polypeptide, ScrR polypeptide, and a TetR polypeptide.

[0029] In some embodiments of these aspects and all such aspects described herein, the at least two input agents are selected from the group consisting of IPTG, galactose, cellobiose, ampicillin, chloramphenicol, kanamycin, and ATc.

[0030] In some embodiments of these aspects and all such aspects described herein, the programmable biological circuit is programmed by assembling a specific combination of effector expression module, first repressor protein expression module and a set of hybrid repressor protein expression modules sensitive to selected input agents.

[0031] In some embodiments of these aspects and all such aspects described herein, the hybrid repressor expression modules and first repressor expression module are on the same nucleic acid construct.

[0032] In some embodiments of these aspects and all such aspects described herein, the hybrid repressor expression modules, first repressor expression module and effector expression module are on the same nucleic acid construct.

[0033] Provided herein, in some aspects, are compositions comprising one or more nucleic acids that alone or together constitute any of the programmable synthetic biological circuits described herein.

[0034] Provided herein, in some aspects, are host cells comprising one or more nucleic acids that alone or together constitute any of the programmable synthetic biological circuits described herein.

[0035] In some embodiments of these aspects and all such aspects described herein, the cell is a bacterial cell, fungal cell, plant cell, animal cell, or synthetic cell.

[0036] In some aspects, provided herein are passively activated biological containment circuits rendering a host cell dependent upon the presence of an exogenous input, the circuit comprising:

- a) an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first modulator protein is present;
- b) a first modulator protein expression module comprising nucleic acid sequence encoding the first modulator protein, operably linked to a second nucleic acid regulatory element, the second nucleic acid regulatory element permitting expression of the first modulator protein unless a second modulator protein is present and binds the second nucleic acid regulatory element; and
- c) a second modulator protein expression module comprising nucleic acid sequence encoding the second modulator protein, operably linked to a third nucleic acid regulatory element, the third nucleic acid regulatory element permitting expression of the second modulator protein, wherein the binding of the second modulator protein to the second nucleic acid regulatory element is modulated by an exogenous input,

wherein, in the presence of the exogenous input, the binding of the second modulator protein to the second nucleic acid regulatory element is inhibited, thereby permitting the expression of the first modulator protein such that expression of the effector protein is inhibited; and

wherein, in the absence of the exogenous input, the binding of the second modulator protein to the second nucleic acid regulatory element is permitted, thereby inhibiting, thereby inhibiting the expression of the first modulator protein such that expression of the effector protein is permitted.

[0037] In some embodiments of these aspects and all such aspects described herein, the effector protein comprises a toxin, a restriction endonuclease, or a protease.

[0038] In some embodiments of these aspects and all such aspects described herein, the effector protein comprises a protease that degrades a cellular polypeptide essential for cell survival.

[0039] In some embodiments of these aspects and all such aspects described herein, the first and second modulator proteins are different repressor proteins that bind different nucleic acid regulatory sequences.

[0040] In some embodiments of these aspects and all such aspects described herein, the passively activated biological containment circuits described herein further comprise a third modulator protein expression module, comprising nucleic acid sequence encoding a third modulator protein, operatively linked to a fourth nucleic acid regulatory element, the fourth nucleic acid regulatory element permitting expression of the third modulator protein unless the first modulator protein is present.

[0041] In some embodiments of these aspects and all such aspects described herein, the third modulator protein degrades the first modulator protein.

[0042] In some embodiments of these aspects and all such aspects described herein, in the absence of the exogenous input, the third modulator protein is expressed, resulting in degradation of the first modulator protein, resulting in the expression of the effector protein.

[0043] In some embodiments of these aspects and all such aspects described herein, the exogenous input comprises a small molecule that binds to the second modulator protein.

[0044] In some embodiments of these aspects and all such aspects described herein, the second modulator protein and the exogenous input are a pair selected from a tetracycline analog that binds TetR and TetR, and a lactose analog that binds LacI and LacI.

[0045] In some embodiments of these aspects and all such aspects described herein, the third modulator protein comprises a protease that degrades the first regulator protein and a cellular polypeptide essential for cell survival, and wherein the effector protein comprises a toxin.

[0046] Provided herein, in some aspects, are isolated nucleic acid comprising sequences encoding any of the passively activated biological containment circuits described herein.

[0047] In some aspects, provided herein are nucleic acid vector constructs comprising sequences encoding any of the passively activated biological containment circuits described herein.

[0048] In some aspects, provided herein are host cells comprising nucleic acid comprising sequences encoding any of the passively activated biological containment circuits described herein.

[0049] In some embodiments of these aspects and all such aspects described herein, the cell is a bacterial, fungal, plant, animal, or synthetic cell.

[0050] In some aspects, provided herein are engineered biological circuits rendering a cellular response sensitive to a predetermined condition, the circuit comprising a nucleic acid construct comprising expression modules that form a deadman regulatory circuit sensitive to the predetermined condition, the construct comprising:

i) a first repressor module M_{R1} comprising a first repressible promoter nucleic acid sequence (rP_1) operably linked to a repressor nucleic acid sequence encoding a first repressor protein (R_A), that binds repressible promoter element rP_E and represses expression from promoters comprising element rP_E ;

ii) a second repressor module M_{R2} comprising a second promoter nucleic acid sequence (P_2) operably linked to a second repressor nucleic acid sequence (R_B) encoding a second repressor protein R_B , wherein transcription from rP_i is inhibited by the second repressor protein R_B and wherein repression activity of R_B is sensitive to inhibition by a first agent A_1 , the presence or absence of A_1 establishing a predetermined condition;

iii) an effector module M_E comprising an effector protein coding sequence E operably linked to a third, repressible promoter comprising repressible promoter element rP_E , that is repressed by first repressor protein R_A ;

the respective modules forming a deadman regulatory circuit such that:

in the absence of agent A_1 , the second repressor protein R_B is expressed and represses transcription from repressor module M_{RI} , such that expression of first repressor protein R_A is repressed, thereby relieving repression of effector module M_E by R_A , such that effector E is expressed by M_E ; and

in the presence of agent A_1 , the activity of the second repressor protein R_B is inhibited, permitting expression of the first repressor protein R_A , which maintains expression from effector module E in the "off" state, such that agent A_1 is required by the circuit to maintain effector protein expression in the "off" state, and in the absence of A_1 , the circuit defaults to expression of the effector protein.

[0051] In some embodiments of these aspects and all such aspects described herein, the effector is a toxin or a protein that induces a cell death program.

[0052] Provided herein, in some aspects, are methods of rendering a cell responsive to a predetermined condition, the method comprising introducing a nucleic acid encoding any of the engineered biological circuits described herein to the cell.

[0053] In some aspects, provided herein are isolated nucleic acids encoding any of the engineered biological circuits described herein.

[0054] In some aspects, provided herein are host cells comprising nucleic acids encoding any of the engineered biological circuits described herein.

Definitions

[0055] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology, and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3); Robert S. Porter *et al.* (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), Taylor & Francis Limited, 2014 (ISBN 0815345305, 9780815345305); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis *et al.*, Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc.,

New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737), the contents of which are all incorporated by reference herein in their entireties.

[0001] As used herein, the term “predetermined input” refers to an agent or condition that influences the activity of a transcription factor polypeptide in a known manner. Generally, such agents can bind to and/or change the conformation of the transcription factor polypeptide to thereby modify the activity of the transcription factor polypeptide. Examples of predetermined inputs include, for example, environmental input agents that are not required for the survival of a given host organism (*i.e.*, in the absence of a synthetic biological circuit as described herein). Conditions that can provide a predetermined input include, for example temperature, *e.g.*, where the activity of one or more factors is temperature-sensitive, the presence or absence of light, including light of a given spectrum of wavelengths, and the concentration of a gas, salt, metal or mineral. Environmental input agents include, for example, a small molecule, biological agents such as pheromones, hormones, growth factors, metabolites, nutrients, and the like and analogs thereof; concentrations of chemicals, environmental byproducts, metal ions, and other such molecules or agents; light levels; temperature; mechanical stress or pressure; or electrical signals, such as currents and voltages.

[0056] It should be understood that the synthetic biological circuits described herein are necessarily exogenous to the host cell in which they are expressed; *i.e.*, the synthetic biological circuits described herein do not occur naturally, nor are they found in host cells naturally. A key point of one embodiment of the disclosed biological circuits is that the host cell cannot survive in the wild, *i.e.*, absent an input controlled by the user of this technology.

[0057] As used herein, the term “effector protein” refers to a polypeptide that provides a detectable read-out, either as, for example, a reporter polypeptide, or more appropriately, as a polypeptide that kills a cell, *e.g.*, a toxin, or an agent that renders a cell susceptible to killing with a chosen agent or lack thereof. Effector proteins include any protein or peptide that directly targets or damages the host cell’s DNA and/or RNA. For example, effector proteins can include, but are not limited to, a restriction endonuclease that targets a host cell DNA sequence (whether genomic or on an extrachromosomal element), a protease that degrades a polypeptide target necessary for cell survival, a DNA gyrase inhibitor, and a ribonuclease-type toxin. In some embodiments, the expression of an effector protein controlled by a synthetic biological circuit as described herein can participate as a factor in another synthetic biological circuit to thereby expand the range and complexity of a biological circuit system’s responsiveness.

[0058] As defined herein, “reporters” refer to proteins that can be used to provide detectable read-outs. Reporters generally produce a measurable signal such as fluorescence, color, or luminescence. Reporter protein coding sequences encode proteins whose presence in the cell or organism is readily observed. For example, fluorescent proteins cause a cell to fluoresce when excited with light of a particular wavelength, luciferases cause a cell to catalyze a reaction that produces light, and enzymes such as β -galactosidase convert a substrate to a colored product. In some embodiments, reporters are used to quantify the strength or activity of the signal received by the modules or programmable synthetic biological circuits of the invention. In some embodiments, reporters can be fused in-frame to other protein coding sequences to identify where a protein is located in a cell or organism.

[0059] Luciferases can be used as effector proteins for various embodiments described herein, for example, measuring low levels of gene expression, because cells tend to have little to no background luminescence in the absence of a luciferase.

[0060] In other embodiments, enzymes that produce colored substrates can be quantified using spectrophotometers or other instruments that can take absorbance measurements including plate readers. Like luciferases, enzymes like β -galactosidase can be used for measuring low levels of gene expression because they tend to amplify low signals.

[0002] In some embodiments, an effector protein can be an enzyme that can degrade or otherwise destroy a given toxin.

[0061] In some embodiments, an effector protein can be an odorant enzyme that converts a substrate to an odorant product.

[0062] In some embodiments, an effector protein can be an enzyme that phosphorylates or dephosphorylates either small molecules or other proteins, or an enzyme that methylates or demethylates other proteins or DNA.

[0063] In some embodiments, an effector protein can be a receptor, ligand, or lytic protein. Receptors tend to have three domains: an extracellular domain for binding ligands such as proteins, peptides or small molecules, a transmembrane domain, and an intracellular or cytoplasmic domain which frequently can participate in some sort of signal transduction event such as phosphorylation. In some embodiments, transporter, channel, or pump gene sequences are used as effector proteins.

[0064] Non-limiting examples and sequences of effector proteins for use with the programmable synthetic biological circuits described herein can be found at the Registry of Standard Biological Parts on the world wide web at parts.igem.org.

[0065] As used herein, a “modulator protein” is a protein that modulates the expression from a target nucleic acid sequence. Modulator proteins include, for example, transcription factors, including transcriptional activators and repressors, among others, and proteins that bind to or modify a transcription factor and influence its activity. In some embodiments, a modulator protein includes, for example, a protease that degrades a protein factor involved in the regulation of expression from a

target nucleic acid sequence. Preferred modulator proteins include modular proteins in which, for example, DNA-binding and input agent-binding or responsive elements or domains are separable and transferrable, such that, for example, the fusion of the DNA binding domain of a first modulator protein to the input agent-responsive domain of a second results in a new protein that binds the DNA sequence recognized by the first protein, yet is sensitive to the input agent to which the second protein normally responds.

[0066] Accordingly, as used herein, the term “modulator polypeptide,” and the more specific “repressor polypeptide” include, in addition to the specified polypeptides, e.g., “a LacI (repressor) polypeptide,” variants, or derivatives of such polypeptides that responds to a different or variant input agent. Thus, for a LacI polypeptide, included are LacI mutants or variants that bind to agents other than lactose or IPTG. A wide range of such agents are known in the art.

[0067] Transcriptional regulators refer to transcriptional activators and repressors that either activate or repress transcription from cognate promoters. Transcriptional activators typically bind nearby to transcriptional promoters and recruit RNA polymerase to directly initiate transcription. Repressors bind to transcriptional promoters and sterically hinder transcriptional initiation by RNA polymerase. Other transcriptional regulators serve as either an activator or a repressor depending on where it binds and cellular conditions. Non-limiting examples of transcriptional regulators

[0068] As used herein, a “repressor protein” is a protein that binds to a regulatory sequence element and represses the transcription of sequences operatively linked to the regulatory sequence element. Preferred repressor proteins as described herein are sensitive to the presence or absence of at least one input agent or environmental input. Preferred repressor proteins as described herein are modular in form, comprising, for example, separable DNA-binding and input agent-binding or responsive elements or domains.

[0069] As used herein, a “hybrid repressor protein” is a repressor protein which comprises a heterologous fusion of DNA binding and input agent-responsive transcription factor domains, such that the activity of the DNA binding domain is rendered responsive to the presence or absence of an input agent that differs from the protein from which the DNA binding domain is taken. The activity of the DNA binding domain of a hybrid repressor protein is shifted to be responsive to the presence or absence of the agent to which the heterologous input agent-responsive domain responds. Methods are described herein to delineate the boundaries of domains so as to permit their heterologous combination to form hybrid repressor proteins that bind a chosen DNA sequence in a manner responsive to the presence or absence of a chosen heterologous input agent.

[0070] As used herein, an “input agent responsive domain” is a domain of a transcription factor that binds to or otherwise responds to a condition or input agent in a manner that renders a linked DNA binding fusion domain responsive to the presence of that condition or input input. In one embodiment, the presence of the condition or input results in a conformational change in the input

agent responsive domain, or in a protein to which it is fused, that modifies the transcription-modulating activity of the transcription factor,

[0071] The modular nature of transcription factors and the ability to redirect the input agent responsive domain to a different DNA sequence, combined with the large variety of input agent-responsive factors permits the engineering of circuits as described herein that respond to a wide variety of chosen combinations of input factors that serve as passcodes. When the effector is a toxin or other protein that renders a cell susceptible to killing, the correct combination of input agents provides a passcode necessary for maintaining the viability of the host cell.

[0072] As used herein, “passively activated” when used in reference to a synthetic biological circuits means that the circuit is “turned on” or active when something required to maintain the circuit in the “off” configuration is not present in an amount sufficient to do so.

[0073] In order to further enhance and expand the range and sensitivity of the programmable synthetic biological circuits described herein, in some embodiments, it is useful to create libraries with multiple interoperable repressors, such as transcriptional repressors. Thus, in some embodiments of the aspects described herein, a library of transcriptional repressors and activators can be targeted towards unique promoters with minimum crossover, using engineered zinc-finger proteins fused to transcriptional activation and repression domains.

[0074] To create such libraries, unique promoters containing sequence sites known to bind to engineered zinc-finger proteins can be synthesized. These sites are made up of three sequences, each of which is at least 3 DNA base pairs long. Each 3 base pair sequence binds to a single zinc-finger domain. Thus, in some embodiments, each complete engineered zinc-finger transcription factor contains three zinc-finger domains to target a total 9 base pair region of DNA. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 1. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 2. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 3. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 4. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 5. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 6. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 7. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 8. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 9. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is 10. In some embodiments, the number of zinc-finger domains used in a complete engineered zinc-finger transcription factor is at least 15, at least 20, at least 25, at least 50, at least 100, or more.

[0075] Representative examples of zinc-finger pools created for the shaded 3 base pair sequences are shown below (ML Maeder et al., *Molecular Cell* 2008: 31, 294–301):

F1				F2				F3			
GAA	GCA	GGA	GTA	GAA	GCA	GGA	GTA	GAA	GCA	GGA	GTA
GAC	CCG	GGC	GTC	GAC	CCC	GGC	GTC	GAC	CCC	GGC	GTC
GAG	CGG	GGG	GTC	GAG	CCG	GGG	GTC	GAG	CGG	GGG	GTC
GAT	GCT	GGT	GTT	GAT	GCT	GGT	GTT	GAT	GCT	GGT	GTT
TAA	TCA	TGA	TTA	TAA	TCA	TGA	TTA	TAA	TCA	TGA	TTA
TAC	TCC	TGC	TTG	TAC	TCC	TGC	TTG	TAC	TCC	TGC	TTG
TAG	TCG	TGG	TTG	TAG	TCG	TGG	TTG	TAG	TCG	TGG	TTG
TAT	TCT	TGT	TTT	TAT	TCT	TGT	TTT	TAT	TCT	TGT	TTT

Using such pools, complete engineered zinc-finger proteins containing at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or more zinc-fingers that can target synthetic promoters can be selected.

[0076] In some embodiments, the engineered zinc-finger proteins are fused to transcriptional activation domains, for *e.g.*, VP16, VP64, p65, Gal4, α -subunit of RNA polymerase, Wild-type CRP (amino acid residues 1–209), CRP D1(residues 1–180), CRP D2 (residues 137–190), CRP D3 (residues 137–180) and CRP D4 (residues 151–168). In other embodiments, the engineered zinc-finger proteins are fused to transcriptional repressor domains *e.g.*, SKD, KRAB (Margolin et al., 1994), SNAG, Kid, Ume6, CRP, SID (Ayer et al., 1996). Thus, an engineered zinc-finger protein can be used a transcriptional activator or transcriptional repressor, depending on the requirements of the various embodiments described herein, by fusing an engineered zinc-finger protein with an appropriate transcriptional activator or transcriptional repressor domain. Non-limiting examples of methods of engineering zinc-finger proteins and transcriptional activation domains for fusion are discussed, for example, at Kwang-Hee B. et al, *Nature Biotechnology* 2003: 21, p. 275-280; R-J Kwon et al., *Biotechnology Letters* (2006) 28: 9–15; P. Blancafort et al., *PNAS*, 2005, 102: 33, p.11716–11721; J. T. Stege et al., *The Plant Journal* (2002) 32, 1077–1086; J.Y. Lee et al., *Nucleic Acids Research*, 2008, 36:16; K-S Park et al., *Nature Biotechnology*, 2003, 21:10, p.1208-1214; R.R. Beerli et al., *PNAS*, 2000, 97:4, p. 1495-1500; P. Blancafort et al., *Nature Biotechnology* 2003: 21, p. 269-274; D-k Lee, et al., *Genome Res.*, 2003, 13: 2708-2716. Interoperability of such fusion engineered zinc-finger proteins can be assessed by combinatorial addition of the different engineered zinc-finger transcription factors to determine how promoter activity is affected.

[0077] To enhance cooperativity of engineered zinc-finger-based transcription factors, in some embodiments, engineered zinc-finger-based transcription factors can be further engineered to dimerize, using dimerization domains such as leucine zipper domains. In some embodiments, the affinity of monomeric engineered-zinc finger proteins can be increased or decreased by site-directed mutagenesis of amino acids known to contact the DNA backbone and/or bases. Non-limiting examples of methods to achieve such affinity modification are discussed, for example, at J. L.

Pomerantz, et al., Biochemistry, 1998, 37: 4, p. 965-970, and S.A. Wolfe et al., Structure, 2000, 8:7, p. 739-750.

[0078] The methods and uses of the synthetic biological circuits described herein can involve *in vivo*, *ex vivo*, or *in vitro* systems. The term "*in vivo*" refers to assays or processes that occur in or within an organism, such as a multicellular animal. In some of the aspects described herein, a method or use can be said to occur "*in vivo*" when a unicellular organism, such as a bacterium, is used. The term "*ex vivo*" refers to methods and uses that are performed using a living cell with an intact membrane that is outside of the body of a multicellular animal or plant, *e.g.*, explants, cultured cells, including primary cells and cell lines, transformed cell lines, and extracted tissue or cells, including blood cells, among others. The term "*in vitro*" refers to assays and methods that do not require the presence of a cell with an intact membrane, such as cellular extracts, and can refer to the introducing of a programmable synthetic biological circuit in a non-cellular system, such as a medium not comprising cells or cellular systems, such as cellular extracts.

[0079] A cell or cellular system to be engineered for use with the synthetic biological circuits described herein can be any cell. As defined herein, a "cell" is the basic structural and functional unit of all known living organisms. It is the smallest unit of life that is classified as a living thing, and is often called the building block of life. Some organisms, such as most bacteria, are unicellular (consist of a single cell). Other organisms, such as humans, are multicellular.

[0080] A "natural cell," as defined herein, refers to any prokaryotic or eukaryotic cell that occurs in nature. A "prokaryotic cell" can comprise a cell envelope and a cytoplasmic region that contains the cell genome (DNA) and ribosomes and various sorts of inclusions. In some embodiments, the cell is a eukaryotic cell. As used herein, a "eukaryotic cell" comprises membrane-bound compartments in which specific metabolic activities take place, such as for example, a nucleus. In some embodiments, the cell is an artificial or synthetic cell. As defined herein, an "artificial cell" or a "synthetic cell" is a minimal cell formed from artificial parts that can do many things a natural cell can do, such as transcribe and translate proteins and generate ATP.

[0081] Host cells of use in the aspects described herein for transformation or transfection with the synthetic biological circuits described herein include any host cell that is capable of supporting the activation and expression of the synthetic biological circuits. In some embodiments of the aspects described herein, the cells are bacterial cells. The term "bacteria" as used herein is intended to encompass all variants of bacteria, for example, prokaryotic organisms and cyanobacteria. Bacteria are small (typical linear dimensions of around 1 μm), non-compartmentalized, with circular DNA and ribosomes of 70S. The term bacteria also includes bacterial subdivisions of *Eubacteria* and *Archaeobacteria*. *Eubacteria* can be further subdivided on the basis of their staining using Gram stain, and both gram-positive and gram-negative eubacteria, which depends upon a difference in cell wall structure are also included, as well as classified based on gross morphology alone (into cocci, bacilli, etc.).

[0082] In some embodiments, the bacterial cells are gram-negative cells and in alternative embodiments, the bacterial cells are gram-positive cells. Non-limiting examples of species of bacterial cells useful for engineering with the synthetic biological circuits described herein include, without limitation, cells from *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. Other examples of bacterial cells that can be genetically engineered for use with the synthetic biological circuits described herein include, but are not limited to, cells from *Yersinia* spp., *Escherichia* spp., *Klebsiella* spp., *Bordetella* spp., *Neisseria* spp., *Aeromonas* spp., *Francisella* spp., *Corynebacterium* spp., *Citrobacter* spp., *Chlamydia* spp., *Hemophilus* spp., *Brucella* spp., *Mycobacterium* spp., *Legionella* spp., *Rhodococcus* spp., *Pseudomonas* spp., *Helicobacter* spp., *Salmonella* spp., *Vibrio* spp., *Bacillus* spp., and *Erysipelothrix* spp. In some embodiments, the bacterial cells are *E.coli* cells. Other examples of organisms from which cells can be transformed or transfected with the synthetic biological circuits described herein include, but are not limited to the following: *Staphylococcus aureus*, *Clostridium butyricum*, *Brevibacterium lactofermentum*, *Streptococcus agalactiae*, *Lactococcus lactis*, *Leuconostoc lactis*, *Streptomyces*, *Actinobacillus actinobycetemcomitans*, *Bacteroides*, *cyanobacteria*, *Helicobacter pylori*, *Selmonas ruminatum*, *Shigella sonnei*, *Zymomonas mobilis*, *Mycoplasma mycoides*, or *Treponema denticola*, *Bacillus thuringiensis*, *Staphylococcus lugdunensis*, *Leuconostoc oenos*, *Corynebacterium xerosis*, *Lactobacillus plantarum*, *Streptococcus faecalis*, *Bacillus coagulans*, *Bacillus ceretus*, *Bacillus popillae*, *Synechocystis* strain PCC6803, *Bacillus liquefaciens*, *Pyrococcus abyssi*, *Selenomonas nominantium*, *Lactobacillus hilgardii*, *Streptococcus ferus*, *Lactobacillus pentosus*, *Bacteroides fragilis*, *Staphylococcus epidermidis*, *Zymomonas mobilis*, *Streptomyces phaeochromogenes*, *Streptomyces ghanaensis*, *Halobacterium* strain GRB, and *Halobaferax* sp. strain Aa2.2.

[0083] Cells of use in the various aspects described herein upon transformation or transfection with the synthetic biological circuits described herein include any cell that is capable of supporting the activation and expression of the synthetic biological circuits. In some embodiments of the aspects described herein, a cell can be from any multicellular or eukaryotic organism. Examples of eukaryotic cells that can be useful in aspects described herein include eukaryotic cells selected from, e.g., mammalian, insect, yeast, or plant cells. The synthetic biological circuits described herein can be introduced into a variety of cells including, e.g., fungal, plant, or animal (nematode, insect, plant, bird, reptile, or mammal (e.g., a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human)). The cells can be primary cells, immortalized cells, stem cells, or transformed cells. In some embodiments, the cells comprise stem cells. Expression vectors for the components of the synthetic biological circuits generally comprise a promoter and/or an enhancer suitable for expression in a particular host cell of interest. Other cell types also contemplated for use with the synthetic biological circuits described herein include, but are not limited to, reproductive cells including sperm, ova and embryonic cells, and non-reproductive cells, such as kidney, lung, spleen,

lymphoid, cardiac, gastric, intestinal, pancreatic, muscle, bone, neural, brain, and epithelial cells. Also contemplated for use with the programmable synthetic biological circuits described herein are stem cells, including human embryonic stem cells, pluripotent stem cells, multipotent stem cells, and induced pluripotent stem cells (iPSCs), as those terms are understood by one of skill in the art.

[0084] In other embodiments of the aspects described herein, synthetic biological circuits can be introduced into a non-cellular system such as a virus or phage, by direct integration of the nucleic acid sequences encoding the various components and modules of the synthetic biological circuit, for example, into the viral genome. A virus for use with the synthetic biological circuits described herein can be a dsDNA virus (e.g. *Adenoviruses*, *Herpesviruses*, *Poxviruses*), a ssDNA viruses ((+)sense DNA) (e.g. *Parvoviruses*); a dsRNA virus (e.g. *Reoviruses*); a (+)ssRNA viruses ((+)sense RNA) (e.g. *Picornaviruses*, *Togaviruses*); (-)ssRNA virus ((-)sense RNA) (e.g. *Orthomyxoviruses*, *Rhabdoviruses*); a ssRNA-Reverse Transcriptase viruses ((+)sense RNA with DNA intermediate in life-cycle) (e.g. *Retroviruses*); or a dsDNA- Reverse Transcriptase virus (e.g. *Hepadnaviruses*).

[0085] Viruses can also include plant viruses and bacteriophages or phages. Examples of phage families that can be used with the synthetic biological circuits described herein include, but are not limited to, *Myoviridae* (*T4-like viruses*; *P1-like viruses*; *P2-like viruses*; *Mu-like viruses*; *SPO1-like viruses*; *φH-like viruses*); *Siphoviridae* (*λ-like viruses* (*T1-like viruses*; *T5-like viruses*; *c2-like viruses*; *L5-like viruses*; *ψM1-like viruses*; *φC31-like viruses*; *N15-like viruses*); *Podoviridae* (*T7-like viruses*; *φ29-like viruses*; *P22-like viruses*; *N4-like viruses*); *Tectiviridae* (*Tectivirus*); *Corticoviridae* (*Corticovirus*); *Lipothrixviridae* (*Alphalipothrixvirus*, *Betalipothrixvirus*, *Gammalipothrixvirus*, *Deltalipothrixvirus*); *Plasmaviridae* (*Plasmavirus*); *Rudoviridae* (*Rudovirus*); *Fuselloviridae* (*Fusellovirus*); *Inoviridae* (*Inovirus*, *Plectrovirus*); *Microviridae* (*Microvirus*, *Spiromicrovirus*, *Bdellovibrio*, *Chlamydia microvirus*); *Leviviridae* (*Levivirus*, *Allolevivirus*) and *Cystoviridae* (*Cystovirus*). Such phages can be naturally occurring or engineered phages.

[0086] In some embodiments of the aspects described herein, the synthetic biological circuits are introduced into a cellular or non-cellular system using a vector or plasmid. Vectors capable of directing the expression of genes and/or nucleic acid sequence to which they are operatively linked are referred to herein as "expression vectors." In general, expression vectors of use in the methods and synthetic biological circuits described herein are often in the form of "plasmids," which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome.

[0087] Other expression vectors can be used in different embodiments, for example, but not limited to, episomes, bacteriophages or viral vectors. Other forms of expression vectors known by those skilled in the art which serve the equivalent functions can also be used. Expression vectors comprise expression vectors for stable or transient expression. A vector can be either a self replicating extrachromosomal vector or a vector which integrates into a host genome. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the chromosomal

DNA of a host cell, cellular system, or non-cellular system. In some embodiments, the nucleic acid sequence or sequences encoding the synthetic biological circuits integrates into the chromosomal DNA of a host cell, cellular system, or non-cellular system along with components of the vector sequence. In other embodiments, the nucleic acid sequence encoding the synthetic biological circuit directly integrates into chromosomal DNA of a host cell, cellular system, or non-cellular system, in the absence of any components of the vector by which it was introduced. In such embodiments, the nucleic acid sequence encoding the synthetic biological circuit can be integrated using targeted insertions, such as knock-in technologies or homologous recombination techniques, or by non-targeted insertions, such as gene trapping techniques or non-homologous recombination. The number of copies of a synthetic biological circuit that integrates into the chromosomal DNA of a cellular or non-cellular system can impact the fidelity of the system, and thus it is preferred that only one copy is integrated per cellular system. Accordingly, in some embodiments of the aspects described herein, only one copy of a synthetic biological circuit is integrated in the chromosomal DNA or RNA of a cellular or non-cellular system. In some embodiments, the number of copies is less than 10, less than 9, less than 8, less than 7, less than 6, less than 6, less than 4, less than 3, or less than 2.

[0088] Another type of vector is an episomal vector, *i.e.*, a nucleic acid capable of extra-chromosomal replication. Such plasmids or vectors can include plasmid sequences from bacteria, viruses or phages. Such vectors include chromosomal, episomal and virus-derived vectors *e.g.*, vectors derived from bacterial plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses, vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids and phagemids. A vector can be a plasmid, bacteriophage, bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC). A vector can be a single or double-stranded DNA, RNA, or phage vector. In some embodiments, the programmable synthetic biological circuits are introduced into a cellular system using a BAC vector.

[0089] The vectors comprising the programmable synthetic biological circuits described herein can be "introduced" into cells as polynucleotides, preferably DNA, by techniques well-known in the art for introducing DNA and RNA into cells. The term "transduction" refers to any method whereby a nucleic acid sequence is introduced into a cell, *e.g.*, by transfection, lipofection, electroporation, biolistics, passive uptake, lipid:nucleic acid complexes, viral vector transduction, injection, contacting with naked DNA, gene gun, and the like. The vectors, in the case of phage and viral vectors can also be introduced into cells as packaged or encapsidated virus by well-known techniques for infection and transduction. Viral vectors can be replication competent or replication defective. In the latter case, viral propagation generally occurs only in complementing host cells. In some embodiments, the synthetic biological circuits are introduced into a cell using other mechanisms known to one of skill in the art, such as a liposome, microspheres, gene gun, fusion proteins, such as a fusion of an antibody moiety with a nucleic acid binding moiety, or other such delivery vehicle.

[0090] The term "transient transformation" or "transiently transformed" refers to the introduction of one or more transgenes into a cell in the absence of integration of the transgene into the host cell's genome.

[0091] In contrast, the term "stable transformation" or "stably transformed" refers to the introduction and integration of one or more transgenes into the genome of a cell or cellular system, preferably resulting in chromosomal integration and stable heritability. Stable transformation of a cell can be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences, which are capable of binding to one or more of the transgenes. Alternatively, stable transformation of a cell can also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify transgene sequences. The term "stable transformant" refers to a cell that has stably integrated one or more transgenes into the genomic DNA. Thus, a stable transformant is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more transgenes, genomic DNA from the transient transformant does not contain a transgene. Transformation also includes introduction of genetic material into plant cells in the form of plant viral vectors involving epichromosomal replication and gene expression, which may exhibit variable properties with respect to meiotic stability. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

[0092] The term "promoter," as used herein, refers to any nucleic acid sequence that regulates the expression of another nucleic acid sequence by driving transcription of the nucleic acid sequence, which can be a heterologous target gene encoding a protein or an RNA. Promoters can be constitutive, inducible, repressible, tissue-specific, or any combination thereof. A promoter is a control region of a nucleic acid sequence at which initiation and rate of transcription of the remainder of a nucleic acid sequence are controlled. A promoter can also contain genetic elements at which regulatory proteins and molecules can bind, such as RNA polymerase and other transcription factors. In some embodiments of the aspects described herein, a promoter can drive the expression of a transcription factor that regulates the expression of the promoter itself, or that of another promoter used in another modular component of the synthetic biological circuits described herein.

[0093] A promoter can be said to drive expression or drive transcription of the nucleic acid sequence that it regulates. The phrases "operably linked," "operatively positioned," "operatively linked," "under control," and "under transcriptional control" indicate that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence it regulates to control transcriptional initiation and/or expression of that sequence. An "inverted promoter," as used herein, refers to a promoter in which the nucleic acid sequence is in the reverse orientation, such that what was the coding strand is now the non-coding strand, and vice versa. Inverted promoter sequences can be used in various embodiments to regulate the state of a module or a switch. In addition, in various embodiments, a promoter can be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence downstream

of the promoter. The enhancer can be located at any functional location before or after the promoter, and/or the encoded nucleic acid.

[0094] A promoter can be one naturally associated with a gene or sequence, as can be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon of a given gene or sequence. Such a promoter can be referred to as “endogenous.” Similarly, in some embodiments, an enhancer can be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence.

[0095] In some embodiments, a coding nucleic acid segment is positioned under the control of a “recombinant promoter” or “heterologous promoter,” both of which refer to a promoter that is not normally associated with the encoded nucleic acid sequence it is operably linked to in its natural environment. A recombinant or heterologous enhancer refers to an enhancer not normally associated with a given nucleic acid sequence in its natural environment. Such promoters or enhancers can include promoters or enhancers of other genes; promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell; and synthetic promoters or enhancers that are not “naturally occurring,” *i.e.*, comprise different elements of different transcriptional regulatory regions, and/or mutations that alter expression through methods of genetic engineering that are known in the art. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, promoter sequences can be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR, in connection with the synthetic biological circuits and modules disclosed herein (see, *e.g.*, U.S. Pat. No. 4,683,202, U.S. Pat. No. 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated that control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

[0096] As described herein, an “inducible promoter” is one that is characterized by initiating or enhancing transcriptional activity when in the presence of, influenced by, or contacted by an inducer or inducing agent. An “inducer” or “inducing agent,” as defined herein, can be endogenous, or a normally exogenous compound or protein that is administered in such a way as to be active in inducing transcriptional activity from the inducible promoter. In some embodiments, the inducer or inducing agent, *i.e.*, a chemical, a compound or a protein, can itself be the result of transcription or expression of a nucleic acid sequence (*i.e.*, an inducer can be a transcriptional modulator protein expressed by another component or module), which itself can be under the control of an inducible promoter. In some embodiments, an inducible promoter is induced in the absence of certain agents, such as a repressor. Examples of inducible promoters include but are not limited to, tetracycline, metallothionine, ecdysone, mammalian viruses (*e.g.*, the adenovirus late promoter; and the mouse mammary tumor virus long terminal repeat (MMTV-LTR)) and other steroid-responsive promoters, rapamycin responsive promoters and the like.

[0097] In some embodiments of the aspects described herein, an inducible promoter is induced by exposure to an externally applied inducer, such as an input agent or an environmental input agent or condition. Exemplary input agents include exposure to heat (*i.e.*, thermal pulses or constant heat exposure), various steroidal compounds, divalent cations (including Cu^{2+} and Zn^{2+}), galactose, tetracycline, IPTG (isopropyl- β -D thiogalactoside), other lactose or galactose analogs, cellobiose, as well as other naturally occurring and synthetic input inducing agents and gratuitous inducers.

[0098] An inducible promoter can be one that is induced by an inducing agent that positively activates a transcriptional activator, or one which is derepressed by an inducing agent that negatively regulates a transcriptional repressor.

[0099] Inducible promoters that are useful in the synthetic biological circuits and methods of use disclosed herein include those controlled by the action of latent transcriptional activators that are subject to induction by the action of environmental inducing agents. Some non-limiting examples include the copper-inducible promoters of the yeast genes CUP1, CRS5, and SOD1 that are subject to copper-dependent activation by the yeast ACE1 transcriptional activator (see *e.g.* Strain and Culotta, 1996; Hottiger et al., 1994; Lapinskas et al., 1993; and Gralla et al., 1991). Alternatively, the copper inducible promoter of the yeast gene CTT1 (encoding cytosolic catalase T), which operates independently of the ACE1 transcriptional activator (Lapinskas et al., 1993), can be utilized. The copper concentrations required for effective induction of these genes are suitably low so as to be tolerated by most cell systems, including yeast and *Drosophila* cells. Alternatively, other naturally occurring inducible promoters can be used including: steroid inducible gene promoters (see *e.g.* Oligino et al. (1998) *Gene Ther.* 5: 491-6); galactose inducible promoters from yeast (see *e.g.* Johnston (1987) *Microbiol Rev* 51: 458-76; Ruzzi et al. (1987) *Mol Cell Biol* 7: 991-7); and various heat shock gene promoters. Many eukaryotic transcriptional activators have been shown to function in a broad range of eukaryotic host cells, and so, for example, many of the inducible promoters identified in yeast can be adapted for use in a mammalian host cell as well. For example, a unique synthetic transcriptional induction system for mammalian cells has been developed based upon a GAL4-estrogen receptor fusion protein that induces mammalian promoters containing GAL4 binding sites (Brasemann et al. (1993) *Proc Natl Acad Sci USA* 90: 1657-61). These and other inducible promoters responsive to transcriptional activators that are dependent upon specific inducers are suitable for use with the synthetic biological circuits described herein.

[00100] Inducible promoters useful in the synthetic biological circuits, methods of use and systems described herein also include those that are repressed by “transcriptional repressors,” which are subject to inactivation by the action of environmental, external agents, or the product of another gene. Such inducible promoters can also be termed “repressible promoters” where it is required to distinguish between other types of promoters in a given module or component of the synthetic biological circuits described herein. Examples include prokaryotic repressor molecules that can

transcriptionally repress prokaryotic or eukaryotic promoters that contain or have been engineered to incorporate appropriate repressor-binding operator sequences. Preferred repressors for use in the modules and methods described herein are sensitive to inactivation by physiologically benign agents. Thus, where a lac repressor protein is used to control the expression of a promoter sequence that has been engineered to contain a lacO operator sequence, treatment of the host cell with IPTG will cause the dissociation of the lac repressor from the engineered promoter containing a lacO operator sequence and allow transcription to occur. Similarly, where a tet repressor is used to control the expression of a promoter sequence that has been engineered to contain a tetO operator sequence, treatment of the host cell with tetracycline will cause dissociation of the tet repressor from the engineered promoter and allow transcription of the sequence downstream of the engineered promoter to occur.

[00101] An inducible promoter useful in the synthetic biological circuits, methods and systems as described herein can be induced by one or more physiological conditions or inducing agents, such as changes in pH, temperature, radiation, osmotic pressure, saline gradients, cell surface binding, and the concentration of one or more extrinsic or intrinsic inducing agents. The extrinsic inducer or inducing agent can comprise amino acids and amino acid analogs, saccharides and polysaccharides, nucleic acids, protein transcriptional activators and repressors, cytokines, toxins, petroleum-based compounds, metal containing compounds, salts, ions, enzyme substrate analogs, hormones, and combinations thereof. In specific embodiments, the inducible promoter is activated or repressed in response to a change of an environmental condition, such as the change in concentration of a chemical, metal, temperature, radiation, nutrient or change in pH. Thus, an inducible promoter useful in the methods and systems as described herein can be, for example, a phage inducible promoter, nutrient inducible promoter, temperature inducible promoter, radiation inducible promoter, metal inducible promoter, hormone inducible promoter, steroid inducible promoter, and/or hybrids and combinations thereof.

[00102] Radiation inducible promoters include the non-limiting examples of fos promoter, c-jun promoter or at least one CArG domain of an Egr-1 promoter. Further non-limiting examples of inducible promoters include promoters from genes such as cytochrome P450 genes, inducible heat shock protein genes, metallothionein genes, hormone-inducible genes, such as the estrogen gene promoter, and such. In further embodiments, an inducible promoter useful in the methods and systems described herein can be Zn²⁺ metallothionein promoter, metallothionein-1 promoter, human metallothionein IIA promoter, lac promoter, lacO promoter, mouse mammary tumor virus early promoter, mouse mammary tumor virus LTR promoter, triose dehydrogenase promoter, herpes simplex virus thymidine kinase promoter, simian virus 40 early promoter or retroviral myeloproliferative sarcoma virus promoter. Examples of inducible promoters also include mammalian probasin promoter, lactalbumin promoter, GRP78 promoter, or the bacterial tetracycline-inducible

promoter. Other examples include phorbol ester, adenovirus E1A element, interferon, and serum inducible promoters.

[00103] Inducible promoters useful in the modules and synthetic biological circuits described herein for *in vivo* uses can include those responsive to biologically compatible agents, such as those that are usually encountered in defined animal tissues. An example is the human PAI-1 promoter, which is inducible by tumor necrosis factor. Further suitable examples include cytochrome P450 gene promoters, inducible by various toxins and other agents; heat shock protein genes, inducible by various stresses; hormone-inducible genes, such as the estrogen gene promoter, and such.

[00104] The administration or removal of an inducer or repressor as described herein results in a switch between the “on” or “off” states of the transcription of the operably linked heterologous target gene. Thus, as defined herein, the “on” state of a promoter operably linked to a nucleic acid sequence, refers to the state when the promoter is actively driving transcription of the operably linked nucleic acid sequence, *i.e.*, the linked nucleic acid sequence is expressed. Several small molecule ligands have been shown to mediate regulated gene expressions, either in tissue culture cells and/or in transgenic animal models. These include the FK1012 and rapamycin immunosuppressive drugs (Spencer et al., 1993; Magari et al., 1997), the progesterone antagonist mifepristone (RU486) (Wang, 1994; Wang et al., 1997), the tetracycline antibiotic derivatives (Gossen and Bujard, 1992; Gossen et al., 1995; Kistner et al., 1996), and the insect steroid hormone ecdysone (No et al., 1996). All of these references are herein incorporated by reference. By way of further example, Yao discloses in U.S. Pat. No. 6,444,871, which is incorporated herein by reference, prokaryotic elements associated with the tetracycline resistance (tet) operon, a system in which the tet repressor protein is fused with polypeptides known to modulate transcription in mammalian cells. The fusion protein is then directed to specific sites by the positioning of the tet operator sequence. For example, the tet repressor has been fused to a transactivator (VP16) and targeted to a tet operator sequence positioned upstream from the promoter of a selected gene (Gussen et al., 1992; Kim et al., 1995; Hennighausen et al., 1995). The tet repressor portion of the fusion protein binds to the operator thereby targeting the VP16 activator to the specific site where the induction of transcription is desired. An alternative approach has been to fuse the tet repressor to the KRAB repressor domain and target this protein to an operator placed several hundred base pairs upstream of a gene. Using this system, it has been found that the chimeric protein, but not the tet repressor alone, is capable of producing a 10 to 15-fold suppression of CMV-regulated gene expression (Deuschle et al., 1995).

[00105] One example of a repressible promoter useful in the modules and synthetic biological circuits as disclosed herein is the Lac repressor (lacR)/operator/inducer system of *E. coli* that has been used to regulate gene expression by three different approaches: (1) prevention of transcription initiation by properly placed lac operators at promoter sites (Hu and Davidson, 1987; Brown et al., 1987; Figge et al., 1988; Fuerst et al., 1989; Deuschle et al., 1989; (2) blockage of transcribing RNA polymerase II during elongation by a LacR/operator complex (Deuschle et al. (1990); and (3)

activation of a promoter responsive to a fusion between LacR and the activation domain of herpes simplex virus (HSV) virion protein 16 (VP16) (Labow et al., 1990; Baim et al., 1991). In one version of the Lac system, expression of lac operator-linked sequences is constitutively activated by a LacR-VP16 fusion protein and is turned off in the presence of isopropyl- β -D-1-thiogalactopyranoside (IPTG) (Labow et al. (1990), cited *supra*). In another version of the system, a lacR-VP16 variant is used that binds to lac operators in the presence of IPTG, which can be enhanced by increasing the temperature of the cells (Baim et al. (1991), cited *supra*). Thus, in some embodiments of the aspects described herein, components of the Lac system are utilized. For example, a lac operator (LacO) can be operably linked to tissue specific promoter, and control the transcription and expression of the heterologous target gene and another repressor protein, such as the TetR. Accordingly, the expression of the heterologous target gene is inversely regulated as compared to the expression or presence of Lac repressor in the system.

[00106] Components of the tetracycline (Tc) resistance system of *E. coli* that have also been found to function in eukaryotic cells and been used to regulate gene expression can also be used in the various aspects described herein. For example, the Tet repressor (TetR), which binds to tet operator (tetO) sequences in the absence of tetracycline and represses gene transcription, has been expressed in plant cells at sufficiently high concentrations to repress transcription from a promoter containing tet operator sequences (Gatz, C. et al. (1992) Plant J. 2:397-404). In some embodiments described herein, the Tet repressor system is similarly utilized.

[00107] A temperature- or heat-inducible gene regulatory system can also be used in the in the synthetic biological circuits and methods described herein, such as the exemplary TIGR system comprising a cold-inducible transactivator in the form of a fusion protein having a heat shock responsive regulator, rheA, fused to the VP16 transactivator (Weber et al., 2003a). The promoter responsive to this fusion thermosensor comprises a rheO element operably linked to a minimal promoter, such as the minimal version of the human cytomegalovirus immediate early promoter. At the permissive temperature of 37°C, the cold-inducible transactivator transactivates the exemplary rheO-CMVmin promoter, permitting expression of the target gene. At 41°C, the cold-inducible transactivator no longer transactivates the rheO promoter. Any such heat-inducible or -regulated promoter can be used in accordance with the modules, synthetic biological circuits, and methods described herein, including but not limited to a heat-responsive element in a heat shock gene (*e.g.*, hsp20-30, hsp27, hsp40, hsp60, hsp70, and hsp90). See Easton et al. (2000) Cell Stress Chaperones 5(4):276-290; Csermely et al. (1998) Pharmacol Ther 79(2): 129-1 68; Ohtsuka & Hata (2000) Int J Hyperthermia 16(3):231-245; and references cited therein. Sequence similarity to heat shock proteins and heat-responsive promoter elements have also been recognized in genes initially characterized with respect to other functions, and the DNA sequences that confer heat inducibility are suitable for use in the disclosed gene therapy vectors. For example, expression of glucose-responsive genes (*e.g.*, grp94, grp78, mortalin/grp75) (Merrick et al. (1997) Cancer Lett 119(2): 185-1 90; Kiang et al. (1998)

FASEB J 12(14):1571-16-579), calreticulin (Szewczenko-Pawlikowski et al. (1997) MoI Cell Biochem 177(1 -2): 145-1 52); clusterin (Viard et al. (1999) J Invest Dermatol 112(3):290-296; Michel et al. (1997) Biochem J 328(Pt1):45-50; Clark & Griswold (1997) J Androl 18(3):257-263), histocompatibility class I gene (HLA-G) (Ibrahim et al. (2000) Cell Stress Chaperones 5(3):207-218), and the Kunitz protease isoform of amyloid precursor protein (Shepherd et al. (2000) Neuroscience 99(2):31 7-325) are upregulated in response to heat. In the case of clusterin, a 14 base pair element that is sufficient for heat-inducibility has been delineated (Michel et al. (1997) Biochem J 328(Pt1):45-50). Similarly, a two sequence unit comprising a 10- and a 14-base pair element in the calreticulin promoter region has been shown to confer heat-inducibility (Szewczenko-Pawlikowski et al. (1997) MoI Cell Biochem 177(1 -2): 145-1 52).

[00108] Other inducible promoters useful in the various embodiments of the aspects described herein include the erythromycin-resistance regulon from *E.coli*, having repressible (E_{off}) and inducible (E_{on}) systems responsive to macrolide antibiotics, such as erythromycin, clarithromycin, and roxithromycin (Weber et al., 2002). The E_{off} system utilizes an erythromycin-dependent transactivator, wherein providing a macrolide antibiotic represses transgene expression. In the E_{on} system, the binding of the repressor to the operator results in repression of transgene expression. Therein, in the presence of macrolides gene expression is induced.

[00109] Fussenegger et al. (Nature Biotechnology 18, 1203 - 1208 (2000)) describe repressible and inducible systems using a Pip (pristinamycin-induced protein) repressor encoded by the streptogramin resistance operon of *Streptomyces coelicolor*, wherein the systems are responsive to streptogramin-type antibiotics (such as, for example, pristinamycin, virginiamycin, and Synercid). The Pip DNA-binding domain is fused to a VP16 transactivation domain or to the KRAB silencing domain, for example. The presence or absence of, for example, pristinamycin, regulates the Pip_{ON} and Pip_{OFF} systems in their respective manners, as described therein.

[00110] Another example of a promoter expression system useful for the modules and synthetic biological circuits described herein utilizes a quorum-sensing (referring to particular prokaryotic molecule communication systems having diffusable signal molecules that prevent binding of a repressor to an operator site, resulting in derepression of a target regulon) system. For example, Weber et al. (2003b) employ a fusion protein comprising the *Streptomyces coelicolor* quorum-sensing receptor to a transactivating domain that regulates a chimeric promoter having a respective operator that the fusion protein binds. The expression is fine-tuned with non-toxic butyrolactones, such as SCB1 and MP133.

[00111] In some embodiments, multiregulated, multigene gene expression systems that are functionally compatible with one another can be utilized in the aspects described herein (see, for example, Kramer et al. (2003)). For example, in Weber et al. (2002), the macrolide-responsive erythromycin resistance regulon system is used in conjunction with a streptogramin (PIP)-regulated and tetracycline-regulated expression systems.

[00112] Other promoters responsive to non-heat stimuli can also be used. For example, the mortalin promoter is induced by low doses of ionizing radiation (Sadekova (1997) *Int J Radiat Biol* 72(6):653-660), the hsp27 promoter is activated by 17- β -estradiol and estrogen receptor agonists (Porter et al. (2001) *J Mol Endocrinol* 26(1):31-42), the HLA-G promoter is induced by arsenite, hsp promoters can be activated by photodynamic therapy (Luna et al. (2000) *Cancer Res* 60(6): 1637-1644). A suitable promoter can incorporate factors such as tissue-specific activation. For example, hsp70 is transcriptionally impaired in stressed neuroblastoma cells (Drujan & De Maio (1999) *Exp Cell Res* 237(1):38-45) and the mortalin promoter is up-regulated in human brain tumors (Takano et al. (1997) *Exp Cell Res* 237(1):38-45). A promoter employed in methods of the present invention can show selective up-regulation in tumor cells as described, for example, for mortalin (Takano et al. (1997) *Exp Cell Res* 237(1):38-45), hsp27 and calreticulin (Szewczenko-Pawlikowski et al. (1997) *Mol Cell Biochem* 177(1-2): 145-152; Yu et al. (2000) *Electrophoresis* 21(14):3058-3068), grp94 and grp78 (Gazit et al. (1999) *Breast Cancer Res Treat* 54(2): 135-146), and hsp27, hsp70, hsp73, and hsp90 (Cardillo et al. (2000) *Anticancer Res* 20(6B):4579-4583; Strik et al. (2000) *Anticancer Res* 20(6B):4457-4552).

[00113] In some embodiments of the various aspects described herein, the promoter sequence that is added to a module or a synthetic biological circuit is an inducible promoter that allows control of the module or synthetic biological circuit using one or more chemical inducers.

[00114] Non-limiting examples of sequences encoding naturally occurring and synthetic inducible promoters for use in the synthetic biological circuits described herein can be found at the Registry of Standard Biological Parts on the world wide web at parts.igem.org.

[00115] A variety of constitutive promoter sequences are available for those embodiments of the modules and synthetic biological circuits described herein where constitutive expression of an operably linked sequence is required. Non-limiting examples of such sequences encoding constitutive promoters for use in the synthetic biological circuits described herein can be found at the Registry of Standard Biological Parts on the world wide web at parts.igem.org.

[00116] Provided herein for use in the synthetic biological circuits described herein, in some embodiments, are ribosome binding site sequences. "Ribosome binding sites" ("RBS") are sequences that promote efficient and accurate translation of mRNAs for protein synthesis, and are also provided for use in the modules and synthetic biological circuits described herein to permit modulation of the efficiency and rates of synthesis of the proteins encoded by the circuits. An RBS affects the translation rate of an open reading frame in two main ways - i) the rate at which ribosomes are recruited to the mRNA and initiate translation is dependent on the sequence of the RBS, and ii) the RBS can also affect the stability of the mRNA, thereby affecting the number of proteins made over the lifetime of the mRNA. Accordingly, in some embodiments, one or more ribosome binding site sequences (RBS) can be added to the modules and synthetic biological circuits described herein to control expression of proteins.

[00117] Translation initiation in prokaryotes is a complex process involving the ribosome, the mRNA, and several other proteins, such as initiation factors, as described in Laursen BS, et al., Microbiol Mol Biol Rev 2005 Mar; 69(1) 101-23. Translation initiation can be broken down into two major steps - i) binding of the ribosome and associated factors to the mRNA, and ii) conversion of the bound ribosome into a translating ribosome lengthening processing along the mRNA. The rate of the first step can be increased by making the RBS highly complementary to the free end of the 16s rRNA and by ensuring that the start codon is AUG. The rate of ribosome binding can also be increased by ensuring that there is minimal secondary structure in the neighborhood of the RBS. Since binding between the RBS and the ribosome is mediated by base-pairing interactions, competition for the RBS from other sequences on the mRNA, can reduce the rate of ribosome binding. The rate of the second step in translation initiation, conversion of the bound ribosome into an initiation complex, is dependent on the spacing between the RBS and the start codon being optimal (5-6bp).

[00118] Thus, a "ribosome binding site" ("RBS"), as defined herein, is a segment of the 5' (upstream) part of an mRNA molecule that binds to the ribosome to position the message correctly for the initiation of translation. The RBS controls the accuracy and efficiency with which the translation of mRNA begins. In prokaryotes (such as *E. coli*) the RBS typically lies about 7 nucleotides upstream from the start codon (*i.e.*, the first AUG). The sequence itself in general is called the "Shine-Dalgarno" sequence after its discoverers, regardless of the exact identity of the bases. Strong Shine-Dalgarno sequences are rich in purines (A's,G's), and the "Shine-Dalgarno consensus" sequence -- derived statistically from lining up many well-characterized strong ribosome binding sites -- has the sequence AGGAGG. The complementary sequence (CCUCCU) occurs at the 3'-end of the structural RNA ("16S") of the small ribosomal subunit and it base-pairs with the Shine-Dalgarno sequence in the mRNA to facilitate proper initiation of protein synthesis. In some embodiments of the aspects described herein, a ribosome binding site (RBS) is added to a synthetic biological circuit to regulate expression of a protein encoded by the circuit.

[00119] For protein synthesis in eukaryotes and eukaryotic cells, the 5' end of the mRNA has a modified chemical structure ("cap") recognized by the ribosome, which then binds the mRNA and moves along it ("scans") until it finds the first AUG codon. A characteristic pattern of bases (called a "Kozak sequence") is sometimes found around that codon and assists in positioning the mRNA correctly in a manner reminiscent of the Shine-Dalgarno sequence, but does not involve base pairing with the ribosomal RNA.

[00120] RBSs can include only a portion of the Shine-Dalgarno sequence. When looking at the spacing between the RBS and the start codon, the aligned spacing rather than just the absolute spacing is important. In essence, if only a portion of the Shine-Dalgarno sequence is included in the RBS, the spacing that matters is between wherever the center of the full Shine-Dalgarno sequence would be and the start codon rather than between the included portion of the Shine-Dalgarno sequence and the start codon.

[00121] While the Shine-Dalgarno portion of the RBS is critical to the strength of the RBS, the sequence upstream of the Shine-Dalgarno sequence is also important. One of the ribosomal proteins, S1, is known to bind to adenine bases upstream from the Shine-Dalgarno sequence. As a result, in some embodiments of the modules and synthetic biological circuits described herein, an RBS can be made stronger by adding more adenines to the sequence upstream of the RBS. A promoter may add some bases onto the start of the mRNA that may affect the strength of the RBS by affecting S1 binding.

[00122] In addition, the degree of secondary structure can affect the translation initiation rate. This fact can be used to produce regulated translation initiation rates, as described in Isaacs FJ *et al.*, Nat Biotechnol 2004 Jul; 22(7) 841-7.

[00123] In addition to affecting the translation rate per unit time, an RBS can affect the level of protein synthesis in a second way. That is because the stability of the mRNA affects the steady state level of mRNA, *i.e.*, a stable mRNA will have a higher steady state level than an unstable mRNA that is being produced at an identical rate. Since the primary sequence and the secondary structure of an RBS (for example, the RBS could introduce an RNase site) can affect the stability of the mRNA, the RBS can affect the amount of mRNA and hence the amount of protein that is synthesized.

[00124] A "regulated RBS" is an RBS for which the binding affinity of the RBS and the ribosome can be controlled, thereby changing the RBS strength. One strategy for regulating the strength of prokaryotic RBSs is to control the accessibility of the RBS to the ribosome. By occluding the RBS in RNA secondary structure, translation initiation can be significantly reduced. By contrast, by reducing secondary structure and revealing the RBS, translation initiation rate can be increased. Isaacs and coworkers engineered mRNA sequences with an upstream sequence partially complementary to the RBS. Base-pairing between the upstream sequence and the RBS 'locks' the RBS off. A 'key' RNA molecule that disrupts the mRNA secondary structure by preferentially base-pairing with the upstream sequence can be used to expose the RBS and increase translation initiation rate.

[00125] Non-limiting examples of RBS sequences for use with the synthetic biological circuits described herein include any of those sequences described in the Examples and any of the RBS sequences described in United States Patent No. US 9,284,562 B2 as SEQ ID NOs: 26-33, 858-1001. In some embodiments of the aspects described herein, novel ribosome binding sites can be generated using automated design of synthetic ribosome sites, as described in Salis HM *et al.*, Nature Biotechnology 27, 946 - 950 (2009).

[00126] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[00127] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[00128] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00129] As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[00130] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean $\pm 1\%$.

[00131] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) difference, above or below a reference value. Additional definitions are provided in the text of individual sections below.

BRIEF DESCRIPTION OF THE FIGURES

[00132] **FIGS. 1A-1C** depict an exemplary embodiment of a "Deadman kill switch." **FIG. 1A.** Deadman circuit control of toxin gene expression. Cell viability was measured by CFU count following removal of the survival signal (anhydrotetracycline, ATc) and is displayed as a ratio of cells without ATc to cells with ATc at each time point. **FIG. 1B.** Deadman circuit control of targeted essential protein degradation. Inclusion of the mf-lon specific pdt#1 tag on the specified essential gene causes mf-Lon-mediated degradation of the essential protein upon Deadman circuit activation. **FIG. 1C.** Combined control of toxin expression and targeted essential protein degradation increases Deadman-induced cell death. In particular, targeted MurC degradation and EcoRI expression reduced cell viability to below the limit of detection ($< 1 \times 10^{-7}$) after 6 hours (indicated by a "0"). All data points represent mean $\pm$ S.D. of three biological replicates.

[00133] **FIG. 2** depicts a fail-safe mechanism for Deadman circuit activation. To demonstrate active control over host cell viability, cells grown under survival conditions (with ATc) were exposed

to 1 mM IPTG to directly induce EcoRI and mf-Lon expression. Cell viability was measured by CFU count and is displayed as a ratio of cell survival with and without IPTG.

[00134] **FIGS. 3A-3B** demonstrate hybrid transcription factor (TF) construction and characterization. **FIG. 3A.** An environmental sensing module (ESM) from one LacI family TF can be combined with the DNA recognition module (DRM) of a second LacI family TF to create a hybrid TF with the specified sensory and regulatory properties. **FIG. 3B.** Using this approach, ESMs from LacI, GalR and CelR were combined with the DRM from LacI or ScrR to control GFP expression from a promoter containing lacO or scrO operator sites as indicated. Plots show GFP expression after 3 hours exposure to IPTG, galactose or cellobiose, and results are presented as a ratio to GFP expression in unexposed cells.

[00135] **FIGS. 4A-4B** depict an exemplary embodiment of a “Passcode kill switch.” **FIG. 4A.** Passcode circuit schematic and logic gate behavior. Cell survival requires the continued presence of inputs a and b and the absence of input c. Loss of input a or b or the addition of input c cause the passcode circuit to activate toxin expression, leading to cell death. **FIG. 4B.** Three embodiments of a passcode kill switch were used to control expression of ecoRI, mf-lon-mediated MurC degradation (mf-lon), or both ecoRI and mf-lon. Cells containing each circuit were placed in each of eight possible combinations of the three input molecules, and cell viability was measured by CFU count after 8 hours. In each condition, cell survival is displayed as a ratio of cells in that condition to cells in the “survival” condition highlighted. Cell survival below the limit of detection ($< 1 \times 10^{-7}$) is indicated by a “0”. All data points represent mean $\pm$ S.D. of three biological replicates.

[00136] **FIGS. 5A-5C** demonstrate long-term circuit stability. **FIGS. 5A-5B.** Cells with Deadman or Passcode circuits containing one toxin (EcoRI) or two toxins (EcoRI and mf-Lon) were passaged under survival conditions for 4 days, and sub-populations of cells were periodically switched to nonpermissive media (Deadman: no ATc, Passcode: no inducer) for eight hours. The survival ratio is the ratio of cells that survive in the death state to those in the survival state. Data points represent the mean $\pm$ S.D. of six biological replicates. The passcode circuit was also passaged in *E. coli* MDS42pdu Δ recA (MDS strain), which lacks recombinogenic and mobile genomic elements¹¹. Deadman and Passcode circuits that do not contain toxin modules displayed increased stability throughout the 4 day experiment. **FIG. 5C.** Cells containing Deadman and Passcode circuits that survived exposure to their respective death states were isolated, and the entire circuit and toxin(s) were sequenced to identify the inactivating mutations. Toxin gene disruption by genome-encoded insertion-sequence (IS) elements and large deletions were the predominant cause of circuit inactivation. In the two-toxin Deadman circuit, inactivating TetR mutations allowed continued LacI expression and repression of toxin genes in non-biocontainment conditions.

[00137] **FIG. 6** depicts conversion of a bistable toggle into the monostable Deadman switch. The toggle switch requires strong reciprocal repression by LacI and TetR to create a bistable circuit. The bistable toggle switch was converted into a monostable switch in a single-copy plasmid by

weakening LacI expression relative to TetR expression. The resulting Deadman switch requires ATc to maintain the circuit in the LacI⁺ state and returns to the TetR⁺ state upon ATc removal. mCherry serves as a fluorescent reporter for the TetR⁺ state.

[00138] FIGS. 7A-7B show LacI and TetR RBS strength analysis. **FIG. 7A.** Toggle circuits with a range of predicted LacI and TetR RBS strengths (L1-L3 and T1-T3, respectively) were tested for relative expression levels. mCherry fusions to the C-terminus of LacI and TetR was used to measure LacI and TetR expression levels under full induction. mCherry spectrometry measurements were normalized to cell growth (OD600), and RBS sequences are listed in Table 1. **FIG. 7B.** Circuit monostability was measured by observing the speed of the shift from the LacI⁺ state to the TetR⁺ state in the absence of inducers. Cells containing each toggle circuit were grown in the presence of ATc, transferred to media without inducer, and measured by flow cytometry after 6 hours. Toggle 5, which showed the fastest change in mCherry fluorescence, was chosen for use in the Deadman circuit. “Null” indicates cells without mCherry. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00139] FIGS. 8A-8C show exemplary Deadman switch dynamics. **FIG. 8A.** Cells containing the Deadman circuit pDM1 were grown in the presence of ATc, transferred to media containing ATc, IPTG, or no input (-), and then measured by flow cytometry after six hours. Cells remained in the LacI⁺/mCherry⁻ state in the presence of ATc but shifted to the TetR⁺/mCherry⁺ state in the absence of ATc or in the presence of IPTG. “Null” indicates cells that do not contain mCherry. **FIG. 8B.** Deadman switch dynamics following ATc removal as described in FIG. 8A. Upon removal of ATc, mCherry expression increased within 4 hours. **FIG. 8C.** Representative flow cytometry analysis of Deadman switch dynamics in the presence (left) or absence (right) of ATc. Data points in FIG. 8A and FIG. 8B represent the mean $\pm$ S.D. of three biological replicates. Where the S.D. is small, error bars are present but inside the data symbol.

[00140] FIGS. 9A-9C show an exemplary Deadman circuit refinement to achieve tight control over mCherry expression. **FIG. 9A.** Schematic representation of the improved Deadman circuit. Three palindromic lacO operator sites were included to reduce leaky expression from the pTrc promoter, and a transcriptional terminator was added to reduce readthrough transcription from the upstream promoter. **FIG. 9B.** Following growth in media containing ATc, strains harboring pDM1 or pDM2 were measured for mCherry expression in the presence or absence of ATc. **FIG. 9C.** In the presence of ATc, cells harboring pDM2 showed reduced mCherry expression levels that are indistinguishable from cells that contain no mCherry (Null), implying that the added terminator and promoter improved regulatory control over the reporter gene. Data points represent the mean $\pm$ S.D. of three biological replicates. Where the S.D. is small, error bars are present but inside the data symbol.

[00141] FIGS. 10A-10C show an exemplary construction of Deadman circuit. **FIG. 10A.** A schematic of Deadman circuits pDM2 and pDM3. Unbalanced reciprocal repression by LacI and TetR

causes strong mCherry expression in the absence of ATc (pDM2). Targeted degradation of LacI by mf-Lon protease speeds the transition to toxin expression upon loss of ATc (pDM3). **FIG. 10B.**

Introduction of mf-Lon-mediated degradation of LacI improved the switching dynamics of the Deadman switch. Cells containing pDM2 or pDM3 were grown in the presence of ATc, transferred to media with and without ATc, and then measured by flow cytometry at the indicated time. Data points represent the mean $\pm$ S.D. of three biological replicates. Where the S.D. is small, error bars are present but inside the data symbol. **FIG. 10C.** Representative flow cytometry plots for cells containing pDM2 and pDM3 at 0 and 6 hours after removal of ATc as shown in FIG. 10B. Cells show monomodal distributions.

[00142] **FIGS. 11A-11B** show RBS strength optimization for Deadman switch toxins. **FIG. 11A.** A range of predicted RBS strengths was used to optimize expression of EcoRI, CcdB, and MazF. Cells with Deadman circuits containing each RBS candidate were grown in the presence of ATc (survival state) or IPTG (induced death state), and the ratio of cell growth in the IPTG-treated and ATc-treated cultures was used to measure the relative killing activity. **FIG. 11B.** Growth rate analysis of ATc-treated cells was used to estimate the cellular burden of leaky toxin expression for each RBS candidate. RBS candidates that showed high killing activity in the induced cell death state and low cellular burden in the survival state were chosen for each toxin; 1500 for EcoRI, 500 for CcdB, and 1000 for MazF. “*” indicates RBS candidates that could not be cloned under survival conditions despite multiple attempts. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00143] **FIG. 12** depicts hybrid TF module prediction. LacI family members have conserved structural features that reflect a common mechanism in which effector binding to the regulatory domain induces structural changes in the hinge-helix (HH) motif that alter the orientation of the helix-turn-helix (HTH) motif to weaken DNA operator binding^{1,2}. Effector binding-induced conformational changes are largely limited to the regulatory domain and the HH motif³⁻⁵, and while the HH motif makes contact with the DNA operator site, only the HTH motif makes direct, sequence-specific contact with nucleobases in the major groove complex⁵⁻⁷. Based on this evidence, the HTH and HH appear to play distinct roles in allosteric regulation – while the HTH mediates operator sequence-specificity, the function of the HH is integrated with the regulatory domain and is involved in receiving and translating the allosteric response. In contrast to work by Meinhardt et al.^{8,9} that uses the boundary between the regulatory and HH motifs to generate hybrid TFs, we reasoned that a boundary between the HH and HTH domains would generate distinct environmental sensing and DNA recognition modules.

[00144] **FIG. 13** depicts protein sequence alignment of relevant LacI/GalR family members. ScrR-V and ScrR-K are ScrR from *Vibrio alginolyticus* and *Klebsiella pneumoniae*, respectively. CelR originates from *Thermobifida fusca*. All other family members are from *E. coli*. Residues 1-70 of LacI and the homologous sequences of the other members are shown.

[00145] **FIG. 14** depicts structures of DNA recognition modules of LacI family members. Crystal structures of the N-terminal region of LacI (left) and PurR (right) are shown, including their helix-turn-helix motif (HTH), hinge helix motif (HH) and part of the regulatory domain connected to the HH motif. The HTH binds to the major groove to interact with nucleobases, and the HH motif sits in the minor grooves to interact with the DNA backbone. The PDB IDs of the LacI and PurR crystal structures are 1EFA and 1QPZ, respectively.

[00146] **FIG. 15** demonstrates determination of the optimal ESM/DRM boundary for the GalR-LacI hybrid TF. Residues 34-48 of LacI are aligned with the homologous GalR residues, and the dotted lines indicate the position between the GalR ESM and LacI DRM used to generate the hybrid TFs, which are designated LG36 to LG46 according to the hybrid site used. The TFs were expressed in cells containing a gfp reporter under control of the pLlacO-1 promoter¹⁰. Cells were grown in the presence or absence of 20 mM galactose for 1 hour, and GFP fluorescence was measured by flow cytometry. Fold-change in fluorescence is the ratio of fluorescence in galactose treated to untreated cells. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00147] **FIGS. 16A-16C** demonstrate a structure-based strategy to identify protein modules that mediate allosteric response and DNA recognition in LacI/GalR family TFs. **FIG. 16A.** A module interchange strategy for engineered hybrid TFs. The environmental sensing module (ESM) of one LacI/GalR family TF can be combined with the DNA recognition module (DRM) of a second LacI/GalR family TF to create a hybrid TF with the specified sensory and regulatory properties. **FIG. 16B.** LacI DRM is combined with ESMs from other LacI/GalR family TFs to create hybrid TFs. Native LacI (LacI-LacI) and the GalR-LacI and CelR-LacI hybrid TFs were expressed in strains containing gfp under control of the pLlacO promoter containing a lacO operator site bound by the LacI DRM. Promoter regions containing the lacO operator and the -35 and -10 elements are shown. Cells containing the TF and reporter constructs were treated with a range of inducer concentrations for 3 hours and assessed for GFP expression by flow cytometry. **FIG. 16C.** DRM from ScrR can also be used to engineer hybrid TFs. Hybrid TFs were constructed by replacing the DRMs of LacI, GalR, and CelR with that of ScrR and were then tested with reporter plasmids that use either the pLscrO-1 or pLscrO-2 promoter to control GFP expression. The promoter region containing the scrO operators and the -10 and -35 elements are shown. GFP fluorescence was determined by flow cytometry 3 hours after exposure to the indicated inducer concentrations. The dotted lines represent the basal GFP fluorescence in cells not exposed to the inducer. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00148] **FIG. 17** depicts GalR-LacI activity in the presence of IPTG. Cells containing GalR-LacI and pLlacO-driven gfp gene were exposed to a range of galactose concentrations in the presence of 0, 1, or 10 mM IPTG. GFP levels in these cells were assessed by flow cytometry after 8 hours. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00149] **FIG. 18** demonstrates GalR-ScrR hybrid regulation is interoperable with LacI regulation. LacI-LacI and GalR-ScrR were expressed in cells containing *pLlacO-1-mcherry* and *pLscrO-1-gfp* reporters. Cells were exposed to IPTG (1 mM) and/or galactose (20 mM) for 1 hour and assayed for GFP and mCherry expression by flow cytometry.

[00150] **FIG. 19** demonstrates generation of AND logic gates using hybrid TFs. For each AND logic gate, two TFs were expressed in cells harboring the *pLlacO-gfp* reporter. Cells were treated with the indicated inducers for 3 hours before GFP fluorescence was measured by flow cytometry. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00151] **FIGS. 20A-20C** demonstrate exemplary passcode circuit control of GFP expression. **FIG. 20A.** Three versions of the Passcode circuit were developed using the indicated circuit architecture. For each Passcode circuit, constitutive expression of hybrid A and hybrid B containing the LacI DRM was used to control expression of hybrid C containing the ScrR DRM which controls *gfp* expression. **FIG. 20B.** Cells containing each Passcode circuit were exposed to all eight combinations of the three small molecule inputs as shown, and GFP expression was assessed by flow cytometry after 3 hours. **FIG. 20C.** Representative flow cytometry plots show cells containing the Passcode circuits in each environmental condition after 3 hours of induction as in FIG. 20B. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00152] **FIG. 21** depicts representative RBS strength analysis for Passcode toxin optimization. For CelR-ScrR dependent toxin expression, RBS sequences with a range of calculated translation initiation rates were used to control EcoRI and mf-Lon expression. Cells containing each RBS candidate were grown in the presence or absence of cellobiose (the death state and survival state, respectively), and the ratio of cell growth in these states was used to measure killing activity (OD600 ratio, top charts). Cell growth rate in the survival state (Cell growth, bottom charts) showed no distinct difference among the RBS candidates, and therefore RBS sequences that showed high killing activity were chosen for each toxin; 200 for EcoRI and 100000 for mf-Lon. "*" indicates RBSs that could not be cloned under survival conditions despite multiple attempts. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00153] **FIG. 22** depicts time-dependent cell killing by the Passcode kill switch. Cells containing the indicated version of the Passcode circuit were exposed to each of the eight combinations of the three small molecule inputs (IPTG, galactose, and cellobiose). Cell viability was measured by CFU count at the indicated times after exposure, and results are presented as the ratio of CFUs in each input condition to that in the survival condition that is unique for each Passcode circuit. Data points represent the mean $\pm$ S.D. of three biological replicates.

[00154] **FIG. 23** depicts effect of long-term growth on the Passcode kill switches. Cells with Passcode kill switches containing one toxin (EcoRI) or two toxins (EcoRI and mf-Lon) were passaged in the survival condition unique to each Passcode circuit, and sub-populations of these cells were periodically switched to the death state by exposure to media with no inducers. Introduction of the

two toxin Passcode circuits into *E. coli* strain MDS42pduArecA (MDS strain), which lacks recombinogenic and mobile genomic elements¹¹, yielded a 3-5 log reduction in escapee frequency after 4 days. Cell viability was measured by CFU count after 8 hours of exposure to the death state and is presented as a ratio of surviving cells in the death state to those in the survival state at each time point. All strains also contain a deletion in *lacI* (Δ lacI) and a genomic *murC*-pdt#1 tag. Data points represent the mean $\pm$ S.D. of six biological replicates.

[00155] **FIGS. 24A-24B** demonstrate effects of long-term growth on the Deadman kill switches without toxin modules. **FIG. 24A.** Cells containing pDM3 with mCherry as the output module were passaged in the presence of ATc for 4 days. Sub-populations of these cells were periodically tested for circuit function by transferring the cells to media with and without ATc for 8 hours. Data points were measured by flow cytometry and represent the mean $\pm$ S.D. of six biological replicates. **FIG. 24B.** Representative flow cytometry plots for each time point in the presence or absence of ATc. Cells passaged for 4 days displayed monomodal population distributions that were very similar to cells tested in day 1.

[00156] **FIG. 25** demonstrates effects of long-term growth on the Passcode kill switches without toxin modules. Cells containing each version of the Passcode circuit were used to control *gfp* expression. These cells were passaged for 4 days under survival conditions unique to each Passcode circuit and periodically tested for circuit function by passage in media with no inducers. GFP expression was assessed with flow cytometry after 8 hours. Representative flow cytometry plots showed a monomodal distribution of cells in both the no inducer and survival conditions for 4 days. Data points represent the mean $\pm$ S.D. of three biological replicates.

DETAILED DESCRIPTION

[00157] Provided herein are novel, engineered circuit-based microbial “kill switches” that restrict host cell survival to an environment defined by specific input signals. Unlike existing biocontainment systems with fixed survival conditions that are difficult to modify, the Deadman and Passcode kill switches described herein are modular and inherently customizable, both in terms of the environmental conditions that control circuit activation and in the output modules that control cell fate. In addition to its use in biocontainment systems, the Passcode circuit has particular utility, in some embodiments, as a tool for intellectual property protection, where unauthorized growth of strains without the appropriate “passcode” molecules would induce cell death. With the proper choice of toxins, including, but not limited to an endonuclease, exemplified herein by EcoRI, embodiments of the Passcode circuits described herein can be used to not only kill the host cell, but also degrade its genome and accompanying plasmids to deter attempts at reverse-engineering the strain of interest. Use of hybrid transcription factors or TFs that respond to proprietary small molecule inputs can further secure the strain against theft, even if its genome is sequenced, in some embodiments.

[00158] The Deadman and Passcode switches described herein provide robust information processing circuits to couple environmental signals with conditional survival of the microbial host. The Deadman kill switch described herein is based, in part, on a monostable circuit that passively activates toxin gene expression in the absence of a small molecule input, such as ATc. Since the small molecule input, such as ATc, is not normally found in nature, engineered cells that escape containment will trigger cell death to prevent the spread of the organism or its genetic content into the surrounding ecosystem. Unlike auxotrophy-based biocontainment where the environmental signal is an intrinsic feature of the system^{9,10}, the environmental sensing and cell killing systems are decoupled in the Deadman switches described herein. These circuits rely on two main elements for functionality: (1) the orthogonality of the TFs to create a toggle switch, and (2) their relative activity under induced expression. As such, the Deadman circuits described herein are highly modular, and the environmental signal detected by the circuit can be altered, for example, by replacing TetR with a wide range of transcription factors, including more than 80,000 annotated TetR family members,³⁸ as well as orthogonal LacI/GalR family members, including hybrid TFs as described for the Passcode switches described herein. In addition, the Deadman circuits described herein have an additional fail-safe mechanism that activates toxin production and cell death in the presence of another molecule, such as IPTG, enabling exogenous control over the microbe's survival even as the cell uses the circuit to monitor its environment.

[00159] Similar to the Deadman switches, the Passcode circuits described herein are based on a two-layered transcriptional repression design. To build hybrid transcription factors (TFs), the conserved boundaries of the ESMs (environmental sensing modules) and DRMs were identified within the LacI/GalR family members LacI, GalR, CelR and ScrR. The resulting environmental sensing and DNA binding modules provide independent control of the sensory input and regulatory output of each hybrid TF. Work by Meinhardt et al.^{27,28} used the boundary between the conserved regulatory domain and HH motif to create hybrid TFs, but some of these hybrids required additional protein engineering and mutagenesis to become functional. Herein, a novel and discrete boundary between the conserved HH and HTH motifs was identified and can be used to create independent environmental sensory and DNA binding domains that can be efficiently combined without further protein engineering. The modularity provided by these hybrid TFs dramatically expands the number and range of environmental signals that can be used to control biocontainment systems such as the Deadman and Passcode circuits described here, as the ESM and DRM boundaries defined in this study can be used to incorporate sensing modules from many of the ~29,000 LacI/GalR family members³⁹ that detect diverse environmental signals.

[00160] The hybrid TFs described herein can also be used to functionalize other synthetic circuits, including the Deadman switch, to respond to different environmental signals. Moreover, the regular use of LacI and TetR in other bacteria^{40,41} indicates that these circuits can be readily transferred to other microbes, including industrial production strains. Replacement of the antibiotic

resistance cassettes in these plasmids with well-characterized selection systems that use toxin-antitoxin modules or auxotrophy complementation also enables their use in biotherapeutic applications^{4,42}.

Deadman Kill Switches

[00161] Provided herein, in some aspects, are engineered, synthetic biological circuits comprising modular components for use as, and with, passively activated biocontainment systems for engineered microbes termed “Deadman kill switches.” “Deadman kill switches” or “Deadman kill circuits,” as these terms are used herein, refer to an engineered, addressable cellular memory module that can be constructed from repressible sequences arranged in a mutually inhibitory network and which exhibits robust monostable behavior. For example, reciprocal repression can be mediated by transcription factors, such as the LacI and TetR transcription factors, which form transcription states that are maintained by the circuit’s linked feedback loops (see, for example, FIG. 6).

[00162] The monostable behavior of the Deadman kill switches, as described herein, arises from a mutually inhibitory arrangement of at least two repressible sequences, such that a small molecule-binding transcription factor is used to produce a 'survival' state in which repression of toxin production is linked to the presence of a specific environmental signal. Upon loss of the environmental signal, the circuit switches permanently to the 'death' state in which the now derepressed toxin production kills the cell in which the Deadman kill switch is present.

[00163] In some aspects, a deadman kill switch is a biological circuit or system rendering a cellular response sensitive to a predetermined condition, such as the lack of an agent in the cell growth environment, e.g., an exogenous agent. Such a circuit or system can comprise a nucleic acid construct comprising expression modules that form a deadman regulatory circuit sensitive to the predetermined condition, the construct comprising expression modules that form a regulatory circuit, the construct including:

i) a first repressor protein expression module, wherein the first repressor protein binds a first repressor protein nucleic acid binding element and represses transcription from a coding sequence comprising the first repressor protein binding element, and wherein repression activity of the first repressor protein is sensitive to inhibition by a first exogenous agent, the presence or absence of the first exogenous agent establishing a predetermined condition;

ii) a second repressor protein expression module, wherein the second repressor protein binds a second repressor protein nucleic acid binding element and represses transcription from a coding sequence comprising the second repressor protein binding element, wherein the second repressor protein is different from the first repressor protein; and

iii) an effector expression module, comprising a nucleic acid sequence encoding an effector protein, operably linked to a genetic element comprising a binding element for the second repressor protein, such that expression of the second repressor protein causes repression of effector expression from the effector expression module, wherein the second expression module comprises a

first repressor protein nucleic acid binding element that permits repression of transcription of the second repressor protein when the element is bound by the first repressor protein, the respective modules forming a regulatory circuit such that in the absence of the first exogenous agent, the first repressor protein is produced from the first repressor protein expression module and represses transcription from the second repressor protein expression module, such that repression of effector expression by the second repressor protein is relieved, resulting in expression of the effector protein, but in the presence of the first exogenous agent, the activity of the first repressor protein is inhibited, permitting expression of the second repressor protein, which maintains expression of effector protein expression in the "off" state,

such that the first exogenous agent is required by the circuit to maintain effector protein expression in the "off" state, and removal or absence of the first exogenous agent defaults to expression of the effector protein.

[00164] In some embodiments, the effector is a toxin or a protein that induces a cell death program. Any protein that is toxic to the host cell can be used. In some embodiments the toxin only kills those cells in which it is expressed. In other embodiments, the toxin kills other cells of the same host organism.

[00165] In the examples described herein, the first repressor protein is the tet repressor, tetR, and the second repressor protein is the lac repressor, LacI, but essentially any pair of different repressor proteins for which the repressor binding element is known can be used. Indeed, where both LacI and TetR are known to be members of large families of related proteins expressed in different species of organism, any of the related members, with their cognate repressor binding elements can be used to construct a deadman kill switch circuit as described herein. A number of repressor proteins and the elements to which they bind are known in the art, and are described, for example in Terpe, Appl. Microbiol. Biotechnol. 72: 211-222 (2006), and in U.S. patent application publication No. 20130034907, which are incorporated herein by reference in their entireties.

[00166] The deadman kill switch circuit can further include an expression module for a targeted protease or a targeted nuclease that degrades the first repressor protein or its message to thereby amplify the effect of the down-regulation of first repressor protein expression. The targeted protease or nuclease can be under the negative control of the second repressor protein, such that loss of the exogenous agent results in degradation of the first repressor protein or its message as well as derepression of expression of the first repressor protein.

[00167] By introducing a construct encoding the respective modules into a host cell, e.g., a host cell that produces a desired agent, a method is provided in which the host cell is rendered sensitive to the presence of the exogenous agent such that when the host cell either escapes containment or is no longer needed, or desired e.g., in a therapeutic use, the removal or absence of the exogenous agent kills the host cell.

[00168] In some embodiments of the aspects described herein, a bistable “toggle switch” circuit, such as those described in U.S. patent application publication No. 20130034907, which is incorporated herein by reference in its entirety, can be converted into a deadman kill switch by manipulating the strength of expression or stability of one of the mutually-regulated repressor proteins. Reducing the efficiency of expression or activity of one of the repressors in a toggle switch circuit can bias the system towards expression or activity of one repressor that results in cell death when that repressor is active. In the toggle switch system, the product of each repressor sequence, *i.e.*, the repressor, can inhibit, at a transcriptional level, a translational level, or a combination thereof, the expression of a product encoded by the other repressor sequence. Thus, in the absence of an appropriate input or inducing agent, such as a transcriptional activating agent, two stable states are possible: a first state in which a first repressor is expressed and inhibits expression of a second repressor sequence, and a second state in which the second repressor is expressed and inhibits expression of the first repressor sequence. This is a bistable system. In some aspects of a bistable system, repressors act at the transcriptional level, whereby a first promoter sequence drives expression of a first repressor sequence that encodes for a repressor specific for a second promoter sequence. The second promoter sequence, in turn, drives expression of a second repressor sequence that encodes for a repressor specific for a second promoter sequence. In such an aspect, switching between the two states (*i.e.*, expression of the first or second repressor) is mediated by the presence of an exogenous or endogenous input agent, such as an agent that prevents repressor binding to the currently inactive promoter. In such an embodiment, the agent permits the opposing repressor to be maximally transcribed until it stably represses the originally active promoter. In other embodiments, repressors in a genetic toggle switch can act at the translational level, whereby a first repressor encodes a product, such as an inhibitory RNA molecule, that inhibits or prevents translation of the second repressor, or causes degradation of the second repressor mRNA. In other embodiments of the aspects described herein, different repressors in a genetic toggle switch can use different mechanisms of repression, *i.e.*, transcriptional, translational, or combinations thereof.

[00169] To create a circuit in which the ‘death’ state is dominant in the absence of the survival signal, *i.e.*, to convert a bistable toggle switch to a monostable deadman kill switch, the expression of one repressor can be manipulated to bias the system either towards or away from expression of that repressor. In the non-limiting examples described herein, the ribosome binding site (RBS) strengths of LacI and TetR were manipulated to favor TetR expression in a single-copy plasmid (FIGS. 7A-7B). In the resulting monostable circuit, the presence of the TetR inhibitor anhydrotetracycline (ATc) is required to maintain the circuit in the subordinate LacI+ ‘survival’ state (FIGS. 8A-8C). Incorporation of toxin genes into the TetR+ state creates a kill switch where the presence of ATc is required to block toxin expression and cell death.

[00170] Additional repressor binding sites can be included to minimize leaky toxin expression, or other steps can be taken to ensure toxin expression occurs only when desired, in some

embodiments. In the Examples described herein, palindromic LacI operator sites were included in the toxin gene promoter for this purpose¹⁹ and a transcriptional terminator was included upstream of the promoter to insulate the gene from spurious transcription (FIGS. 9A-9C). To accelerate the circuit's switching dynamics, a degradation tag can be placed on a repressor protein. In the Examples, a tag was fused to the C-terminus of LacI that is specifically recognized by *mf*-Lon²⁰, a heterologous protease under control of a LacI-dependent promoter (FIGS. 10A-10C). Upon removal of ATc, TetR repression of *lacI* allows expression of *mf*-Lon, which targets LacI for degradation to create a positive feedback loop that accelerates the switch to the TetR⁺ state (FIG. 10B). Other proteases can be targeted in a similar manner, or the message encoding the repressor can also be targeted. Importantly, single-cell analysis of these circuits by flow cytometry showed a monomodal distribution of cells in the LacI⁺ and TetR⁺ state, demonstrating stable circuit expression across the cell population (see 0 and 6 hour data in FIG. 10C).

[00171] As noted above, any of a large number of products that will lead to cell death can be employed in a deadman kill switch. Agents that inhibit DNA replication, protein translation or other processes or, e.g., that degrade the host cell's nucleic acid, are of particular usefulness. To identify an efficient mechanism to kill the host cells upon circuit activation, several toxin genes were tested that directly damage the host cell's DNA or RNA. The endonuclease *ecoRI*²¹, the DNA gyrase inhibitor *ccdB*²² and the ribonuclease-type toxin *mazF*²³ were tested because they are well-characterized, are native to *E. coli*, and provide a range of killing mechanisms. The toxin genes were independently incorporated into the Deadman circuit, and a range of RBS strengths were tested for each toxin to optimize cell death upon circuit activation²⁴ (FIGS. 11A-11B). Upon removal of ATc, the toxins produced 3–5 logs of killing within 6 hours as measured by colony forming units (CFUs) (FIG. 1A). To increase the robustness of the circuit and provide an independent method of circuit-dependent cell death, the system can be further adapted to express, e.g., a targeted protease or nuclease that further interferes with the repressor that maintains the death gene in the “off” state. Upon loss or withdrawal of the survival signal, death gene repression is even more efficiently removed by, e.g., active degradation of the repressor protein or its message. As non-limiting examples, *mf*-Lon protease was used to not only degrade LacI but also target essential proteins for degradation (FIG. 1B). The *mf*-Lon degradation tag pdt#1 was attached to the 3' end of five essential genes whose protein products are particularly sensitive to *mf*-Lon degradation²⁰, and cell viability was measured following removal of ATc (FIG. 1B). Among the tested essential gene targets, the peptidoglycan biosynthesis gene *murC* provided the strongest and fastest cell death phenotype (survival ratio $< 1 \times 10^{-4}$ within 6 hours).

[00172] To determine if the toxin- and *mf*-Lon-mediated killing mechanisms produce synergistic effects, Deadman circuits were created containing each of the toxins in combination with the *mf*-Lon-MurC targeting module (FIG. 1C). In each instance, the combinatorial approach provided more effective biocontainment, and in particular, coordinated EcoRI expression and *mf*-Lon-mediated MurC degradation resulted in cell killing below the limit of detection (survival ratio $< 1 \times 10^{-7}$) 6

hours after removal of ATc (FIG. 1C). Furthermore, the Deadman circuit's design provides an additional fail-safe mechanism which bypasses the circuit's sensor system to directly activate toxin expression to cause cell death. Direct derepression of the subordinate TF, in this case derepression of LacI with isopropyl β -D-1-thiogalactopyranoside (IPTG), activates toxin production and cell death irrespective of the presence of the programmed survival signal (FIG. 2).

Passcode Kill Switches

Hybrid transcription factor design

[00173] To extend the versatility and modularity of this system, a second circuit, called the Passcode circuit, was built which uses hybrid TFs to expand the range and complexity of environmental signals used to define biocontainment conditions. This survival "passcode" can be easily reprogrammed to restrict cell growth to a new environment or to limit knowledge of the growth conditions to authorized personnel.

[00174] In one aspect, a "passcode" system that renders cell growth restricted to the presence of a predetermined set of at least two selected agents, includes one or more nucleic acid constructs encoding expression modules comprising: i) a toxin expression module that encodes a toxin that is toxic to a host cell, wherein sequence encoding the toxin is operably linked to a promoter P1 that is repressed by the binding of a first hybrid repressor protein hRP1; ii) a first hybrid repressor protein expression module that encodes the first hybrid repressor protein hRP1, wherein expression of hRP1 is controlled by an AND gate formed by two hybrid transcription factors hTF1 and hTF2, the binding or activity of which is responsive to agents A1 and A2, respectively, such that *both* agents A1 and A2 are required for expression of hRP1, wherein in the absence of either A1 or A2, hRP1 expression is insufficient to repress toxin promoter module P1 and toxin production, such that the host cell is killed. In this system, hybrid factors hTF1, hTF2 and hRP1 each comprise an environmental sensing module from one transcription factor and a DNA recognition module from a different transcription factor that renders the binding of the respective DRM sensitive to the presence of an environmental agent, A1, or A2, that is different from that which the respective DRM binds in nature.

[00175] The passcode approach was tested using hybrid TFs designed from members of the LacI/GalR families. To build hybrid LacI family TFs, the boundaries of the environmental sensing modules (ESMs) and DNA recognition modules (DRMs) found in LacI family members were first identified. (FIG. 3A and FIGS. 12-15). Hybrid TFs were generated that use the small molecule input defined by the hybrid's ESM to regulate the promoter defined by the hybrid's DRM^{25,26} (FIG. 3A and FIGS. 16A-16C).

[00176] To construct the hybrid TFs, we used the cellobiose-responsive TF CelR from *Thermobifida fusca* and the galactose-responsive TF GalR and IPTG-responsive LacI from *E. coli*. We fused the ESMs from CelR and GalR to the DRM of LacI to generate the hybrid TFs CelR-LacI and GalR-LacI. To test their functionality, these hybrid TFs or native LacI were used to control GFP expression from a promoter containing *lacO* operator sites recognized by the LacI DRM. The hybrid

TFs allowed strong GFP expression upon exposure to the small molecule input defined by their ESM and showed almost no response to the other inputs (FIG. 3A and FIG. 16B). We fused the LacI, GalR and CelR ESMs to the DRM of ScrR from *Klebsiella pneumoniae* and used the resulting hybrid TFs to regulate a promoter containing *scrO* operator sites. As predicted from their design, these hybrid TFs only respond to the input defined by their ESM (FIG. 3B and FIG. 16C), although it is interesting to note that the GalR ESM shows distinct inhibition by high levels of IPTG as seen by Shis *et al.*²⁷ (FIG. 17). Importantly, the DRMs used in these hybrid TFs provided similar specificity, as they regulated promoters containing their cognate operator sites but not other LacI family operator sites (FIG. 18). Similar to work by Shis *et al.*²⁷, we found that co-expression of hybrid TFs containing the same DRM could be used to regulate a single promoter, creating an AND logic gate function (FIG. 19).

Development of the Passcode kill switches

[00177] We used these hybrid TFs to create a series of Passcode circuits that contain a single transcriptional architecture but respond to distinct combinations of environmental inputs to control gene expression and cell survival. As shown in FIGS. 20A-20C, the Passcode circuits contain the output module (in this case, *gfp*) under control of a TF (hybrid C) whose expression is controlled by an AND gate formed by two TFs (hybrid A and hybrid B). This serial arrangement, made possible by the orthogonality of the hybrid DRMs and ESMs, creates the condition that both of the inducers recognized by hybrid A and hybrid B (inputs a and b, respectively) must be present to allow expression of hybrid C to repress *gfp* expression. Loss of input a or input b or the presence of input c allows *gfp* expression, causing cell death if *gfp* is replaced by a toxin gene.

[00178] To test the functionality and modularity of this circuit architecture, we created three exemplary embodiments of the Passcode circuit that respond to different combinations of input signals to control output expression (FIG. 4A). For example, in one Passcode circuit (FIG. 4B, left column), we used GalR-LacI (A) and CelR-LacI (B) to control expression of LacI-ScrR (C), which in turn represses toxin expression. In this circuit, loss of galactose (input a) or cellobiose (input b) allows GalR-LacI or CelR-LacI to bind the *lacO* operator, blocking LacI-ScrR expression, thereby enabling toxin expression and causing cell death. Any exposure to IPTG (input c) releases LacI-ScrR repression of toxin expression, thereby killing the cell as well. Importantly, the passcode combinations for cell survival and cell death can be reprogrammed by rearranging the ESMs of the three TFs to rewire the connections between the environmental sensing and transcriptional regulation, in different embodiments.

[00179] These Passcode circuits were first evaluated with GFP as the output module in all eight combinations of the three environmental inputs. All three circuits allowed high level GFP expression in all conditions except that designated by the desired three input combination (FIG. 20B), and single-cell fluorescence showed a monomodal population distribution under all conditions (FIG. 20C). GFP was then replaced with the *ecoRI* and *mf*-Lon-MurC toxin modules described for the

Deadman switch above (FIG. 4A), and toxin expression levels were optimized by testing a range of calculated RBS strengths²⁴ (FIG. 21). Hybrid C, which directly controls toxin expression in the circuit, was also engineered in the same manner to optimize circuit performance (see Methods). Each kill switch circuit was tested in *E. coli* using eight combinations of input signals, and cell survival was measured by CFU count at multiple time points (FIG. 22). As seen in FIG. 4B, only circuits that received the proper survival code allowed the host cells to survive (each survival condition is highlighted in green). Furthermore, inclusion of both the *ecoRI* and *mf-Lon* toxin modules in the Passcode circuit caused the cell survival ratio to drop below 1×10^{-6} for all non-passcode conditions.

Circuit stability

[00180] To measure the long-term stability and robustness of the Passcode and Deadman kill switches, we passaged cells containing the circuits for four days under survival conditions and periodically tested subsets of cells for circuit function under non-permissive conditions. Both the Deadman and Passcode circuits showed reduced killing efficiency over time, and sequence analysis of cells that escaped biocontainment predominantly showed inactivating mutations in the toxin genes (FIGS. 5A-5C and FIG. 23). The noted exception was independent TetR mutations in the two-toxin Deadman circuit where TetR inactivation repressed toxin expression even in the absence of the ATc survival signal. It is important to note, however, that these ‘escapees’ are still sensitive to IPTG-mediated fail-safe circuit activation as described above (FIG. 2). Genome-encoded insertion-sequence (IS) elements³⁷, particularly IS1 and IS5, caused a large percentage of inactivating mutations in the one-toxin and two-toxin Passcode circuits. Deletion of these IS elements and other genome repair mechanisms in *E. coli* reduced the Passcode ‘escapee’ rate by 3-5 logs after four days, demonstrating that increased stability of the host genome will augment the functionality of these biocontainment systems (FIG. 5B and FIG. 23). As the toxin genes were the main target for circuit inactivation, inclusion of additional redundant killing systems into each circuit should further reduce the escapee rate.

[00181] Described herein are two safe-guard systems, demonstrated in *Escherichia coli*, but generalizable across host cells in part due to the modularity of they systems’ constituent parts. The systems include a “Deadman” kill switch that requires a specific input signal to block cell death and a “Passcode” circuit that uses hybrid transcription factors to detect multiple environmental inputs. These circuits efficiently kill *E. coli* and can be reprogrammed to change the input signal, regulatory architecture and killing mechanism.

[00182] The systems, compositions and methods described provide a biocontainment system for engineered bacteria. Examples include engineered probiotic bacteria in the human intestine, engineered bacteria or eukaryotes used in production facilities for fuels, chemicals and materials, and engineered bacteria or eukaryotes used in environmental applications, among others. The circuits are designed to kill any cells that are released from the intended environment.

[00183] The described systems also provide a tool for intellectual property protection. Unauthorized growth of a protected strain without the appropriate “passcode” molecules will induce cell death, and with the proper choice of toxins, such as endonucleases like EcoRI described here, the Passcode circuit can be used to not only kill the host cell but degrade its genome and accompanying plasmids to deter attempts at reverse-engineering. The use of hybrid TFs that respond to proprietary small molecule inputs will further secure the strain against theft even if its genome is sequenced.

[00184] The described systems also provide a tool to control the proliferation of pathogen used in research facilities. Unauthorized growth of the strain without a specific molecule or appropriate “passcode” molecules will activate a killing mechanism.

[00185] Existing biocontainment systems have used metabolic auxotrophy and the induction of toxin proteins to control cell survival, and recent strategies include the introduction of synthetic auxotrophy, enzyme redesign, orthogonal control of essential gene functions, and engineered addiction modules and riboregulated auxotrophy. However, many of these systems are intrinsically difficult to reprogram for different environmental conditions, potentially limiting their application.

[00186] Described herein is a circuit-based approach to develop versatile biocontainment systems that incorporate modularity into both the circuit designs and the environmental sensors that control them. Additionally, the high degree of modularity in both the Deadman and Passcode circuits dramatically expands the number and range of environmental signals that the circuits can detect. The ESM and DRM boundaries defined in the studies described herein can be used incorporate the sensing modules from many of the ~29,000 LacI family members into the hybrid TFs to detect other environmental signals, thereby increasing the specificity and complexity of the programmed “passcode”. These hybrid TFs may also be used to “functionalize” existing synthetic circuits to respond to different environmental signals without having to modify the transcription regulatory architecture.

[00187] Biocontainment systems that couple environmental sensing with circuit-based control of cell viability can prevent escape of engineered microbes into the environment.

[00188] Described herein is the use of a monostable toggle design to control an output module. This design allows passive activation of the genetic circuit in the absence of the input molecule, and upon circuit activation, it provides a positive feedback loop that increases the speed of expression of the output module.

[00189] In the case of the deadman switch, this output module uses toxin genes to control cell survival, but the output module could be used to control any cell process.

[00190] Also described is the development of hybrid transcription factors that use the boundary region homologous to the Escherischia coli LacI protein region from aa36 to aa46 to create hybrid TFs containing the N-terminal DNA-binding domain and the C terminal sensor domain that are defined by that boundary. The resulting hybrid TFs recognize the small molecule defined by the C-

terminal sensor domain and respond by binding or releasing the DNA region defined by the hybrid TF's N-terminal DNA binding domain.

[00191] Also described is the use of hybrid transcription factors to create biosensors in which the C-terminal sensor domains from diverse LacI family members are fused to the N-terminal DNA-binding domain from well-characterized transcription factors such as *E. coli* LacI to allow transcriptional activation from a well-characterized promoter upon detection of the small molecule by the C-terminal sensor domain.

[00192] Also described is the use of hybrid transcription factors to create a 'Passcode' circuit that requires the presence and/or absence of specific small molecules to activate the output module. By placing the genes that encode for cellular toxins in the output module, this circuit may be used to create a kill switch mechanism in which the circuit kills the cell if the cell leaves the specific environment defined by the sensor domains. The modularity of the hybrid TFs, the circuit architecture, and the output module allows the circuit to be reconfigured to sense other environmental signals, to react to the environmental signals in other ways, and to control other functions in the cell in addition to induced cell death.

[00193] The deadman switch can use alternative transcription factors to create the positive feedback loop or can use alternative methods including transcriptional, post-transcriptional, translational, or post-translational systems.

[00194] The output module can be reconfigured to use different cellular toxins to kill the cell or may be used to cause an alternative outputs such as degrading specific genetic components with or without killing the cell. The output module can be used to regulate other genetic circuits of endogenous genes with or without killing the cell. The output module can be an RNA-based circuit.

[00195] The deadman and passcode circuits can be used in other organisms, including other bacteria or eukaryotes, including mammalian cells.

[00196] For the deadman switch, replacement of TetR or LacI and their regulated promoters with repressors that sense other environmental signals would allow this circuit to sense a wide range of environmental cues.

[00197] The ESM and DRM boundary may be in any amino acid within the region defined by homology to *E. coli* LacI amino acids 36-46.

[00198] The ESM and DRM boundaries defined in this study can be used incorporate the sensing modules from many of the ~29,000 LacI family members into the hybrid TFs to detect other environmental signals.

[00199] The hybrid TFs can be used in alternative circuit architectures to control the circuit output. Additional hybrid TFs could be used to respond to different environmental signals to control the same promoter or hybrid TFs could be used to respond to the same signal to activate or repress different promoters.

[00200] More than two hybrid TFs can be used to control the same promoter.

[00201] Two or more hybrid TFs that sense the same molecule can be used in a circuit to control multiple promoters.

[00202] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A programmable synthetic biological circuit responsive to a selected combination of predetermined inputs, the circuit comprising:
 - a. an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first modulator protein is present;
 - b. a first modulator protein expression module comprising nucleic acid sequence encoding the first modulator protein, operably linked to a second nucleic acid regulatory element comprising an AND circuit, whereby expression of the first modulator protein requires the presence of each of a selected set of at least two predetermined input agents, and in the absence of any one of the at least two predetermined input agents, expression of the first modulator protein is altered, resulting in expression of the effector protein.
2. The programmable synthetic biological circuit of paragraph 1, wherein the second nucleic acid regulatory element comprises a DNA binding site for a second modulator protein.
3. The programmable synthetic biological circuit of paragraph 1, wherein the first modulator protein is a repressor protein, and where, in the absence of any one the at least two predetermined input agents, expression of the repressor protein is inhibited, resulting in expression of the effector protein.
4. The programmable synthetic biological circuit of paragraph 2, further comprising a set of at least two hybrid modulator protein expression modules, each of which comprises nucleic acid sequence encoding a hybrid modulator protein and operatively linked regulatory sequences that permit the expression thereof, each hybrid modulator protein comprising:
 - a. an input agent-responsive domain of an input agent-sensitive transcription factor; and
 - b. the DNA binding domain of the second modulator protein,
 - c. wherein the binding of each respective hybrid modulator protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is altered by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid modulator protein, and
 - d. wherein each respective hybrid modulator protein alters expression from the first modulator protein expression module in the absence of its respective input agent.
5. The programmable synthetic biological circuit of paragraph 4, wherein the at least two hybrid modulator proteins are each hybrid repressor proteins, where the binding of each respective

- hybrid repressor protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is altered by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid modulator protein, and
- a. wherein each respective hybrid modulator protein alters expression from the first modulator protein expression module in the absence of its respective input agent.
6. A composition comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of paragraphs 1-5.
 7. The composition of paragraph 6, wherein the expression modules are comprised by a single nucleic acid construct.
 8. The programmable synthetic biological circuit of any one of paragraphs 4-6, wherein the hybrid modulator expression modules and first modulator expression module are on the same nucleic acid construct.
 9. The programmable synthetic biological circuit of any one of paragraphs 4-6, wherein the hybrid repressor expression modules, first repressor expression module and effector expression module are on the same nucleic acid construct.
 10. A host cell comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of paragraphs 1-9.
 11. The host cell of paragraph 10, wherein the cell is a bacterial, fungal, plant or animal cell.
 12. A programmable synthetic biological circuit responsive to a selected combination of predetermined inputs, the circuit comprising:
 - a. an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first repressor protein is present;
 - b. a first repressor protein expression module comprising nucleic acid sequence encoding the first repressor protein, operably linked to a second nucleic acid regulatory element comprising an AND circuit, whereby expression of the first repressor protein requires the presence of each of a selected set of at least two predetermined input agents, and in the absence of any one of the at least two predetermined input agents, expression of the first repressor protein is repressed, resulting in expression of the effector protein.
 13. The programmable synthetic biological circuit of paragraph 12, wherein the second nucleic acid regulatory element comprises a DNA binding site for a second repressor protein.
 14. The programmable synthetic biological circuit of any one of paragraphs 12 and 13, wherein the first nucleic acid regulatory element comprises a DNA binding site for the first repressor protein.

15. The programmable synthetic biological circuit of any one of paragraphs 12-14, wherein the predetermined input agent comprises an environmental input agent.
16. The programmable synthetic biological circuit of paragraph 13, further comprising a set of at least two hybrid repressor protein expression modules, each of which comprises nucleic acid sequence encoding a hybrid repressor protein and operatively linked regulatory sequences that permit the expression thereof, each hybrid repressor protein comprising:
 - a. an input agent-responsive domain of an input agent-sensitive transcription factor; and
 - b. the DNA binding domain of the second repressor protein,
 - c. wherein the binding of each respective hybrid repressor protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is inhibited by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid repressor protein, and
 - d. wherein each respective hybrid repressor protein represses expression from the first repressor protein expression module in the absence of its respective input agent.
17. The programmable synthetic biological circuit of any one of paragraphs 12-16, wherein the binding and activity of the first repressor protein is inhibited by the binding of an input agent different from members of the selected set of at least two input agents, thereby establishing a NOT circuit, such that in the absence of the different input agent, the first repressor protein binds the first nucleic acid regulatory element and represses expression of the effector protein, but in the presence of the different input agent, the first repressor protein does not bind the first nucleic acid regulatory element and permits expression of the effector protein.
18. The programmable synthetic biological circuit of any one of paragraphs 12-17, wherein the first repressor protein is a hybrid repressor protein comprising an input agent-responsive domain of an input agent-sensitive transcription factor and the DNA binding domain of the second repressor protein, wherein the input agent responsive domain and the DNA binding domain are heterologous to each other.
19. The programmable synthetic biological circuit of any one of paragraphs 12-18, wherein the effector is a toxin or a reporter protein.
20. The programmable synthetic biological circuit of paragraph 19, wherein the toxin is selected from the group consisting of an endonuclease, a DNA gyrase, and a ribonuclease.
21. The programmable synthetic biological circuit of any one of paragraphs 12-20, wherein the first repressor protein is selected from a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CelR polypeptide, a TetR polypeptide, or a hybrid comprising the DNA binding domain of one such polypeptide fused to the input agent-responsive domain of another such polypeptide.

22. The programmable synthetic biological circuit of any one of paragraphs 13-21, wherein the second repressor protein is selected from a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CeiR polypeptide, a TetR polypeptide, or a hybrid comprising the DNA binding domain of one such polypeptide fused to the input agent-responsive domain of another such polypeptide.
23. The programmable synthetic biological circuit of any one of paragraphs 16-22, wherein the input agent-responsive domain of each hybrid repressor protein is selected from the environmental agent binding domains of LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CeiR polypeptide, and a TetR polypeptide.
24. The programmable synthetic biological circuit of any one of paragraphs 12-23, wherein the at least two input agents are selected from the group consisting of IPTG, galactose, cellobiose, ampicillin, chloramphenicol, kanamycin, and ATc.
25. The programmable synthetic biological circuit of any one of paragraphs 16-24, wherein the biological circuit is programmed by assembling a specific combination of effector expression module, first repressor protein expression module and a set of hybrid repressor protein expression modules sensitive to selected input agents.
26. The programmable synthetic biological circuit of any one of paragraphs 16-25, wherein the hybrid repressor expression modules and first repressor expression module are on the same nucleic acid construct.
27. The programmable synthetic biological circuit of any one of paragraphs 16-26, wherein the hybrid repressor expression modules, first repressor expression module and effector expression module are on the same nucleic acid construct.
28. A composition comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of paragraphs 12-27.
29. A host cell comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of paragraphs 12-27.
30. The host cell of paragraph 29, wherein the cell is a bacterial cell, fungal cell, plant cell, animal cell, or synthetic cell.
31. A passively activated biological containment circuit rendering a host cell dependent upon the presence of an exogenous input, the circuit comprising:
 - a. an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first modulator protein is present;

- b. a first modulator protein expression module comprising nucleic acid sequence encoding the first modulator protein, operably linked to a second nucleic acid regulatory element, the second nucleic acid regulatory element permitting expression of the first modulator protein unless a second modulator protein is present and binds the second nucleic acid regulatory element; and
 - c. a second modulator protein expression module comprising nucleic acid sequence encoding the second modulator protein, operably linked to a third nucleic acid regulatory element, the third nucleic acid regulatory element permitting expression of the second modulator protein, wherein the binding of the second modulator protein to the second nucleic acid regulatory element is modulated by an exogenous input,
 - d. wherein, in the presence of the exogenous input, the binding of the second modulator protein to the second nucleic acid regulatory element is inhibited, thereby permitting the expression of the first modulator protein such that expression of the effector protein is inhibited; and
 - e. wherein, in the absence of the exogenous input, the binding of the second modulator protein to the second nucleic acid regulatory element is permitted, thereby inhibiting, thereby inhibiting the expression of the first modulator protein such that expression of the effector protein is permitted.
32. The passively activated biological containment circuit of paragraph 31, wherein the effector protein comprises a toxin, a restriction endonuclease, or a protease.
 33. The passively activated biological containment circuit of paragraph 31, wherein the effector protein comprises a protease that degrades a cellular polypeptide essential for cell survival.
 34. The passively activated biological containment circuit of paragraph 31, wherein the first and second modulator proteins are different repressor proteins that bind different nucleic acid regulatory sequences.
 35. The passively activated biological containment circuit of paragraph 31, further comprising a third modulator protein expression module, comprising nucleic acid sequence encoding a third modulator protein, operatively linked to a fourth nucleic acid regulatory element, the fourth nucleic acid regulatory element permitting expression of the third modulator protein unless the first modulator protein is present.
 36. The passively activated biological containment circuit of paragraph 35 wherein the third modulator protein degrades the first modulator protein.
 37. The passively activated biological containment circuit of paragraph 36, wherein, in the absence of the exogenous input, the third modulator protein is expressed, resulting in degradation of the first modulator protein, resulting in the expression of the effector protein.

38. The passively activated biological containment circuit of paragraph 31, wherein the exogenous input comprise a small molecule that binds to the second modulator protein.
39. The passively activated biological containment circuit of paragraph 31, wherein the second modulator protein and the exogenous input are a pair selected from a tetracycline analog that binds TetR and TetR, and a lactose analog that binds LacI and LacI.
40. The passively activated biological containment circuit of paragraph 35, wherein the third modulator protein comprises a protease that degrades the first regulator protein and a cellular polypeptide essential for cell survival, and wherein the effector protein comprises a toxin.
41. One or more isolated nucleic acid comprising sequences encoding a passively activated biological containment circuit of paragraph 31.
42. A nucleic acid vector construct comprising sequences encoding a passively activated biological containment circuit of paragraph 31.
43. A host cell comprising nucleic acid comprising sequences encoding a passively activated biological containment circuit of paragraph 31.
44. The host cell of paragraph 43, wherein the cell is a bacterial, fungal, plant, animal, or synthetic cell.
45. A biological circuit rendering a cellular response sensitive to a predetermined condition, the circuit comprising a nucleic acid construct comprising expression modules that form a deadman regulatory circuit sensitive to the predetermined condition, the construct comprising:
 - a. a first repressor module M_{R1} comprising a first repressible promoter nucleic acid sequence (rP_1) operably linked to a repressor nucleic acid sequence encoding a first repressor protein (R_A), that binds repressible promoter element rP_E and represses expression from promoters comprising element rP_E ;
 - b. a second repressor module M_{R2} comprising a second promoter nucleic acid sequence (P_2) operably linked to a second repressor nucleic acid sequence (R_B) encoding a second repressor protein R_B , wherein transcription from rP_i is inhibited by the second repressor protein R_B and wherein repression activity of R_B is sensitive to inhibition by a first agent A_1 , the presence or absence of A_1 establishing a predetermined condition;
 - c. an effector module M_E comprising an effector protein coding sequence E operably linked to a third, repressible promoter comprising repressible promoter element rP_E , that is repressed by first repressor protein R_A ; the respective modules forming a deadman regulatory circuit such that:
 - d. in the absence of agent A_1 , the second repressor protein R_B is expressed and represses transcription from repressor module M_{R1} , such that expression of first repressor protein R_A is repressed, thereby relieving repression of effector module M_E by R_A , such that effector E is expressed by M_E ; and

- e. in the presence of agent A_1 , the activity of the second repressor protein R_B is inhibited, permitting expression of the first repressor protein R_A , which maintains expression from effector module E in the "off" state, such that agent A_1 is required by the circuit to maintain effector protein expression in the "off" state, and in the absence of A_1 , the circuit defaults to expression of the effector protein.
46. The circuit of paragraph 45 wherein the effector is a toxin or a protein that induces a cell death program.
 47. A method of rendering a cell responsive to a predetermined condition, the method comprising introducing nucleic acid encoding a biological circuit of paragraph 45 to the cell.
 48. Isolated nucleic acid encoding a biological circuit of paragraph 45.
 49. A host cell comprising nucleic acid of paragraph 48.
 50. A biological circuit rendering a cellular response sensitive to a predetermined condition, the circuit comprising a nucleic acid construct comprising expression modules that form a deadman regulatory circuit sensitive to the predetermined condition, the construct comprising:
 - a. a first repressor module M_{R1} comprising a first repressible promoter nucleic acid sequence (rP_1) operably linked to a repressor nucleic acid sequence encoding a first repressor protein (R_A), that binds repressible promoter element rP_E and represses expression from promoters comprising element rP_E ;
 - b. a second repressor module M_{R2} comprising a second promoter nucleic acid sequence (P_2) operably linked to a second repressor nucleic acid sequence (R_B) encoding a second repressor protein R_B , wherein transcription from rP_1 is inhibited by the second repressor protein R_B and wherein repression activity of R_B is sensitive to inhibition by a first agent A_1 , the presence or absence of A_1 establishing a predetermined condition;
 - c. an effector module M_E comprising an effector protein coding sequence E operably linked to a third, repressible promoter comprising repressible promoter element rP_E , that is repressed by first repressor protein R_A ;
 51. the respective modules forming a deadman regulatory circuit such that:
 - a. in the absence of agent A_1 , the second repressor protein R_B is expressed and represses transcription from repressor module M_{R1} , such that expression of first repressor protein R_A is repressed, thereby relieving repression of effector module M_E by R_A , such that effector E is expressed by M_E ; and
 - b. in the presence of agent A_1 , the activity of the second repressor protein R_B is inhibited, permitting expression of the first repressor protein R_A , which maintains expression from effector module E in the "off" state, such that agent A_1 is required by the circuit to maintain effector protein expression in the "off" state, and in the absence of A_1 , the circuit defaults to expression of the effector protein.

52. The circuit of paragraph 50, wherein the effector is a toxin or a protein that induces a cell death program.
53. A method of rendering a cell responsive to a predetermined condition, the method comprising introducing nucleic acid encoding a biological circuit of paragraph 51 to the cell.
54. An isolated nucleic acid encoding a biological circuit of paragraph 51.
55. A system to render cell growth restricted to the presence of a predetermined set of at least two selected agents, the system comprising a nucleic acid construct encoding expression modules comprising:
- a. a toxin expression module that encodes a toxin that is toxic to a host cell, wherein sequence encoding the toxin is operably linked to a promoter P1 that is repressed by the binding of a first hybrid repressor protein hRP1;
 - b. a first hybrid repressor protein expression module that encodes the first hybrid repressor protein hRP1, wherein expression of hRP1 is controlled by an AND gate formed by two hybrid transcription factors hTF1 and hTF2, the binding or activity of which is responsive to agents A1 and A2, respectively, such that that are both agents A1 and A2 required for expression of hRP1,
 - c. wherein in the absence of either A1 or A2, hRP1 expression is insufficient to repress toxin promoter module P1 and toxin production, such that the host cell is killed; and
 - d. wherein hybrid factors hTF1, hTF2 and hRP1 each comprise an environmental sensing module from one transcription factor and a DNA recognition module from a different transcription factor that renders the binding of the respective DRM sensitive to the presence of an environmental agent, A1, or A2, that is different from that which the respective DRM binds in nature.
56. A method of restricting cell growth to require the presence of a predetermined set of at least two selected agents, the system comprising introducing to a host cell nucleic acid encoding a system as described in paragraph 54.
57. An isolated nucleic acid encoding a system of paragraph 55.

[00203] This invention is further illustrated by the following examples which should not be construed as limiting. It is understood that the foregoing description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such

disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

EXAMPLES

EXAMPLE 1

[00204] As demonstrated herein, biocontainment systems that couple environmental sensing with circuit-based control of cell viability can be used to prevent escape of genetically modified microbes into the environment. Two exemplary, novel engineered safe-guard systems are described herein: the Deadman and Passcode kill switches. The Deadman kill switch uses unbalanced reciprocal transcriptional repression to couple a specific input signal with cell survival. The Passcode kill switch uses a similar two-layered transcription design and incorporates hybrid LacI/GalR family transcription factors to provide diverse and complex environmental inputs to control circuit function. These exemplary synthetic gene circuits efficiently kill *Escherichia coli* and can be readily reprogrammed to change their environmental inputs, regulatory architecture and killing mechanism.

[00205] With the advent of synthetic biology, genetically modified microorganisms have been increasingly used for biomedical, industrial and environmental applications¹⁻⁶. Deployment of these engineered microbes in large scales and open environments calls for the development of safe and secure means to restrain their proliferation. Pioneering biocontainment systems used metabolic auxotrophy in which target cells could only grow in the presence of an exogenously supplied metabolite^{7,8}, and the recent creation of an *E. coli* strain with an altered genetic code enabled production of synthetic auxotrophy strains in which an exogenous supply of non-natural amino acids is required for cell survival^{9,10}. Traditional metabolic auxotrophy strains are hampered by the potential for inadvertent complementation by crossfeeding or by the presence of the metabolite in heterogenous environments, and synthetic auxotrophy systems rely on extensive genome-wide engineering that can be impractical for many industrial production and biotherapeutic microbes. Furthermore, they are intrinsically difficult to reprogram for different environmental conditions, limiting their application.

[00206] As described herein, an alternative approach to biocontainment is to use gene circuits to maintain essential gene expression or block toxin gene expression under the assigned biocontainment conditions^{7,11-14}. Upon loss of the biocontainment signal, the circuit blocks essential gene expression or induces toxin gene expression to kill the cell. These circuits offer the promise of complex environmental signal integration but are typically hindered by a relative lack of programmable environment sensors to enable their use under non-laboratory conditions¹⁵.

[00207] Provided herein are programmable biocontainment circuits in *E. coli* – in some embodiments, a switch termed herein as a “Deadman kill switch” that uses, in part, a transcription-

based monostable toggle design to provide rapid and robust target cell killing is used, and, in some embodiments, a circuit termed herein a “Passcode circuit” that uses hybrid LacI/GalR family transcription factors (TFs) to construct complex environmental requirements for cell survival is provided. As described herein, a tripartite strategy of (i) TF protein engineering to detect diverse input signals, (ii) robust circuit design to provide signal processing, and (iii) redundant toxin-induced and protease-mediated cell killing mechanisms was used. The resulting biocontainment systems described herein are modular, flexible and extensible, and are useful across many industrial and biotherapeutic applications.

Deadman circuit development

[00208] We developed the Deadman kill switch to serve as a passively activated biocontainment system for engineered microbes. Similar to biocontainment systems in *E. coli*¹² and *Pseudomonas putida*¹⁶, the Deadman circuit uses a small molecule binding transcription factor to produce a ‘survival’ state in which repression of toxin production is linked to the presence of a specific environmental signal. Upon loss of the environmental signal, the circuit switches to the ‘death’ state in which de-repressed toxin production kills the cell. To increase the robustness of these biocontainment states, the Deadman circuit uses a genetic ‘toggle switch’ architecture in which reciprocal repression by the LacI and TetR transcription factors form transcription states that are maintained by the circuit’s linked feedback loops^{17,18} (FIG. 6). To create a circuit in which the ‘death’ state is dominant in the absence of the survival signal, we altered the ribosome binding site (RBS) strengths of LacI and TetR to favor TetR expression in a single-copy plasmid (FIGS. 7A-7B and Methods). In the resulting monostable circuit, the presence of the TetR inhibitor anhydrotetracycline (ATc) is required to maintain the circuit in the subordinate LacI+ ‘survival’ state (FIGS. 8A-8C). Incorporation of toxin genes into the TetR+ state creates a kill switch where the presence of ATc is required to block toxin expression and cell death.

[00209] We included additional palindromic LacI operator sites in the toxin gene promoter to minimize leaky toxin expression¹⁹ and introduced a transcriptional terminator upstream of the promoter to insulate the gene from spurious transcription (FIGS. 9A-9C). To accelerate the circuit’s switching dynamics, we fused a degradation tag to the C-terminus of LacI that is specifically recognized by *mf*-Lon²⁰, a heterologous protease under control of a LacI-dependent promoter (FIGS. 10A-10C). Upon removal of ATc, TetR repression of *lacI* allows expression of *mf*-Lon, which targets LacI for degradation to create a positive feedback loop that accelerates the switch to the TetR+ state (FIG. 10B). Importantly, single-cell analysis of these circuits by flow cytometry showed a monomodal distribution of cells in the LacI+ and TetR+ state, demonstrating stable circuit expression across the cell population (see 0 and 6 hour data in FIG. 10C).

Deadman kill switch characterization

[00210] To identify an efficient mechanism to kill the host cells upon circuit activation, we tested several toxin genes that directly damage the host cell’s DNA or RNA. We chose to test the

endonuclease *ecoRI*²¹, the DNA gyrase inhibitor *ccdB*²² and the ribonuclease-type toxin *mazF*²³ because they are well-characterized, are native to *E. coli*, and provide a range of killing mechanisms. The toxin genes were independently incorporated into the Deadman circuit, and a range of RBS strengths were tested for each toxin to optimize cell death upon circuit activation²⁴ (FIGS. 11A-11B). Upon removal of ATc, the toxins produced 3–5 logs of killing within 6 hours as measured by colony forming units (CFUs) (FIG. 1A). To increase the robustness of the circuit and provide an independent method of circuit-dependent cell death, we used *mf*-Lon protease to not only degrade LacI but also target essential proteins for degradation (FIG. 1B). We attached the *mf*-Lon degradation tag pdt#1 to the 3' end of five essential genes whose protein products are particularly sensitive to *mf*-Lon degradation²⁰, and we then measured cell viability following removal of ATc (FIG. 1B). Among the tested essential gene targets, the peptidoglycan biosynthesis gene *murC* provided the strongest and fastest cell death phenotype (survival ratio $< 1 \times 10^{-4}$ within 6 hours).

[00211] To determine if the toxin- and *mf*-Lon-mediated killing mechanisms produce synergistic effects, we created Deadman circuits containing each of the toxins in combination with the *mf*-Lon-MurC targeting module (FIG. 1C). In each instance, the combinatorial approach provided more effective biocontainment, and in particular, coordinated EcoRI expression and *mf*-Lon-mediated MurC degradation resulted in cell killing below the limit of detection (survival ratio $< 1 \times 10^{-7}$) 6 hours after removal of ATc (FIG. 1C). Furthermore, the Deadman circuit's design provides an additional fail-safe mechanism which bypasses the circuit's sensor system to directly activate toxin expression to cause cell death. Direct derepression of the subordinate TF, in this case derepression of LacI with isopropyl β -D-1-thiogalactopyranoside (IPTG), activates toxin production and cell death irrespective of the presence of the programmed survival signal (FIG. 2).

Hybrid transcription factor design

[00212] To extend the versatility and modularity of this system, we built a second circuit, called the Passcode circuit, which uses hybrid LacI/GalR family TFs to expand the range and complexity of environmental signals used to define biocontainment conditions. This survival "passcode" can be easily reprogrammed to restrict cell growth to a new environment or to limit knowledge of the growth conditions to authorized personnel. To build hybrid LacI family TFs, we first identified the boundaries of the environmental sensing modules (ESMs) and DNA recognition modules (DRMs) found in LacI family members (FIG. 3A and FIGS. 12-15). We generated hybrid TFs that use the small molecule input defined by the hybrid's ESM to regulate the promoter defined by the hybrid's DRM^{25,26} (FIG. 3A and FIGS. 16A-16C).

[00213] To construct the hybrid TFs, we used the cellobiose-responsive TF CelR from *Thermobifida fusca* and the galactose-responsive TF GalR and IPTG-responsive LacI from *E. coli*. We fused the ESMs from CelR and GalR to the DRM of LacI to generate the hybrid TFs CelR-LacI and GalR-LacI. To test their functionality, these hybrid TFs or native LacI were used to control GFP expression from a promoter containing *lacO* operator sites recognized by the LacI DRM. The hybrid

TFs allowed strong GFP expression upon exposure to the small molecule input defined by their ESM and showed almost no response to the other inputs (FIG. 3A and FIG. 16B). We fused the LacI, GalR and CelR ESMs to the DRM of ScrR from *Klebsiella pneumoniae* and used the resulting hybrid TFs to regulate a promoter containing *scrO* operator sites. As predicted from their design, these hybrid TFs only respond to the input defined by their ESM (FIG. 3B and FIG. 16C), although it is interesting to note that the GalR ESM shows distinct inhibition by high levels of IPTG as seen by Shis *et al.*²⁷ (FIG. 17). Importantly, the DRMs used in these hybrid TFs provided similar specificity, as they regulated promoters containing their cognate operator sites but not other LacI family operator sites (FIG. 18). Similar to work by Shis *et al.*²⁷, we found that co-expression of hybrid TFs containing the same DRM could be used to regulate a single promoter, creating an AND logic gate function (FIG. 19).

Development of the Passcode kill switches

[00214] We used these hybrid TFs to create a series of Passcode circuits that contain a single transcriptional architecture but respond to distinct combinations of environmental inputs to control gene expression and cell survival. As shown in FIGS. 20A-20C, the Passcode circuits contain the output module (in this case, *gfp*) under control of a TF (hybrid C) whose expression is controlled by an AND gate formed by two TFs (hybrid A and hybrid B). This serial arrangement, made possible by the orthogonality of the hybrid DRMs and ESMs, creates the condition that both of the inducers recognized by hybrid A and hybrid B (inputs a and b, respectively) must be present to allow expression of hybrid C to repress *gfp* expression. Loss of input a or input b or the presence of input c allows *gfp* expression, causing cell death if *gfp* is replaced by a toxin gene.

[00215] To test the functionality and modularity of this circuit architecture, we created three exemplary embodiments of the Passcode circuit that respond to different combinations of input signals to control output expression (FIG. 4A). For example, in one Passcode circuit (FIG. 4B, left column), we used GalR-LacI (A) and CelR-LacI (B) to control expression of LacI-ScrR (C), which in turn represses toxin expression. In this circuit, loss of galactose (input a) or cellobiose (input b) allows GalR-LacI or CelR-LacI to bind the *lacO* operator, blocking LacI-ScrR expression, thereby enabling toxin expression and causing cell death. Any exposure to IPTG (input c) releases LacI-ScrR repression of toxin expression, thereby killing the cell as well. Importantly, the passcode combinations for cell survival and cell death can be reprogrammed by rearranging the ESMs of the three TFs to rewire the connections between the environmental sensing and transcriptional regulation, in different embodiments.

[00216] These Passcode circuits were first evaluated with GFP as the output module in all eight combinations of the three environmental inputs. All three circuits allowed high level GFP expression in all conditions except that designated by the desired three input combination (FIG. 20B), and single-cell fluorescence showed a monomodal population distribution under all conditions (FIG. 20C). GFP was then replaced with the *ecoRI* and *mf*-Lon-MurC toxin modules described for the

Deadman switch above (FIG. 4A), and toxin expression levels were optimized by testing a range of calculated RBS strengths²⁴ (FIG. 21). Hybrid C, which directly controls toxin expression in the circuit, was also engineered in the same manner to optimize circuit performance (see Methods). Each kill switch circuit was tested in *E. coli* using eight combinations of input signals, and cell survival was measured by CFU count at multiple time points (FIG. 22). As seen in FIG. 4B, only circuits that received the proper survival code allowed the host cells to survive (each survival condition is highlighted in green). Furthermore, inclusion of both the *ecoRI* and *mf*-Lon toxin modules in the Passcode circuit caused the cell survival ratio to drop below 1×10^{-6} for all non-passcode conditions.

Circuit stability

[00217] To measure the long-term stability and robustness of the Passcode and Deadman kill switches, we passaged cells containing the circuits for four days under survival conditions and periodically tested subsets of cells for circuit function under non-permissive conditions. Both the Deadman and Passcode circuits showed reduced killing efficiency over time, and sequence analysis of cells that escaped biocontainment predominantly showed inactivating mutations in the toxin genes (FIGS. 5A-5C and FIG. 23). The noted exception was independent TetR mutations in the two-toxin Deadman circuit where TetR inactivation repressed toxin expression even in the absence of the ATc survival signal. It is important to note, however, that these ‘escapees’ are still sensitive to IPTG-mediated fail-safe circuit activation as described above (FIG. 2). Genome-encoded insertion-sequence (IS) elements³⁷, particularly IS1 and IS5, caused a large percentage of inactivating mutations in the one-toxin and two-toxin Passcode circuits. Deletion of these IS elements and other genome repair mechanisms in *E. coli* reduced the Passcode ‘escapee’ rate by 3-5 logs after four days, demonstrating that increased stability of the host genome will augment the functionality of these biocontainment systems (FIG. 5B and FIG. 23). As the toxin genes were the main target for circuit inactivation, inclusion of additional redundant killing systems into each circuit should further reduce the escapee rate.

[00218] The Deadman and Passcode switches provide robust information processing circuits to couple environmental signals with conditional survival of the microbial host. The Deadman kill switch described above is based on a monostable circuit that passively activates toxin gene expression in the absence of the small molecule input ATc. Since ATc is not normally found in nature, engineered cells that escape biocontainment will trigger cell death to prevent the spread of the organism or its genetic content into the surrounding ecosystem. Unlike auxotrophy-based biocontainment where the environmental signal is an intrinsic feature of the system^{9,10}, the environmental sensing and cell killing systems are decoupled in the Deadman switch. This circuit relies on two main elements for functionality: (1) the orthogonality of the TFs to create a toggle switch, and (2) their relative activity under induced expression. As such, the Deadman circuit is highly modular, and the environmental signal detected by the circuit may be altered by replacing TetR with a wide range of transcription factors, including more than 80,000 annotated TetR family

members³⁸ as well as orthogonal LacI/GalR family members including hybrid TFs as described for the Passcode switch. In addition, the Deadman circuit has an additional fail-safe mechanism which activates toxin production and cell death in the presence of IPTG, enabling exogenous control over the microbe's survival even as the cell uses the circuit to monitor its environment.

[00219] Similar to the Deadman switch, the Passcode circuits are based on a two-layered transcriptional repression design. To build hybrid TFs, we identified the conserved boundaries of the ESMs and DRMs within the LacI/GalR family members LacI, GalR, CelR and ScrR. The resulting environmental sensing and DNA binding modules provide independent control of the sensory input and regulatory output of each hybrid TF. Pioneering work by Meinhardt et al.^{27,28} used the boundary between the conserved regulatory domain and HH motif to create hybrid TFs, but some of these hybrids required additional protein engineering and mutagenesis to become functional. Here we identify a discrete boundary between the conserved HH and HTH motifs to create independent environmental sensory and DNA binding domains that can be efficiently combined without further protein engineering. The modularity provided by these hybrid TFs dramatically expands the number and range of environmental signals that can be used to control biocontainment systems such as the Deadman and Passcode circuits described here, as the ESM and DRM boundaries defined in this study may be used to incorporate sensing modules from many of the ~29,000 LacI/GalR family members³⁹ that detect diverse environmental signals.

[00220] These hybrid TFs may also be used to functionalize other synthetic circuits, including the Deadman switch, to respond to different environmental signals. Moreover, the regular use of LacI and TetR in other bacteria^{40,41} suggests that these circuits may be readily transferred to other microbes, including industrial production strains. Replacement of the antibiotic resistance cassettes in these plasmids with well characterized selection systems that use toxin-antitoxin modules or auxotrophy complementation should also enable their use in biotherapeutic applications^{4,42}.

[00221] In summary, we have established two exemplary circuit-based microbial kill switches that constrict host cell survival to an environment defined by specific input signals. Unlike existing biocontainment systems with fixed survival conditions that are difficult to modify, the Deadman and Passcode kill switches are inherently customizable, both in the environmental conditions that control circuit activation and in the output modules that control cell fate. In addition to its use as a biocontainment system, the Passcode circuit may find particular utility as a tool for intellectual property protection, where unauthorized growth of strains without the appropriate "passcode" molecules would induce cell death. With the proper choice of toxins, such as the endonuclease EcoRI described here, the Passcode circuit could be used to not only kill the host cell but also degrade its genome and accompanying plasmids to deter attempts at reverse-engineering the strain of interest. Use of hybrid TFs that respond to proprietary small molecule inputs may further secure the strain against theft, even if its genome is sequenced.

EXAMPLE 1 METHODS

[00222] Strains. *E. coli* MG1655 Δ *lacI* was the parental strain for all circuit characterization and was created through P1 phage transduction of *lacI::kanR* from the Keio collection⁴³ into *E. coli* MG1655 (ATCC 47076). Flp recombinase, expressed on pCP20, was used to remove the *kanR* cassette⁴⁴. To construct *E. coli* strains containing *mf*-Lon recognition tags on the essential genes *dxs*, *cysS*, *fldA*, *plsB* or *murC*, the pdt#1 *mf*-Lon recognition tag from each corresponding gene in the EPD library²⁰ was transferred to MG1655 Δ *lacI* by P1 phage transduction and the *kanR* cassette was removed as above. P1 phage transduction was used to convert *E. coli* MDS42pdu¹¹ (Scarab Genomics) for use in the Passcode switch analysis. Specifically, *lacI::kanR* and *recA::kanR* deletions from the Keio collection¹⁶ and *murC-pdt#1* from the EPD library¹⁷ were independently transferred to MDS42pdu by P1 phage transduction, and the accompanying kanamycin cassettes were removed by FlpE-mediated excision using pECA102.

[00223] Cell growth and media. Luria-Bertani (LB) media was used for all experiments, and the following antibiotics and inducers were included when appropriate: ampicillin (50 μ g/ml), chloramphenicol (10 μ g/ml), kanamycin (50 μ g/ml), ATc (100 ng/ml), IPTG (1 mM), galactose (20 mM) and cellobiose (5 mM). For the Deadman switch, single colonies grown on LB agar plates containing ATc were inoculated into liquid cultures containing ATc for growth overnight at 37°C with shaking. Similarly, cells harboring each of the three Passcode switches were picked from plates with the survival combination of inputs and inoculated into their respective survival liquid media. Overnight cultures were inoculated 1:20,000 into 96-well plates and grown at 37°C and 900 rpm for further tests.

[00224] Plasmid construction. All plasmids were constructed using conventional molecular cloning protocols⁴⁵ and Gibson Assembly⁴⁶. *E. coli* NEB Turbo (New England BioLabs Inc.) was used for cloning purposes, and all primers were purchased from IDT. To create the Deadman switch pDM1 (Genbank accession number KT893253), genetic elements from the toggle pECJ3²⁰ were cloned into the conditionally amplified single-copy plasmid pBAC/oriV⁴⁷, and the *lacI* and *tetR* RBS strengths were modified as described in FIGS. 7A-7B. To provide increased control over the promoter controlling mCherry expression, the T1 terminator from *rnpB* (Registry of Standard Biological Parts BBa_J61048) was inserted upstream (FIG. 9A), and three palindromic *lac* operator sites¹⁹ were inserted around the -35 and -10 region of the promoter (pDM2, GenBank accession number KT893254). Finally, the *M. florum* protease gene *mf-lon* was cloned under control of this LacI-regulated promoter (pDM2L; GenBank accession number KT893255). The resulting plasmid served as the base Deadman circuit, and *mCherry* was cloned to yield pDM3 and *ecoRI*, *ccdB* and *mazF* to make the toxin variants (see Table 1).

[00225] Hybrid TF genes (*lacI-galR* LG36-LG46, *galR-lacI*, *celR-lacI*, *lacI-scrR*, *galR-scrR*, and *celR-scrR*) were constructed by overlap extension PCR to fuse the environmental sensing modules (ESMs) and the DNA recognition modules (DRMs) of the designated genes. The hybrid TFs were cloned into pTR, a derivative of pKE2-MCS containing the *pLtetO-1* promoter and T0

terminator from pZA11³⁴, using restriction sites BamHI and BsrGI. Transcription from the *pLtetO-1* promoter driving TF expression is constitutive because the *E. coli* strains used in this study did not contain *tetR*. Reporter plasmids (pREPORT) were constructed from the plasmid pZA12³⁴, with *mcherry* or *gfp* inserted downstream of the *pLlacO* promoter using KpnI and HindIII. To test hybrid TFs that contain the ScrR DRM, *pLlacO-1* was replaced with *pLscrO-1* or *pLscrO-2* using the Gibson Assembly method⁴⁶. For implementation of both LacI/*pLlacO-1* and GalR-ScrR/*pLscrO* inducible expression systems in the same cells (FIG. 18), the *pLlacO-1-mCherry-T1* cassette was subcloned into pTR using NheI and SalI.

[00226] The Passcode circuit was developed using a two-plasmid system. Plasmid pPasscode (GenBank accession number KT895272, KT895273 and KT895274), derived from pKE2_MCS¹⁷, was constructed to contain the hybrid TF circuit, and pToxin (GenBank accession number KT895275, KT895276 and KT895277), derived from pZA12³⁴, was constructed to contain the toxin output module under control of the *pLscrO* promoter. For pPasscode, three promoter-hybrid TF-terminator fragments were used to construct each hybrid TF circuit version, as listed in Table 1. For version 1 of pPasscode (pPasscode1), in which LacI-ScrR is used as hybrid C, the promoter *pLscrO-2* was utilized to control the expression of toxin gene(s) in pToxin. For pPasscode2, which contains GalR-ScrR, the promoter *pLscrO-1* was used for toxin control in pToxin. For pPasscode3, which contains CelR-ScrR, the promoter *pLscrO-2* was used to control the expression of *ecoRI*. For Passcode circuits that contain two toxin gene systems, the DNA fragments *pLscrO-mf-Lon-terminator* and *pLscrO-ecoRI-terminator* were incorporated into pToxin using Gibson Assembly (Table 1). For Passcode circuit characterization, pPasscode was first transformed into the desired *E. coli* strain and grown in media containing the “passcode” combination of the three inputs (IPTG, galactose and cellobiose). Plasmid pToxin, which contains the toxin gene(s), was then transformed into the cells to complete the Passcode circuit.

[00227] **Flow cytometry assay.** Cells containing Passcode circuits were grown as described for each experiment, and at the appropriate time were fixed in 2% paraformaldehyde in PBS and then diluted 1:10 in PBS for analysis. GFP fluorescence measurements were performed using a BD FACSAriaII (BD BIOSCIENCES) or a BD LSRFortessa™ flow cytometer (BD BIOSCIENCES). Flow cytometry data were gated by forward and side scatter to eliminate multi-cell aggregates, and the geometric mean of GFP fluorescence distributions were calculated using FLOWJO software (TREESTAR). At least 10,000 events were collected for each measurement.

[00228] **Survival assays.** Colony forming unit (CFU) cell viability assays were used to measure functionality of the Deadman and Passcode circuits. Overnight cultures were grown under the survival conditions (Deadman: with ATc, Passcode: with survival “passcode” inputs) and were transferred into fresh LB medium with or without the survival signal(s). For the Passcode circuit, all eight combinations of the three inputs were tested (+/- IPTG, +/- galactose and +/- cellobiose). Samples were collected every two hours, serially diluted in PBS over a 7-log range, and spotted (5

μL) onto a square plate containing LB agar with the appropriate survival signal(s). CFU and survival ratios were calculated as previously reported¹¹: CFU/mL = (number of colonies) x (dilution factor)/0.005 mL, survival ratio (log₁₀) = log {(CFU/mL without the survival signal)/(CFU/mL with the survival signal)}.

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EXAMPLE 2

Methods

[00229] Analysis of protein sequences and crystal structures. ClustalW2¹² was used for protein sequence alignment of GalS, GalR, AscG, RbsR, PurR, GntR, LacI, and Mall from *E. coli*; CelR from *T. fusca*; ScrR from *V. alginolyticus* (ScrR-V); and ScrR from *K. pneumonia* (ScrR-K). Protein crystal structure analysis was performed with PyMol 1.5.x using Protein Data Bank (PDB) entries 1EFA, 1LBG, 1LBI, 1LBH, 1QPZ, and 1TLF^{5-7,13,14}.

[00230] Strain construction. *E. coli* MG1655*AlacI* and *E. coli* MG1655Pro^{10,15} were used for Deadman and Passcode switch construction. *E. coli* MG1655*AlacI* was used to perform functional analysis of hybrid TFs as shown in FIGS. 15-17. In this strain, transcription from the *pLtetO-1* promoter driving TF expression is constitutive because it does not contain *tetR*. *E. coli* MG1655Pro, which produces high levels of LacI and TetR¹⁵, was used in hybrid TF analysis when LacI regulation of *pLlacO-1* was a desired feature (FIGS. 18-19). In these assays, the TetR inhibitor anhydrotetracycline (ATc; 100 ng/mL) was included in the media to ensure TF expression from the *pLtetO* promoter. P1 phage transduction was used to convert *E. coli* MDS42pdu¹¹ (Scarab Genomics) for use in the Passcode switch analysis. Specifically, *lacI::kanR* and *recA::kanR* deletions from the Keio collection¹⁶ and *murC-pdt#1* from the EPD library¹⁷ were independently transferred to MDS42pdu by P1 phage transduction, and the accompanying kanamycin cassettes were removed by FlpE-mediated excision using pECA102.

[00231] Deadman monostable toggle construction. To construct the monostable toggle, an RBS calculator algorithm¹⁸ was used to identify RBS variants that produce a range of LacI and TetR expressions (Table 1). Cells containing each toggle RBS variant were grown overnight in the presence of ATc, transferred to media without ATc, and then measured for mCherry expression by flow cytometry after 6 hours. Toggle variant 5, which showed the largest change in mCherry fluorescence

upon loss of ATc, was chosen for use in the Deadman circuit (FIGS. 7A-7B). To quantify the relative LacI and TetR expression levels, mCherry was fused to the C-terminus of LacI or TetR to yield pBAC-LC and pBAC-TC, respectively (GenBank accession numbers KT893256 and KT893257). RBS variants for LacI and TetR were then cloned into pBAC-LC and pBAC-TC, respectively, and a SpectraMax M5 microplate reader (Molecular Devices) was used to measure mCherry fluorescence with excitation and emission wavelengths of 587 nm and 610 nm, respectively, with an emission filter cutoff at 610 nm. mCherry fluorescence was normalized to cell growth (OD_{600}).

[00232] RBS strength optimization for toxin expression. To optimize cell death dynamics upon Deadman or Passcode circuit activation, a range of predicted RBS strength variants¹⁸ was generated for each toxin (Table 1). For the Deadman kill switches (FIGS. 11A-11B), RBS variants and the corresponding toxin genes *ecoRI*, *ccdB*, and *mazF*, were cloned into pDM2L using Gibson Assembly (Table 1). Overnight cultures were grown in the presence of ATc and then transferred into media with ATc (survival condition) or with IPTG (induced death condition). A SpectraMax M5 microplate reader (Molecular Devices) was used to measure cell growth (OD_{600}) every 15 min for 15 hours, and the cell growth ratios of the induced death state to the survival state were calculated at 15 hours.

[00233] For Passcode kill switches, RBS variants (Table 1) and the corresponding toxin genes *ecoRI* and *mf-lon* were cloned into pREPORT to replace *gfp* and tested for optimal expression under regulation by the hybrid TFs LacI-ScrR, GalR-ScrR and CelR-ScrR. Plasmids containing each RBS-toxin variant were transformed into cells constitutively expressing LacI-ScrR, GalR-ScrR, or CelR-ScrR, grown overnight without inducers, and then transferred into media with or without the appropriate inducer (1 mM IPTG, 20 mM galactose, or 5 mM cellobiose for cells containing LacI-ScrR, GalR-ScrR, or CelR-ScrR, respectively). Cell growth analysis was performed as described for the Deadman circuit above, and the cell growth ratio was calculated at 12 hours. Representative data are shown in FIG. 21.

[00234] RBS strength optimization for ScrR ESM-containing TFs. A range of RBS variants was tested to optimize the expression of ScrR ESM-containing TFs (see TF 'C' in FIG. 4A) in the Passcode circuits (Table 1). Cells with the Passcode circuit harboring RBS variants were transformed with the indicated pToxin plasmid, grown overnight under survival conditions (see FIGS. 20A-20C for the appropriate inducers for each circuit), and then transferred to media with all 8 combinations of the three inducers (IPTG, galactose, and cellobiose). Performance of each circuit was determined by CFU count after 8 hours of exposure, as described herein.

[00235] Long-term growth analysis. Cells containing the Deadman and Passcode kill switches were passaged under survival conditions for 4 days (Deadman: 100 ng/mL ATc; Passcode: unique inducer for each Passcode circuit; see FIGS. 20A-20C). Sub-populations of these cells were transferred 1:20,000 into media with or without the survival signal(s) (Deadman: no ATc; Passcode: no inducers), and samples were collected at 8 hours after inoculation, serially diluted 1:10 in PBS

over a 7-log range, and spotted (5 μ L) onto LB agar plates with the appropriate survival signal(s). CFU and survival ratios were calculated as previously reported¹⁵: $\text{CFU/mL} = (\text{number of colonies}) \times (\text{dilution factor})/0.005 \text{ mL}$; $\text{survival ratio} (\log_{10}) = \log \{(\text{CFU/mL without the survival signal(s)})/(\text{CFU/mL with the survival signal(s)})\}$.

[00236] Escapee genetic analysis. Cells containing independent Deadman and Passcode circuit transformants ($n=20$ for each circuit) were grown under survival conditions (Deadman: 100 ng/mL ATc; Passcode: unique inducer for each Passcode circuit; see FIGS. 20A-20C). The cells were then transferred to media without the survival signal(s) for 8 hours and then placed on LB agar plates containing the appropriate survival signal(s). Deadman circuits were isolated from surviving cells by amplification with Phusion high-fidelity DNA polymerase (NEB), and Passcode circuits were isolated by plasmid DNA purification, and the circuits were then sequenced by QUINTARA BIOSCIENCES (Boston, MA).

[00237] Flow cytometry. Cells containing Deadman and Passcode circuits were grown as described for each experiment, and at the appropriate time they were fixed in 2% paraformaldehyde in PBS and then diluted 1:10 in PBS for analysis. GFP and mCherry fluorescence measurements were performed using a BD FACSARIAII (FIGS. 7A-9B, 15, 17, 18, 24A-25; BD BIOSCIENCES) or a BD LSRFORTESSATM flow cytometer (FIGS. 16A-16C, 19, and 20; BD BIOSCIENCES). Flow cytometry data were gated by forward and side scatter to eliminate multi-cell aggregates, and the geometric mean of GFP and mCherry fluorescence distributions were calculated using FLOWJO software (TREESTAR). At least 10,000 events were collected for each measurement.

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Table 1. DNA sequences of genes, promoters, and ribosomal binding site.

DNA fragments used to construct the Deadman kill switch		
Name	Sequence (5' to 3') (Cloned into pDM2L between 9396 to 9397)	Source
<i>rnpB</i> T1 terminator + <u>pTrc-2 derivative promoter</u> +RBS (bold) + <i>mCherry</i> +3' UTR	ccggcttatcggtcagttcacctgatttacgtaaaaaccgcttcggcgggttttgcctttggaggggcagaaagatga atgactgtccacgacgctatacccaaaagaaagagctcggactggactcgaattgtgagcgctcacaattactagcgg ccgcatggaattgtgagcgctcacaattttgacaattaatcatccggctcgtataatgtgtgggaattgtgagcgctcaca atttcacaccaattgataatatacaagattaaggaggtaaagaat ggtgagcaagggcgaggaggataacatgg ccatcatcaaggagttcatgcgcttcaagggtcacatggagggtccgtgaacggccacgagttcagatcgagggc gagggcgaggggcccccctacgagggcaccagaccgccaagctgaaggtgaccaaggtggccccctgccctt cgcttgggacatcctgtccccctcagttcatgtacggctccaaggcctacgtgaagcacccccgccgacatccccgacta ctggaagctgtccttccccgagggtcgaagtgggagcgcgtgatgaacttcgaggacggcgcggtggtagaccgtga cccaggactcctccctgcaagacggcgagttcatctacaaggtgaagctcgcgccaccaacttccccctccgacggc cccgtaatgcagaagaagactatgggctgggagcctcctccgagcggtatgacccgaggacggcgcgctgaag ggcgagatcaagcagaggtgaagctgaaggacggcgccactacgacgtgaggtcaagaccactacaaggc caagaagcccggtcaactgcccggcgtacaacgtcaacatcaagttggacatcacctccacaacgaggactac accatcgtggaacagtacgaacgcggaggcgccactccaccggcgcatggacgagctgtataagtaagat atctatgccctagggaccgt	<i>rnpB</i> T1 terminator, BBa_J61048; pTrc-2 derivative and RBS, this study; <i>mCherry</i> , pECJ3 ¹⁷
<i>rnpB</i> T1 terminator + <u>pTrc-2 derivative promoter</u> +RBS (bold) + <i>ecoRI</i> +3' UTR	ccggcttatcggtcagttcacctgatttacgtaaaaaccgcttcggcgggttttgcctttggaggggcagaaagatga atgactgtccacgacgctatacccaaaagaaagagctcggactggactcgaattgtgagcgctcacaattactagcgg ccgcatggaattgtgagcgctcacaattttgacaattaatcatccggctcgtataatgtgtgggaattgtgagcgctcaca atttcacaccaattgtaacagtggaaacatggattc atgtctaataaaaaacagtc aaataggctaactgaacaacat aagttatctcaagggtgaattgggattttggggattatgcaaaagctcatgctcgtgttggtgaggtttcaaaattagt aaagaaagctcttagcaacgaataccctcaattatcatttcgatatagatagatataaagaaaacagaataaatgaag ctttaaaaaaattgacctgatcttggcggtactttatttgcataaattccagcatcaaacctgatggtggaattgtagag gtcaaaagatgattatggtgaatggagagttgtacttgttgcgaagccaaacaccaaggtaagataattataaatataag gaatggttggtagttgggaaaagaggagatcaagatttaattggtcgtgtaagctatcgaagatctcataagaatat atcagagatagcgaattttatgctctctgagagccacttccctacgtccttttcttagaggggtctaacttttaacagaaa atatctcaataacaagaccagatggaagggttgtaattcttgatataattctggtatattaaataggttagatcgactaact gcagctaattatggaatgctataaatagtaattctgtatatacaaaattgtaaatcataaagacaaaagcattatgctac aagcagcatctatatactcaaggagatgggaggagtggtgattcgaaaatcatgtttgaataatgtttgatataatcaa cgacttcgctcagagtggtggggcgtgacttggtgaacagcttacatctaagtactcaggggtcttgataat	<i>ecoRI</i> , pSCC2 (Addgene plasmid # 39993)

<i>rnpB</i> T1 terminator + <i>pTrc-2</i> derivative promoter +RBS (bold) + <i>ccdB</i> +3' UTR	ccggcttatcggtcagttcacctgatttacgtaaaaccgcttcggcggttttgcctttggaggggcagaaagatgatgactgtccacgacgctatacccaaaagaaagagctcggactggactcgaattgtgagcgctcacaattactagcggccgcatggaattgtgagcgctcacaattttgacaattaatcatccggctcgtataatgtgtgggaattgtgagcgctcacaatttcacaccaattgattaaagcccataaacgtaccatgcagtttaagggttacacctataaaagagagagccgttatcgtctgttggatgtacagagtgtatattgacacgcccggcgacggatggtgatccccctggccagtgcacgtctgctgtcagataaagtctcccgtgaactttaccgggtgtgcatatcggggatgaaagctggcgcagatgaccaccgatggccagtgtccggtctccgttatcggggaagaagtggctgatctcagccaccgcgaaatgacatcaaaaacgccattaacctgatgttctggggaatataactcagggtcttgataat	<i>ccdB</i> , RR12(14)Cc dB ¹⁵
<i>rnpB</i> T1 terminator + <i>pTrc-2</i> derivative promoter +RBS (bold) + <i>mazF</i> +3' UTR	ccggcttatcggtcagttcacctgatttacgtaaaaccgcttcggcggttttgcctttggaggggcagaaagatgatgactgtccacgacgctatacccaaaagaaagagctcggactggactcgaattgtgagcgctcacaattactagcggccgcatggaattgtgagcgctcacaattttgacaattaatcatccggctcgtataatgtgtgggaattgtgagcgctcacaatttcacaccaattgtaactgggaagataaacggagactggtaattgtaagccgatacgtacccgatatggcgatctgatttgggtgattttgaccgacaaaagtagcgagcaagctggacatcgtccagctgttgcctgagtcctttcatgtacaacaacaaacaggtatgtgtctgtgttcttgtacaacgcaatcaaaaggatattccgtcgaagtgttttatccggtcaggaacgtgatggcgtagcgttagctgatcaggtataaaagtatcgctggcgggcaaggaggaacgaagaaggacagtgccccagagggaattacaactcataaagccaaaattaacgtactgattgggtaaf	<i>mazF</i> , <i>Escherichia coli</i> K-12 MG1655

RBS used to design monostable toggle

L1	(NcoI)aacggcgccgcttcctatcagtgatagagattgacatccctatcagtgatagatatactgagcacatcagcaggacgcactgaccggaggatagaaaatcaagaattaaggaggtacaatATG	This study
L2	(NcoI)aactctagtgtttccctatcagtgatagagattgacatccctatcagtgatagatatactgagcacatcagcaggacgcactgaccgctgataagccgaggatagaaaatcaagaattaaggaggtacaatATG	This study
L3	(NcoI)aacggcgccgcttcctatcagtgatagagattgacatccctatcagtgatagatatactgagcacatcagcaggacgcactgaccgctcagatgacgaggatagaaaatcaagaattaaggaggtacaatATG	This study
T1	(NcoI)ctgaaatgagctgttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataacaatttcacacgtcgaccgcttcggcgaagctaggagcagagagctagcATG	This study
T2	(NcoI)ctgaaatgagctgttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataacaatttcacacgtcgacgcaccggaggcggaagtaaggaggttagatATG	This study
T3	(NcoI)ctgaaatgagctgttgacaattaatcatccggctcgtataatgtgtggaattgtgagcggataacaatttcacacgtcgacgcaccataaagcggaagtaaggaggttagatATG	This study

RBS tested for EcoRI expression

Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>ecoRI</i> -50	(MfeI)ctaggagctacagataacctttctattagaaggATG	This study
<i>ecoRI</i> -200	(MfeI)acgcaattcgtaagaacagatatattccagtaatATG	This study
<i>ecoRI</i> -1500	(MfeI)taacagtggaaacatggattcATG	This study
<i>ecoRI</i> -5000	(MfeI)tatcgcatctaaagcggaattaacgataaaagcATG	This study
<i>ecoRI</i> -15000	(MfeI)ataaagagaattaagatcaataaaggatatctcttATG	This study

RBS tested for CcdB expression

Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>ccdB</i> -50	(MfeI)gcgagaaagtaatcatccgcattatagggttaATG	This study

<i>ccdB</i> -500	(MfeI) attaaagcccataacagtacc ATG	This study
<i>ccdB</i> -1500	(MfeI) tttacaaaataatttattaacatcgcgcgtat ATG	This study
<i>ccdB</i> -5000	(MfeI) tcatacacaataatcagatactaagagccccc aaATG	This study
RBS tested for MazF expression		
Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>mazF</i> -50	(MfeI) ggagaaataccactcagactctcatccataa ATG	This study
<i>mazF</i> -200	(MfeI) ataaaaattacaacatagacctacaggaatct ATG	This study
<i>mazF</i> -1000	(MfeI) taactgggaaagataacggagactggta ATG	This study
<i>mazF</i> -5000	(MfeI) gacacaacgctaacataaggacgcaatttcaa ATG	This study
<i>mazF</i> -15000	(MfeI) cagactaatctaaaataattaagccggaggcgcaa ATG	This study
DNA fragments used to construct the Passcode circuit		
Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>pLlacO</i>	(XhoI)ttgacaattgtgagcgtcacaagatactgagcacatcagcaggacgcactgacc(AvrII)	This study
<i>pLscrO-1</i>	(EagI)ttattaaaccggttttagcattgacaattaaaccggttttagcagatactgagcacatcagcaggacgcactgacc(MfeI)	This study
<i>pLscrO-2</i>	(EagI)catttattaaaccggtttattgacataaaccggttttagcatagatactgagcacatcagcaggacgcactgacc(MfeI)	This study
RBS+ <i>lacI-lacI</i>	(SacI) atcagcaggacgcactgaccggatccatga aaccagtaacgttatacgtatcgcagagtatgccggtgtctctatcagaccgttcccgcgtggtgaaccaggccagccacgttctcgcaaacgcgggaaaaagtggagcggcgatggcggagctgaattacattcccaaccgcgtggcacaacaactggcgggcaaacagtcgttgctgattggcgttgccacctccagctcgtccctgcagcgcgcgtcgcaattgtcgcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgctgatggtagaacgaagcggcgtcgaagcctgtaaagcggcggtgcacaatcttctgcgcaacgcgtcagtgggctgatcattaactatccgctggatgaccaggatgccattgctgtggaagctgcctgcactaatgtccggcgttatttctgatgtcttgaccagacacctatcaacagatattatttctcccatgaagacggtacgcgactgggcgtggagcatctgctgcattgggtcaccagcaaatcgcgctgttagcgggccattagttctgtctcggcgcgtctgcgtctggctggctggcataaataatctcactcgcaatcaaatcagccgatagcggaaacgggaaggcactggagtgccatgtccggttttcaaaaaccatgcaaatgctgaatgagggcatcgttcccactgcgatgctggttgccaacgatcagatggcgtcggcgcaatgcgcgccattaccagtcgggctgcgcgttggtgcggatctcggtagtggtgatacgaacgataccgaagacagctcatgttatatcccgcgttaaccacctcaaacaggattttcgcctgctggggcaaacacgcgtggaccgttgcgtgcaactctcagggccaggcgggtgaagggaacatcagctgttcccgtctcactggtgaaaagaaaaaccacctggcgcccaatacgaacaccgctctccccgcgcgttgccgattcattaatgcagctggcacgacaggtttcccgactggaaagcgggcagtga(BsrGI)	<i>Escherichia coli</i> K-12 MG1655
RBS+ <i>galR-lacI</i>	(SacI) atcagcaggacgcactgaccggatccatga aaccagtaacgttatacgtatcgcagagtatgccggtgtctctatcagaccgttcccgcgtggtgaaccaggccagccacgttctcgcaaacgcgggaaaaagtggagcggcgatggagctctttagctatcacccgaacgccaacgccgtgcgtggcgcagcagaccactgaaacggtcggtctggtcgttggtgatgttccgatccgttttccggtgcaatggtgaaagcggcgaacaggtggcttatcacaccgtaatttttattgattggcaacggttaccacaacgaacaaaagagcgtcaggccattgagcaactgatccgccatcgtctgctgcgttggtggtcctatgccaaatgatccggatgctgatttagcctcattaatgaacaaatgcccggtatggtgctgataaccgtatcctgcctgcttgaaaaccgttgattgctctggacgatcgttacggtgcctggctggcaacgcgtcatttaattcagcaaggtcataccgcatgttattctgtgcttaaccacttatttctgacgccgaagatcgtctgcaagggtattacgatccctgtctgaaagtggattgctggccaatgaccggcgtggtgacattggcgaaccagacgaagcggcgcgcaacaggcaatgaccgagcttttggacagggaagaatttactgcggtagcctgttataacgattcaatgcgggcgggtgcgatggcgcttctcaatgataatggtattgatgaccgggtgagatttctgtaattggctttgatgatgtgctggtgtcacgctatgtgcgtccgcgcctgaccaccgtgcgttacccaatcgtgacgatggcgaccagcgtccggaactggcgttggcgtggtggataatcgcctctccggaaatcactaatgtctttatgctcgacgctggtacgtctgctattcagtcgaactccgtcgtgagggaagtcacatcatgaaccagcgactaa(BsrGI)	This study

[illegible]

RBS+ <i>celR-scrR</i>	(AvrII) ctctagccattttataggatctta agatgaagacgaaacgcgtaaccattaaagatatcgcggaattagctgggggtgagtaaagcaacggcaagctctgttcttaatggctgtggtaaagaactgcgtgtcggccaggaaacccgtgagcgcgtgctggctattatcaagaactcgggttacgtcccgaatcgcgcggcacgcacattggttacacggcgacggacaccgtggccttgggtgtgccgaaataaccagaaactgttgcggaaccgtttatgcaggtatcgtgctgggtgtcgtgtggctctgagtgaaacgtggttccagttcgtcctggctacgggtcgttcgggcattgagcacgaacgcctggggggtctatctggcaggccagcatgtggacggcgtgctgcttcttagttgcaccgcgacgatccgtgccgcagatgctggacgaagctggagtagcgtatgtatgtggccgcccgtgggtgtgccggaagagcaggtcagctatgtcgatcgcacataattgggtggcggcgccagggtaccagcgtctgacgaaacgggtcatcgtcgtatcgcgactattgccggcccgaagatatgggtggcaggagtagagcgtctgcaaggataccgtgaggcattattagccgggcatggaatagatgaaacattagatcatatggtgactttacgtatgattcggcgctgccgccatgcgcgaacttctggaccgcgcacgggatgtggatgccgtattcgacatctgattctatggggcgtggcggttacgtgactgctgcatcggggcgccgtgtcctgaagatgtcggcgtgtgggtacgacgattcagcgggtggcgaacacgcggaacccccatgacgagcgtcaaccagccgacagaattaatgggtcgtgagatggctcgttgcctgtagatcgtattacaggcgaaactacggaaacgggtcgttgggtactcgaaactcatttaattggtcgcgaaagtgggtaa(XmaI)	This study
Promoter and RBS for toxin expression regulation used with pPasscode1 (CelR-LacI, GalR-LacI, and CelR-ScrR)		
<i>ecoRI</i>	(EagI)catttattaaccggtttattgacataaaccggttagcatagatactgagcacatcagcaggacgcactgacc caattgctcacaaccacgaaggaaca (BamHI)	This study
<i>mf-lon</i>	(XhoI)catttattaaccggtttattgacataaaccggttagcatagatactgagcacatcagcaggacgcactgacc gaattcgtatctagga (KpnI)	This study
Promoter and RBS for toxin expression regulation used with pPasscode2 (LacI-LacI, CelR-LacI, and GalR-ScrR)		
Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in red)	Source
<i>ecoRI</i>	(EagI)ttattaaccggttagcattgacaattaaaccggttagcagatactgagcacatcagcaggacgcactgacc caattgctcaggaataggcccgctgcc (BamHI)	This study
<i>mf-lon</i>	(XhoI)ttattaaccggttagcattgacaattaaaccggttagcagatactgagcacatcagcaggacgcactgac cgaattccagttacaggtcac (KpnI)	This study
Promoter and RBS for toxin expression regulation used with pPasscode3 (GalR-LacI, LacI-LacI, and CelR-ScrR)		
Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>ecoRI</i>	(EagI)catttattaaccggtttattgacataaaccggttagcatagatactgagcacatcagcaggacgcactgacc caattgctcaggaataggcccgctgcc (BamHI)	This study
<i>mf-lon</i>	(XhoI)ttattaaccggttagcattgacaattaaaccggttagcagatactgagcacatcagcaggacgcactgac cgaattcgtatctagga (KpnI)	This study
RBS tested for EcoRI expression		
Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>ecoRI</i> -200	(MfeI) tcaggaataggcccgctgcc (BamHI)	This study
<i>ecoRI</i> -1000	(MfeI) taccgataacgaccgaaag (BamHI)	This study
<i>ecoRI</i> -10000	(MfeI) tggcacaacctaagtagggc (BamHI)	This study
<i>ecoRI</i> -40000	(MfeI) ctcacaaccacgaaggaaca (BamHI)	This study
<i>ecoRI</i> -80000	(MfeI) ctaaggatcgaacacgggag (BamHI)	This study
RBS tested for <i>mf-Lon</i> expression		
Name	Sequence (5' to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>mf-lon</i> -500	(EcoRI) cagttacaggtcac (KpnI)	This study
<i>mf-lon</i> -1000	(EcoRI) gcggccgcgactc (KpnI)	This study
<i>mf-lon</i> -10000	(EcoRI) gacgcaaaaggcaa (KpnI)	This study
<i>mf-lon</i> -30000	(EcoRI) ggtcggtcttcggg (KpnI)	This study

<i>mf-lon</i> -100000	(EcoRI) gtatctagga (KpnI)	This study
RBS tested for TF C (ScrR ESM-containing TFs)		
Name	Sequence (5'to 3') (Restriction sites in parentheses, RBS region in bold)	Source
<i>scrR</i> -2000	(AvrII) ggcgcaaccaagtcctaggt (AflII)	This study
<i>scrR</i> -10000	(AvrII) ctctagccattttataggat (AflII)	This study
<i>scrR</i> -25000	(AvrII) gggccgcattaggagggtac (AflII)	This study
<i>scrR</i> -100000	(AvrII) ggtaagagtcaagaggagg (AflII)	This study
<i>scrR</i> -150000	(AvrII) acttacttataataaggagg (AflII)	This study

CLAIMS

We claim:

1. A programmable synthetic biological circuit responsive to a selected combination of predetermined inputs, the circuit comprising:
 - i. an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first modulator protein is present;
 - ii. a first modulator protein expression module comprising nucleic acid sequence encoding the first modulator protein, operably linked to a second nucleic acid regulatory element comprising an AND circuit, whereby expression of the first modulator protein requires the presence of each of a selected set of at least two predetermined input agents, and in the absence of any one of the at least two predetermined input agents, expression of the first modulator protein is altered, resulting in expression of the effector protein.
2. The programmable synthetic biological circuit of claim 1, wherein the second nucleic acid regulatory element comprises a DNA binding site for a second modulator protein.
3. The programmable synthetic biological circuit of claim 1, wherein the first modulator protein is a repressor protein, and where, in the absence of any one the at least two predetermined input agents, expression of the repressor protein is inhibited, resulting in expression of the effector protein.
4. The programmable synthetic biological circuit of claim 2, further comprising a set of at least two hybrid modulator protein expression modules, each of which comprises nucleic acid sequence encoding a hybrid modulator protein and operatively linked regulatory sequences that permit the expression thereof, each hybrid modulator protein comprising:
 - i. an input agent-responsive domain of an input agent-sensitive transcription factor; and
 - ii. the DNA binding domain of the second modulator protein,wherein the binding of each respective hybrid modulator protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is altered by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid modulator protein, and
wherein each respective hybrid modulator protein alters expression from the first modulator protein expression module in the absence of its respective input agent.

5. The programmable synthetic biological circuit of claim 4, wherein the at least two hybrid modulator proteins are each hybrid repressor proteins, where the binding of each respective hybrid repressor protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is altered by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid modulator protein, and

wherein each respective hybrid modulator protein alters expression from the first modulator protein expression module in the absence of its respective input agent.

6. A composition comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of claims 1-5.

7. The composition of claim 6, wherein the expression modules are comprised by a single nucleic acid construct.

8. The programmable synthetic biological circuit of any one of claims 4-6, wherein the hybrid modulator expression modules and first modulator expression module are on the same nucleic acid construct.

9. The programmable synthetic biological circuit of any one of claims 4-6, wherein the hybrid repressor expression modules, first repressor expression module and effector expression module are on the same nucleic acid construct.

10. A host cell comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of claims 1-9.

11. The host cell of claim 10, wherein the cell is a bacterial, fungal, plant or animal cell.

12. A programmable synthetic biological circuit responsive to a selected combination of predetermined inputs, the circuit comprising:

- i. an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first repressor protein is present;
- ii. a first repressor protein expression module comprising nucleic acid sequence encoding the first repressor protein, operably linked to a second nucleic acid regulatory element comprising an AND circuit, whereby expression of the first repressor protein requires the presence of each of a selected set of at least two predetermined input agents, and in the

absence of any one of the at least two predetermined input agents, expression of the first repressor protein is repressed, resulting in expression of the effector protein.

13. The programmable synthetic biological circuit of claim 12, wherein the second nucleic acid regulatory element comprises a DNA binding site for a second repressor protein.

14. The programmable synthetic biological circuit of any one of claims 12 and 13, wherein the first nucleic acid regulatory element comprises a DNA binding site for the first repressor protein.

15. The programmable synthetic biological circuit of any one of claims 12-14, wherein the predetermined input agent comprises an environmental input agent.

16. The programmable synthetic biological circuit of claim 13, further comprising a set of at least two hybrid repressor protein expression modules, each of which comprises nucleic acid sequence encoding a hybrid repressor protein and operatively linked regulatory sequences that permit the expression thereof, each hybrid repressor protein comprising:

- i. an input agent-responsive domain of an input agent-sensitive transcription factor; and
- ii. the DNA binding domain of the second repressor protein,

wherein the binding of each respective hybrid repressor protein to the DNA binding site for the second repressor protein in the second nucleic acid regulatory element is inhibited by the binding of the respective input agent to the input agent-responsive domain of each respective hybrid repressor protein, and

wherein each respective hybrid repressor protein represses expression from the first repressor protein expression module in the absence of its respective input agent.

17. The programmable synthetic biological circuit of any one of claims 12-16, wherein the binding and activity of the first repressor protein is inhibited by the binding of an input agent different from members of the selected set of at least two input agents, thereby establishing a NOT circuit, such that in the absence of the different input agent, the first repressor protein binds the first nucleic acid regulatory element and represses expression of the effector protein, but in the presence of the different input agent, the first repressor protein does not bind the first nucleic acid regulatory element and permits expression of the effector protein.

18. The programmable synthetic biological circuit of any one of claims 12-17, wherein the first repressor protein is a hybrid repressor protein comprising an input agent-responsive domain of an input agent-sensitive transcription factor and the DNA binding domain of the second repressor protein,

wherein the input agent responsive domain and the DNA binding domain are heterologous to each other.

19. The programmable synthetic biological circuit of any one of claims 12-18, wherein the effector is a toxin or a reporter protein.

20. The programmable synthetic biological circuit of claim 19, wherein the toxin is selected from the group consisting of an endonuclease, a DNA gyrase, and a ribonuclease.

21. The programmable synthetic biological circuit of any one of claims 12-20, wherein the first repressor protein is selected from a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CelR polypeptide, a TetR polypeptide, or a hybrid comprising the DNA binding domain of one such polypeptide fused to the input agent-responsive domain of another such polypeptide.

22. The programmable synthetic biological circuit of any one of claims 13-21, wherein the second repressor protein is selected from a LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CelR polypeptide, a TetR polypeptide, or a hybrid comprising the DNA binding domain of one such polypeptide fused to the input agent-responsive domain of another such polypeptide.

23. The programmable synthetic biological circuit of any one of claims 16-22, wherein the input agent-responsive domain of each hybrid repressor protein is selected from the environmental agent binding domains of LacI polypeptide, an ScrR polypeptide, a GalR polypeptide, a GalS polypeptide, an AscG polypeptide, an RbsR polypeptide, a PurR polypeptide, a GntR polypeptide, a CelR polypeptide, and a TetR polypeptide.

24. The programmable synthetic biological circuit of any one of claims 12-23, wherein the at least two input agents are selected from the group consisting of IPTG, galactose, cellobiose, ampicillin, chloramphenicol, kanamycin, and ATc.

25. The programmable synthetic biological circuit of any one of claims 16-24, wherein the biological circuit is programmed by assembling a specific combination of effector expression module, first repressor protein expression module and a set of hybrid repressor protein expression modules sensitive to selected input agents.

26. The programmable synthetic biological circuit of any one of claims 16-25, wherein the hybrid repressor expression modules and first repressor expression module are on the same nucleic acid construct.
27. The programmable synthetic biological circuit of any one of claims 16-26, wherein the hybrid repressor expression modules, first repressor expression module and effector expression module are on the same nucleic acid construct.
28. A composition comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of claims 12-27.
29. A host cell comprising one or more nucleic acids that alone or together constitute a programmable synthetic biological circuit of any one of claims 12-27.
30. The host cell of claim 29, wherein the cell is a bacterial cell, fungal cell, plant cell, animal cell, or synthetic cell.
31. A passively activated biological containment circuit rendering a host cell dependent upon the presence of an exogenous input, the circuit comprising:
- i. an effector expression module comprising nucleic acid sequence encoding an effector protein, operably linked to a first nucleic acid regulatory element, the first nucleic acid regulatory element permitting expression of the effector protein unless a first modulator protein is present;
 - ii. a first modulator protein expression module comprising nucleic acid sequence encoding the first modulator protein, operably linked to a second nucleic acid regulatory element, the second nucleic acid regulatory element permitting expression of the first modulator protein unless a second modulator protein is present and binds the second nucleic acid regulatory element; and
 - iii. a second modulator protein expression module comprising nucleic acid sequence encoding the second modulator protein, operably linked to a third nucleic acid regulatory element, the third nucleic acid regulatory element permitting expression of the second modulator protein, wherein the binding of the second modulator protein to the second nucleic acid regulatory element is modulated by an exogenous input,
- wherein, in the presence of the exogenous input, the binding of the second modulator protein to the second nucleic acid regulatory element is inhibited, thereby permitting the expression of the first modulator protein such that expression of the effector protein is inhibited; and

wherein, in the absence of the exogenous input, the binding of the second modulator protein to the second nucleic acid regulatory element is permitted, thereby inhibiting, thereby inhibiting the expression of the first modulator protein such that expression of the effector protein is permitted.

32. The passively activated biological containment circuit of claim 31, wherein the effector protein comprises a toxin, a restriction endonuclease, or a protease.

33. The passively activated biological containment circuit of claim 31, wherein the effector protein comprises a protease that degrades a cellular polypeptide essential for cell survival.

34. The passively activated biological containment circuit of claim 31, wherein the first and second modulator proteins are different repressor proteins that bind different nucleic acid regulatory sequences.

35. The passively activated biological containment circuit of claim 31, further comprising a third modulator protein expression module, comprising nucleic acid sequence encoding a third modulator protein, operatively linked to a fourth nucleic acid regulatory element, the fourth nucleic acid regulatory element permitting expression of the third modulator protein unless the first modulator protein is present.

36. The passively activated biological containment circuit of claim 35 wherein the third modulator protein degrades the first modulator protein.

37. The passively activated biological containment circuit of claim 36, wherein, in the absence of the exogenous input, the third modulator protein is expressed, resulting in degradation of the first modulator protein, resulting in the expression of the effector protein.

38. The passively activated biological containment circuit of claim 31, wherein the exogenous input comprise a small molecule that binds to the second modulator protein.

39. The passively activated biological containment circuit of claim 31, wherein the second modulator protein and the exogenous input are a pair selected from a tetracycline analog that binds TetR and TetR, and a lactose analog that binds LacI and LacI.

40. The passively activated biological containment circuit of claim 35, wherein the third modulator protein comprises a protease that degrades the first regulator protein and a cellular polypeptide essential for cell survival, and wherein the effector protein comprises a toxin.

41. One or more isolated nucleic acid comprising sequences encoding a passively activated biological containment circuit of claim 31.
42. A nucleic acid vector construct comprising sequences encoding a passively activated biological containment circuit of claim 31.
43. A host cell comprising nucleic acid comprising sequences encoding a passively activated biological containment circuit of claim 31.
44. The host cell of claim 43, wherein the cell is a bacterial, fungal, plant, animal, or synthetic cell.
45. A biological circuit rendering a cellular response sensitive to a predetermined condition, the circuit comprising a nucleic acid construct comprising expression modules that form a deadman regulatory circuit sensitive to the predetermined condition, the construct comprising:
- i. a first repressor module M_{R1} comprising a first repressible promoter nucleic acid sequence (rP_1) operably linked to a repressor nucleic acid sequence encoding a first repressor protein (R_A), that binds repressible promoter element rP_E and represses expression from promoters comprising element rP_E ;
 - ii. a second repressor module M_{R2} comprising a second promoter nucleic acid sequence (P_2) operably linked to a second repressor nucleic acid sequence (R_B) encoding a second repressor protein R_B , wherein transcription from rP_i is inhibited by the second repressor protein R_B and wherein repression activity of R_B is sensitive to inhibition by a first agent A_1 , the presence or absence of A_1 establishing a predetermined condition;
 - iii. an effector module M_E comprising an effector protein coding sequence E operably linked to a third, repressible promoter comprising repressible promoter element rP_E , that is repressed by first repressor protein R_A ; the respective modules forming a deadman regulatory circuit such that:
in the absence of agent A_1 , the second repressor protein R_B is expressed and represses transcription from repressor module M_{R1} , such that expression of first repressor protein R_A is repressed, thereby relieving repression of effector module M_E by R_A , such that effector E is expressed by M_E ; and
in the presence of agent A_1 , the activity of the second repressor protein R_B is inhibited, permitting expression of the first repressor protein R_A , which maintains expression from effector module E in the "off" state, such that agent A_1 is required by the circuit to maintain effector protein expression in the "off" state, and in the absence of A_1 , the circuit defaults to expression of the effector protein.

46. The circuit of claim 45 wherein the effector is a toxin or a protein that induces a cell death program.
47. A method of rendering a cell responsive to a predetermined condition, the method comprising introducing nucleic acid encoding a biological circuit of claim 45 to the cell.
48. Isolated nucleic acid encoding a biological circuit of claim 45.
49. A host cell comprising nucleic acid of claim 48.
50. A biological circuit rendering a cellular response sensitive to a predetermined condition, the circuit comprising a nucleic acid construct comprising expression modules that form a deadman regulatory circuit sensitive to the predetermined condition, the construct comprising:
- i. a first repressor module M_{R1} comprising a first repressible promoter nucleic acid sequence (rP_1) operably linked to a repressor nucleic acid sequence encoding a first repressor protein (R_A), that binds repressible promoter element rP_E and represses expression from promoters comprising element rP_E ;
 - ii. a second repressor module M_{R2} comprising a second promoter nucleic acid sequence (P_2) operably linked to a second repressor nucleic acid sequence (R_B) encoding a second repressor protein R_B , wherein transcription from rP_i is inhibited by the second repressor protein R_B and wherein repression activity of R_B is sensitive to inhibition by a first agent A_1 , the presence or absence of A_1 establishing a predetermined condition;
 - iii. an effector module M_E comprising an effector protein coding sequence E operably linked to a third, repressible promoter comprising repressible promoter element rP_E , that is repressed by first repressor protein R_A ;

the respective modules forming a deadman regulatory circuit such that:

in the absence of agent A_1 , the second repressor protein R_B is expressed and represses transcription from repressor module M_{R1} , such that expression of first repressor protein R_A is repressed, thereby relieving repression of effector module M_E by R_A , such that effector E is expressed by M_E ; and

in the presence of agent A_1 , the activity of the second repressor protein R_B is inhibited, permitting expression of the first repressor protein R_A , which maintains expression from effector module E in the "off" state, such that agent A_1 is required by the circuit to maintain effector protein expression in the "off" state, and in the absence of A_1 , the circuit defaults to expression of the effector protein.

51. The circuit of claim 50, wherein the effector is a toxin or a protein that induces a cell death program.
52. A method of rendering a cell responsive to a predetermined condition, the method comprising introducing nucleic acid encoding a biological circuit of claim 51 to the cell.
53. An isolated nucleic acid encoding a biological circuit of claim 51.
54. A system to render cell growth restricted to the presence of a predetermined set of at least two selected agents, the system comprising a nucleic acid construct encoding expression modules comprising:
- i. a toxin expression module that encodes a toxin that is toxic to a host cell, wherein sequence encoding the toxin is operably linked to a promoter P1 that is repressed by the binding of a first hybrid repressor protein hRP1;
 - ii. a first hybrid repressor protein expression module that encodes the first hybrid repressor protein hRP1, wherein expression of hRP1 is controlled by an AND gate formed by two hybrid transcription factors hTF1 and hTF2, the binding or activity of which is responsive to agents A1 and A2, respectively, such that that are both agents A1 and A2 required for expression of hRP1,
- wherein in the absence of either A1 or A2, hRP1 expression is insufficient to repress toxin promoter module P1 and toxin production, such that the host cell is killed; and
- wherein hybrid factors hTF1, hTF2 and hRP1 each comprise an environmental sensing module from one transcription factor and a DNA recognition module from a different transcription factor that renders the binding of the respective DRM sensitive to the presence of an environmental agent, A1, or A2, that is different from that which the respective DRM binds in nature.
55. A method of restricting cell growth to require the presence of a predetermined set of at least two selected agents, the system comprising introducing to a host cell nucleic acid encoding a system as described in claim 54.
56. An isolated nucleic acid encoding a system of claim 55.

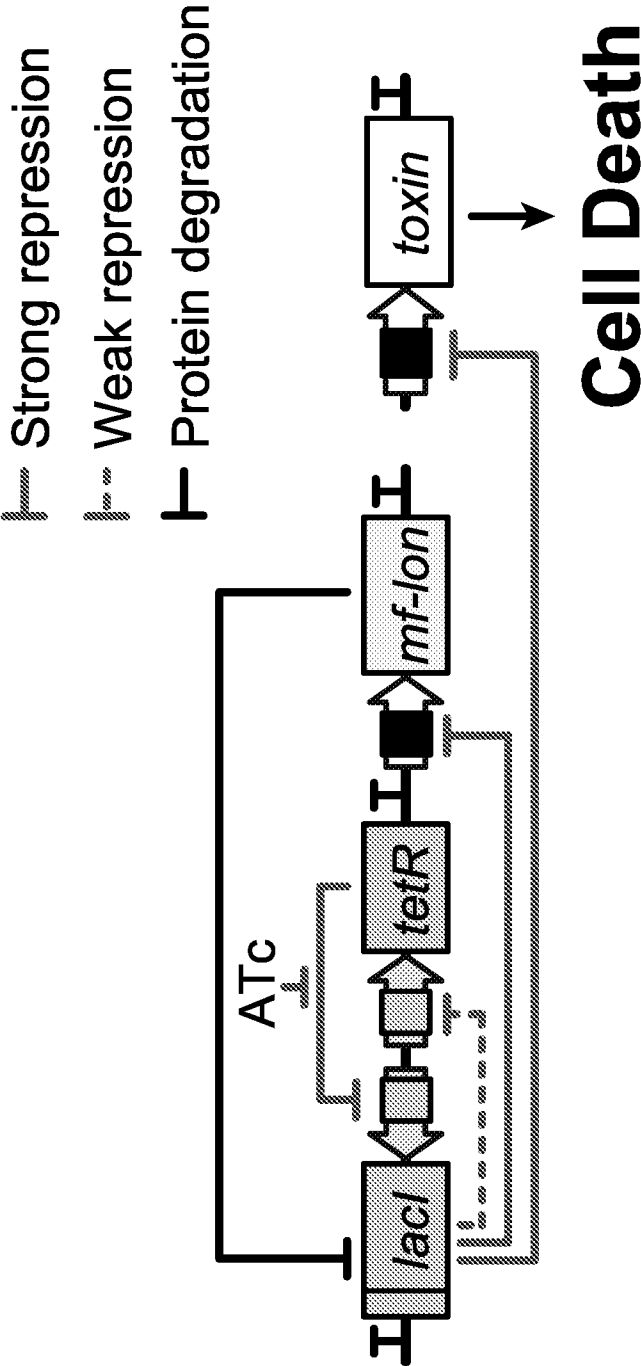


FIG. 1A

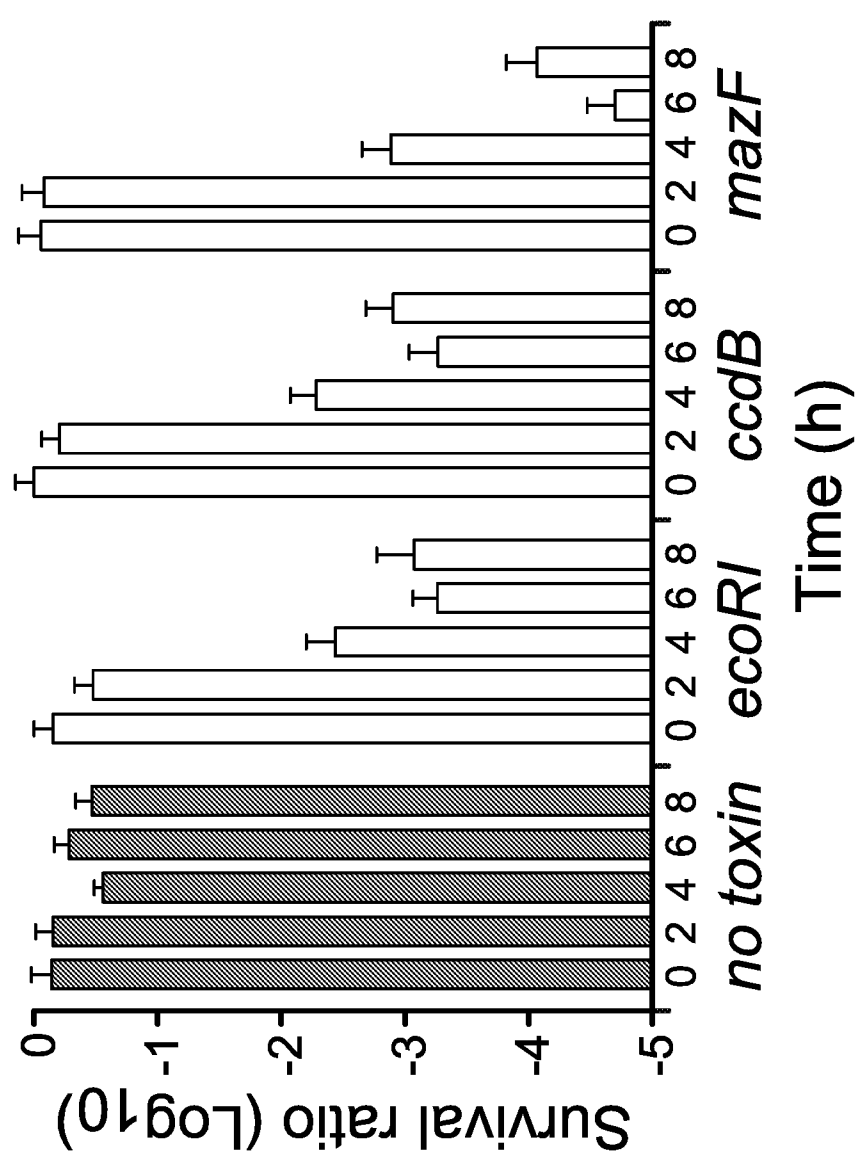


FIG. 1A (cont.)

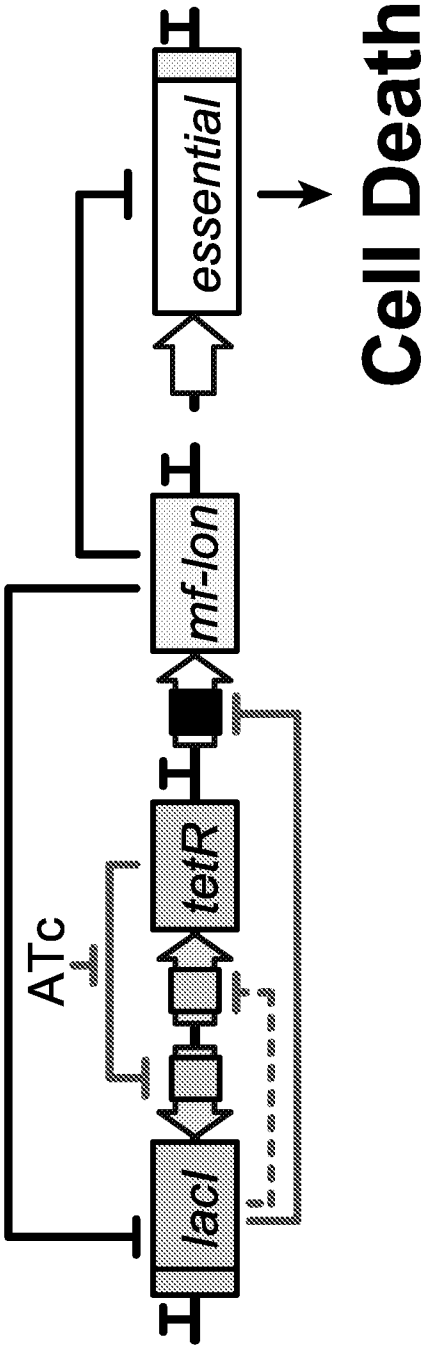


FIG. 1B

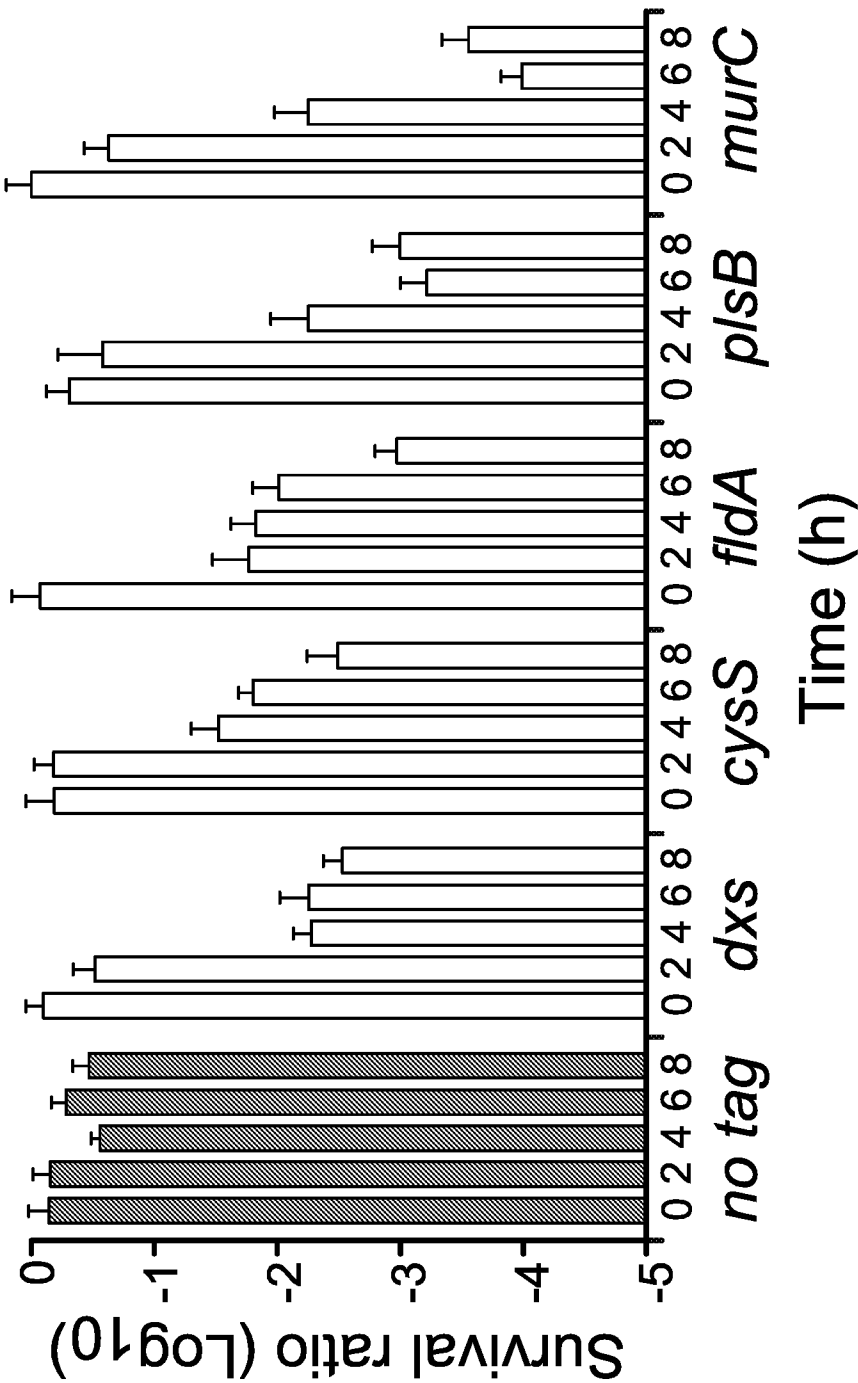


FIG. 1B (cont.)

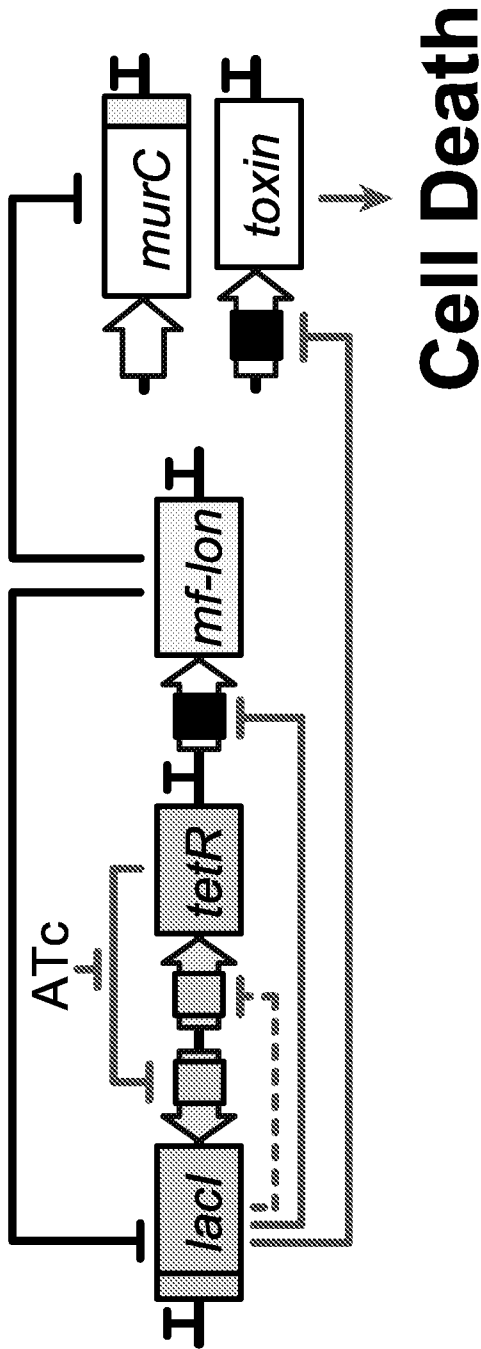


FIG. 1C

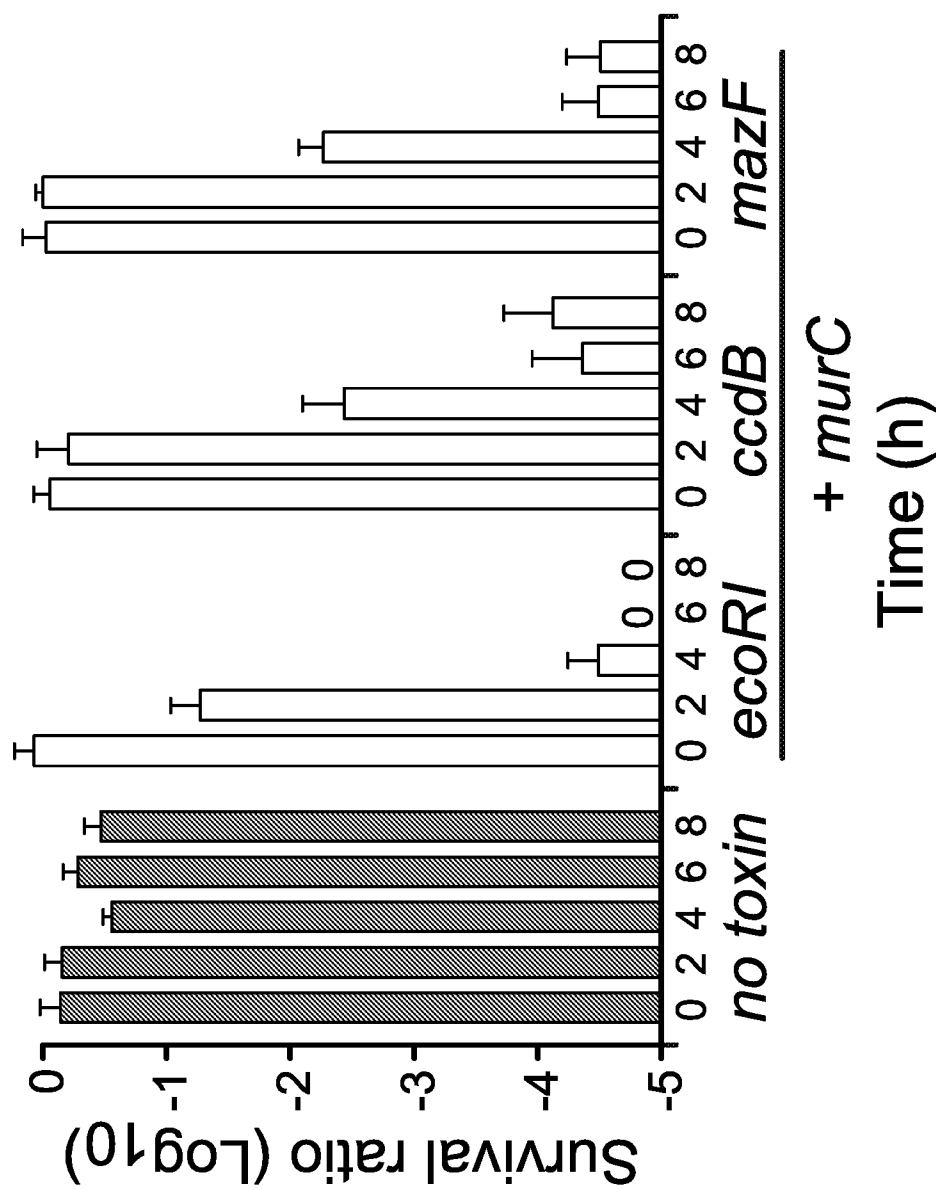


FIG. 1C (cont.)

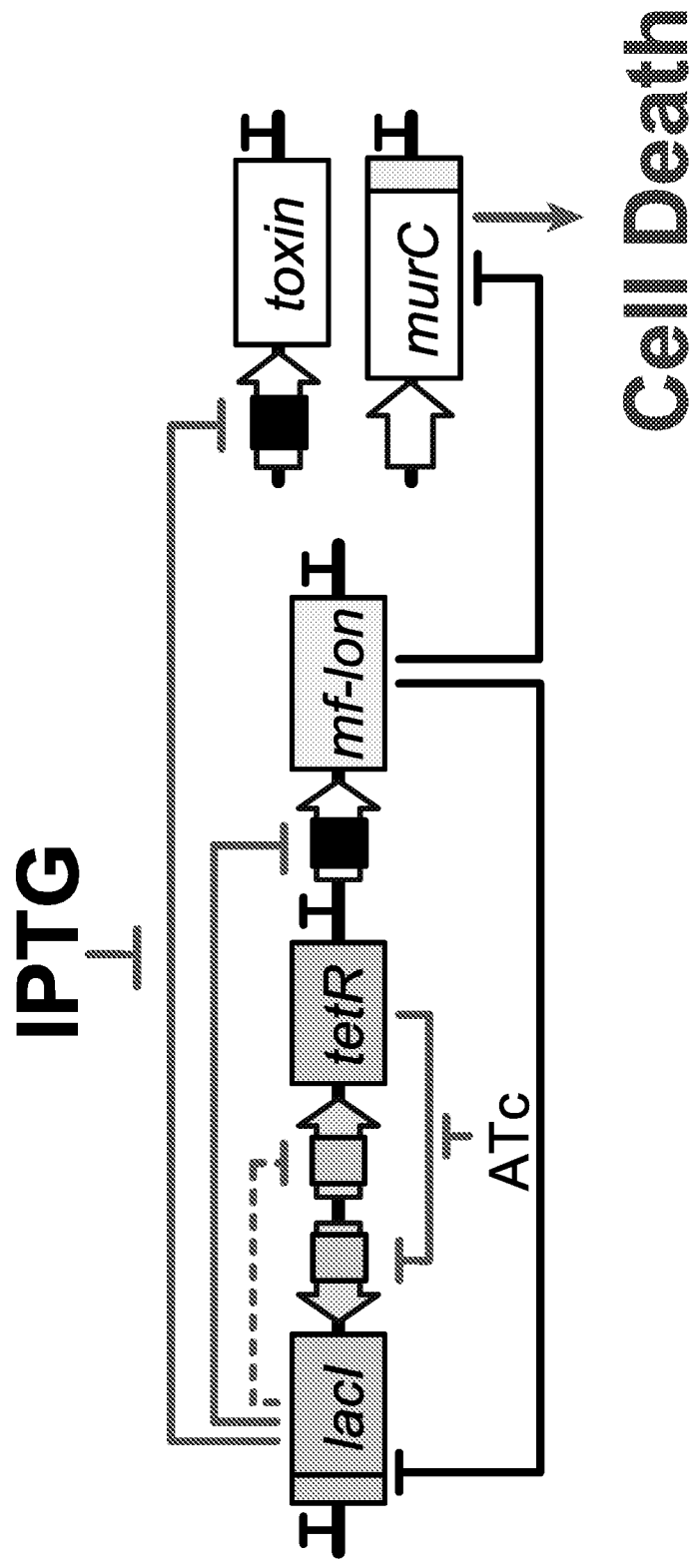


FIG. 2

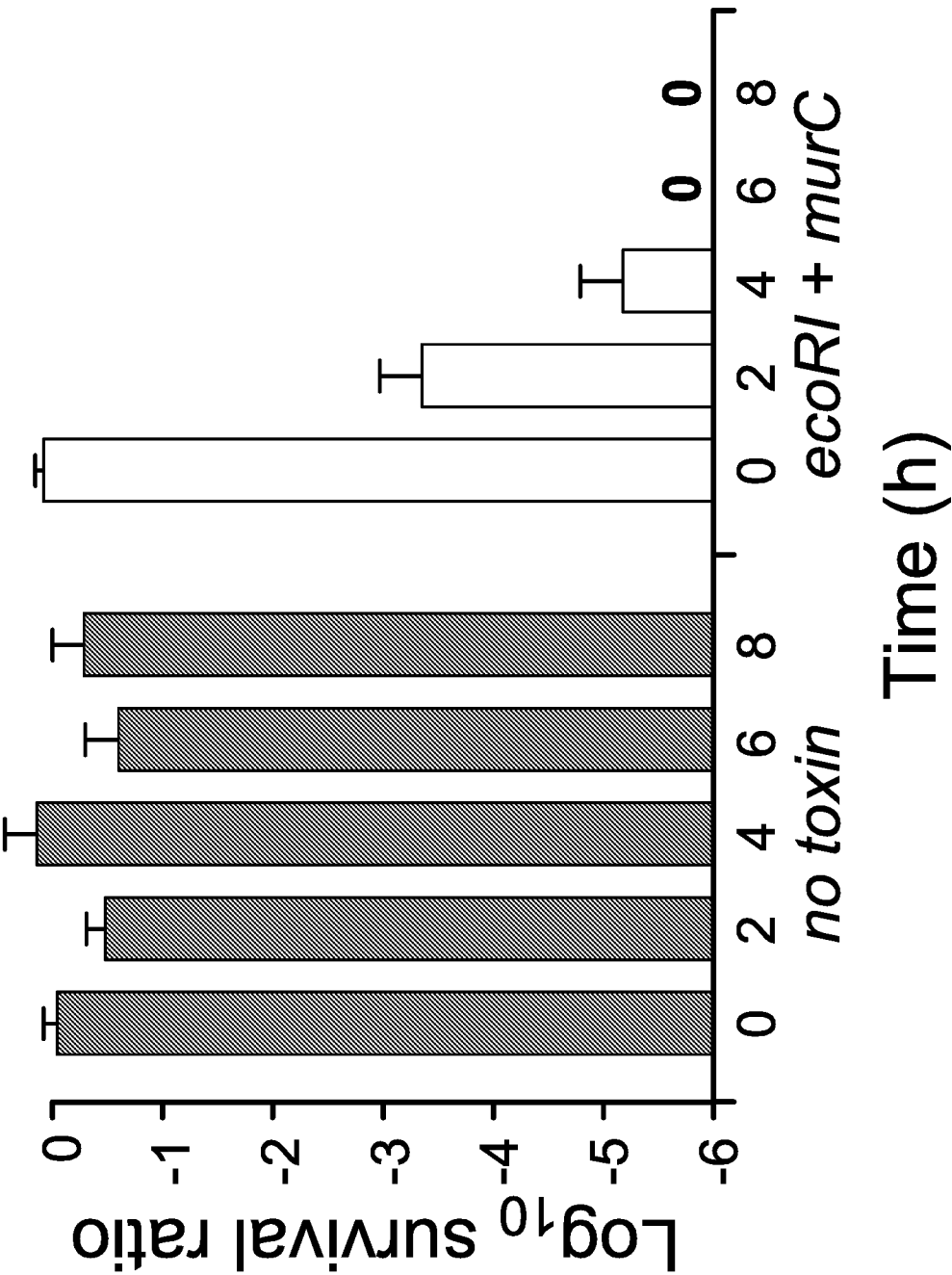


FIG. 2 (cont.)

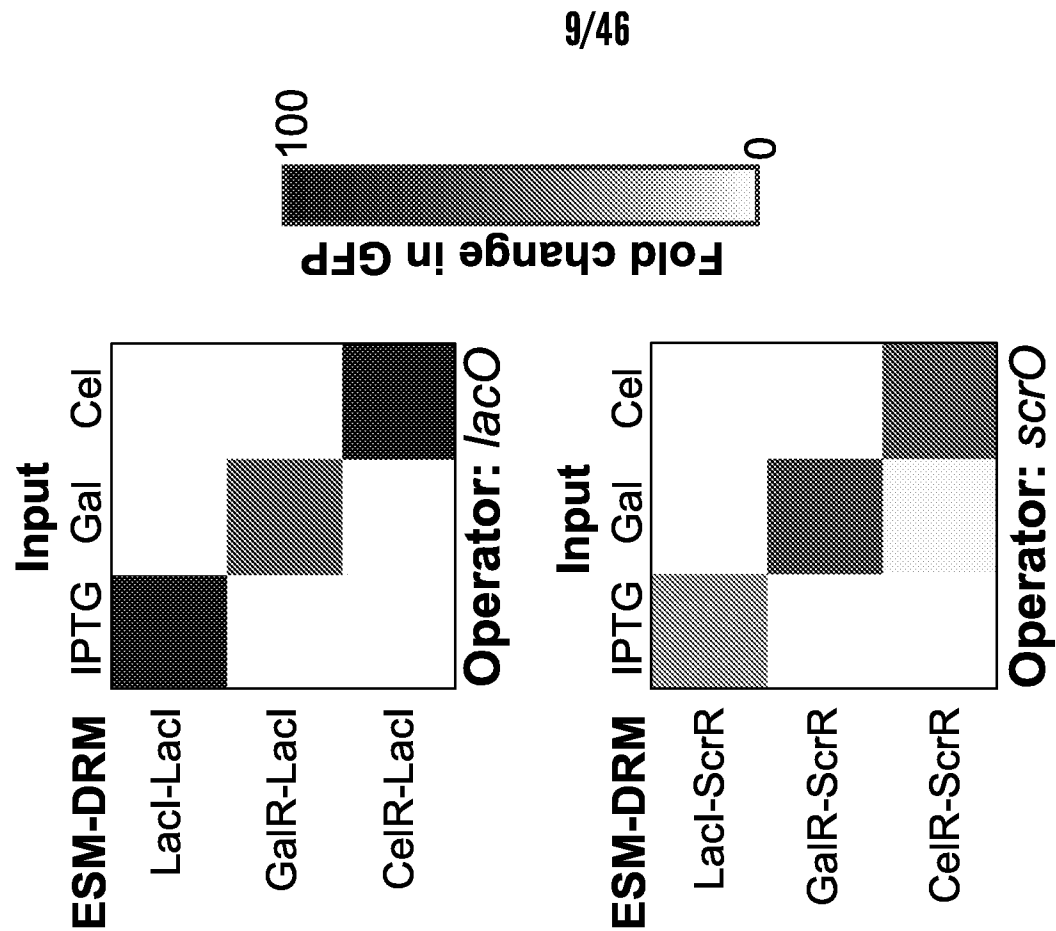


FIG. 3B

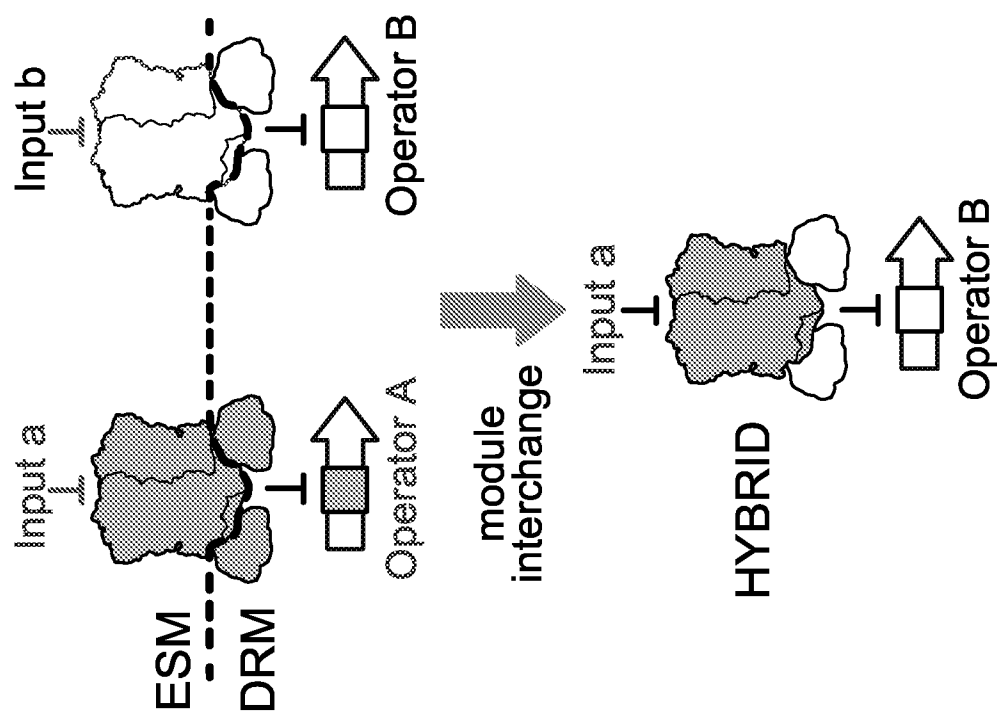


FIG. 3A

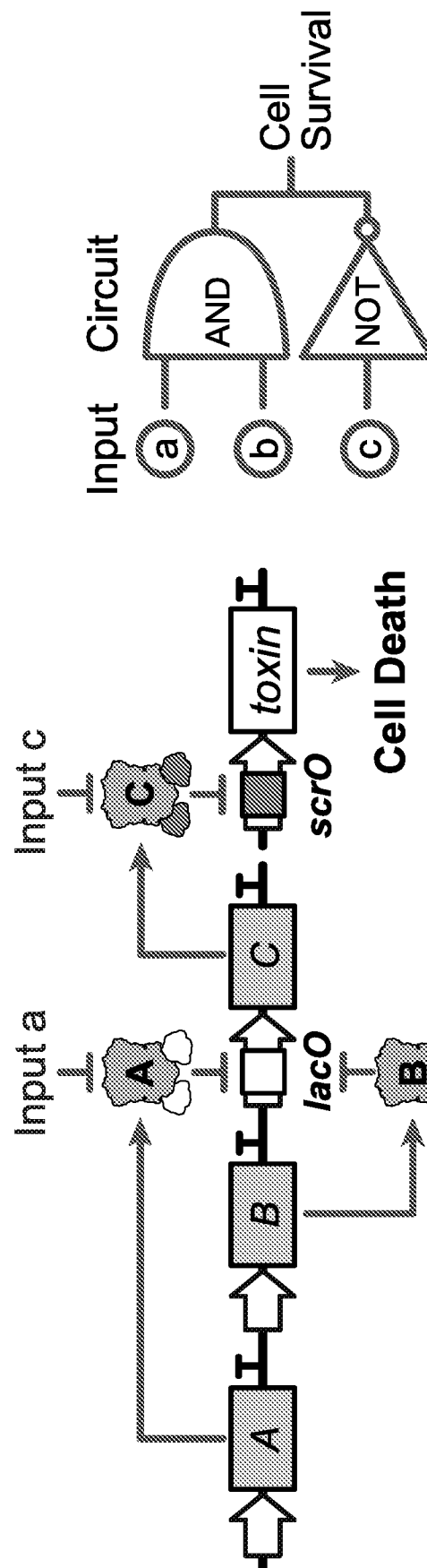


FIG. 4A

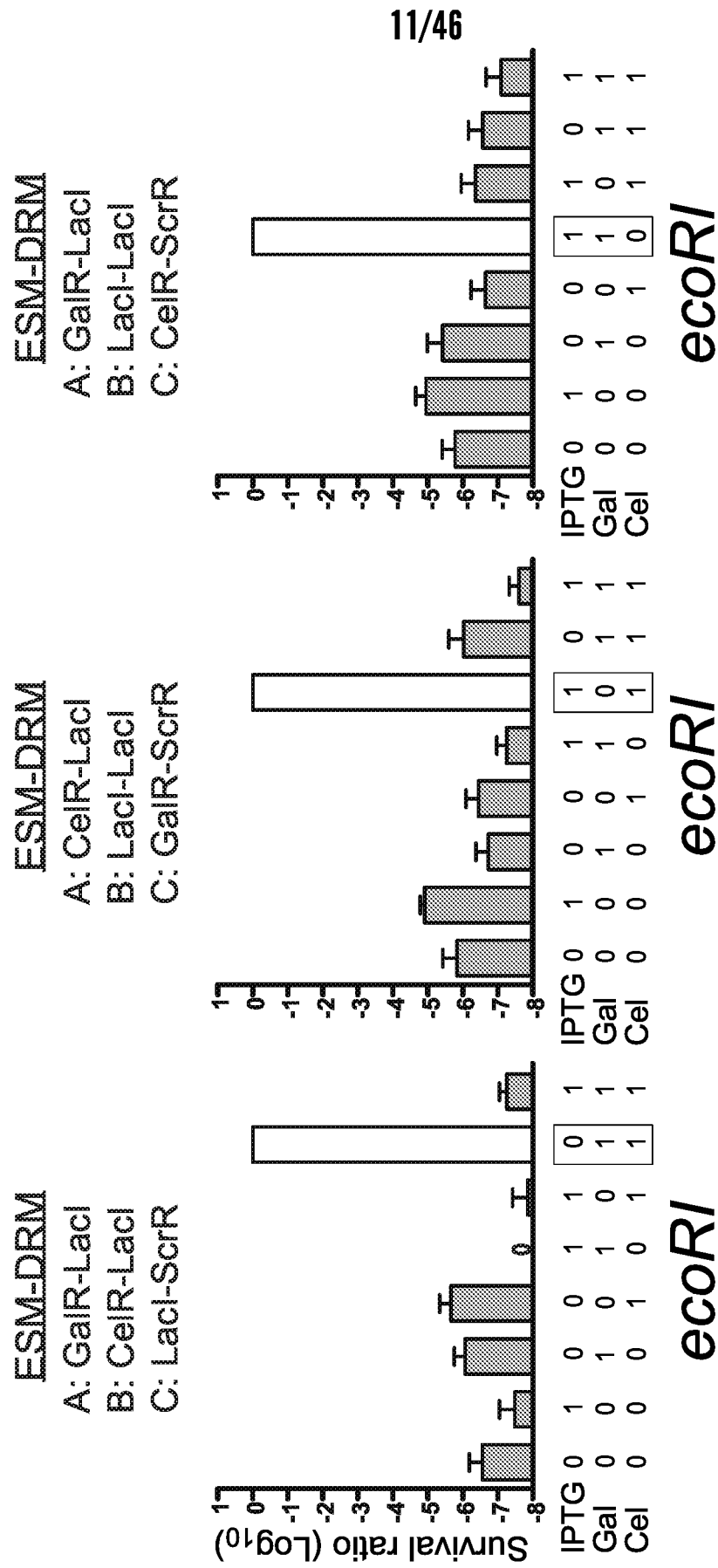


FIG. 4B

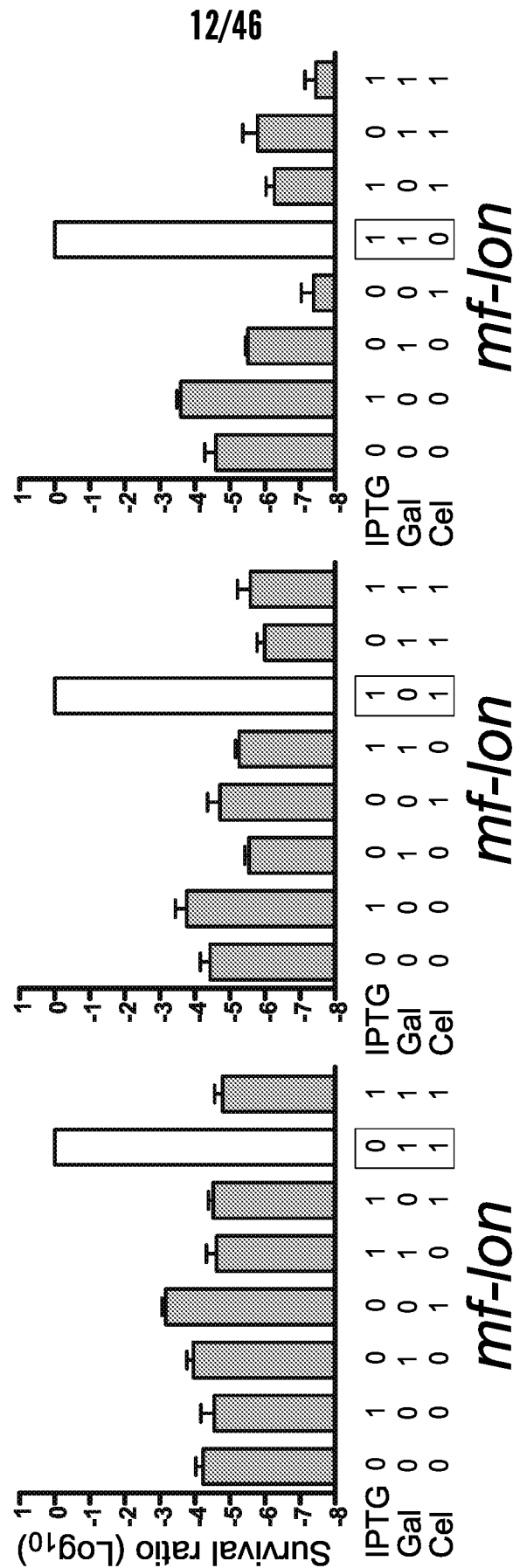


FIG. 4B (cont.)

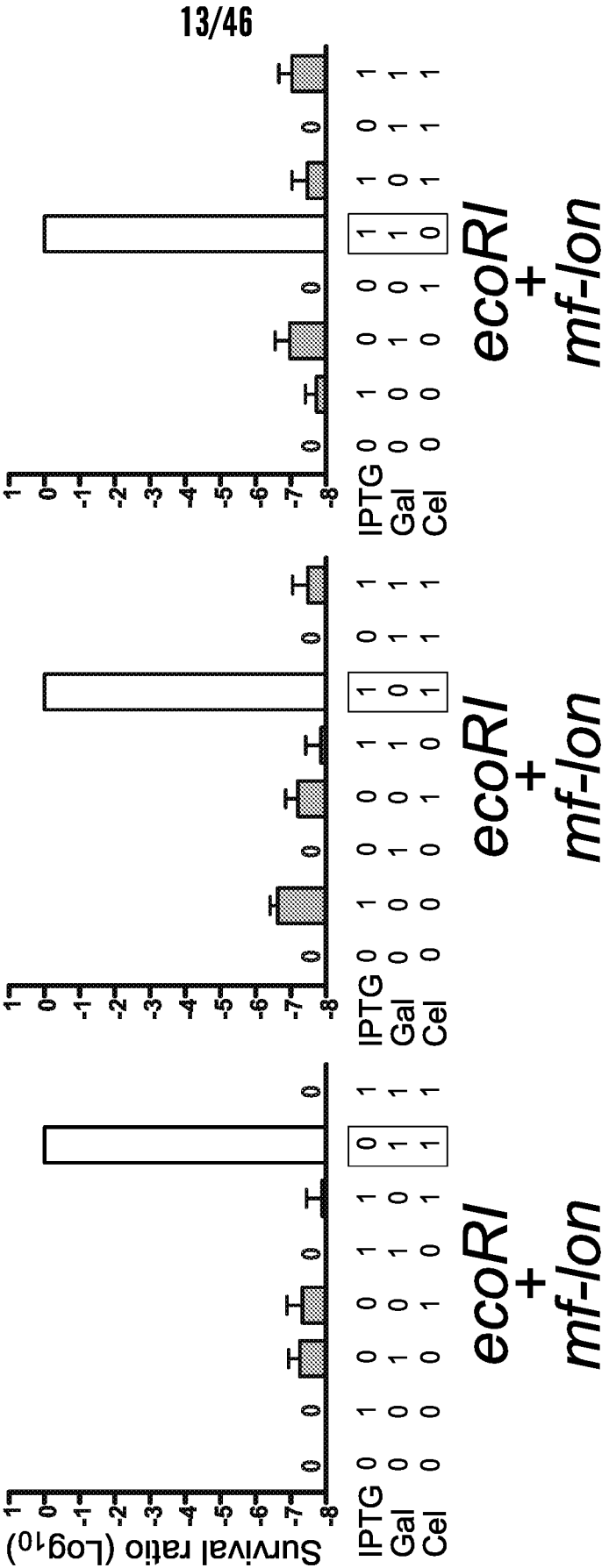


FIG. 4B (cont.)

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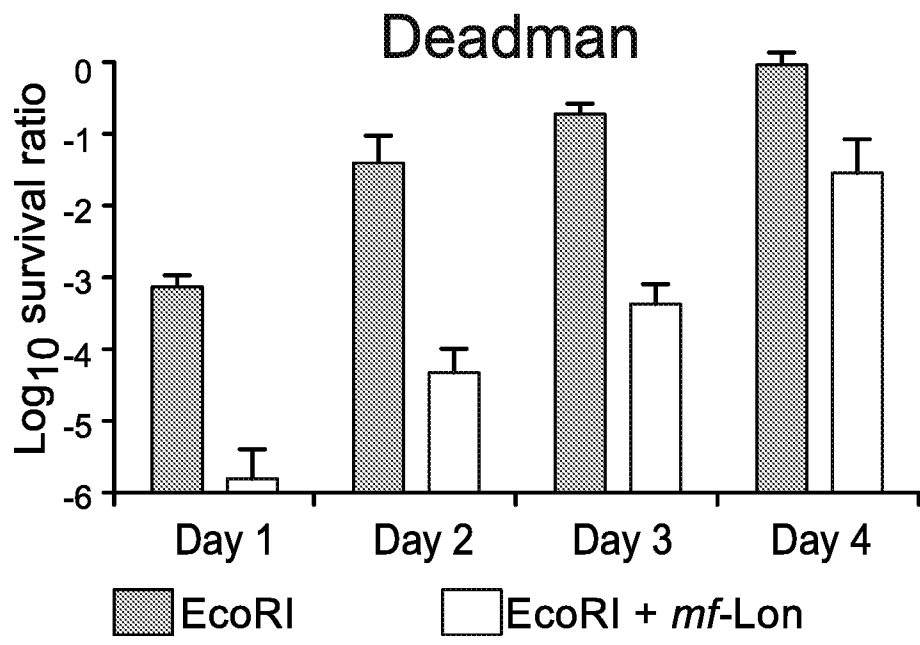


FIG. 5A

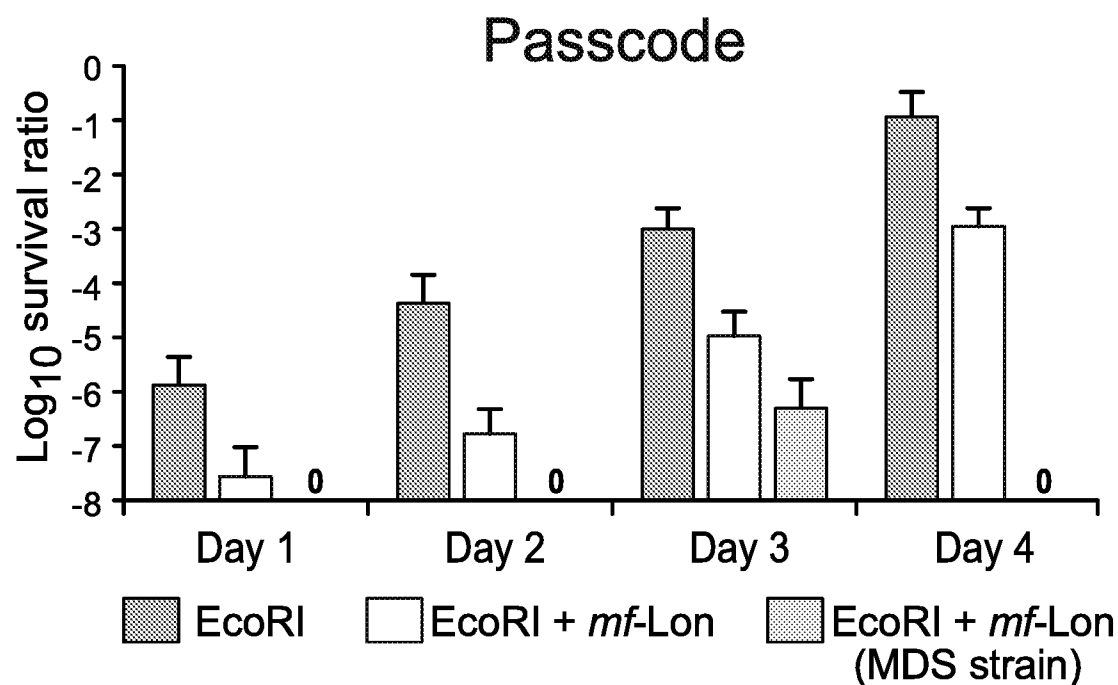


FIG. 5B

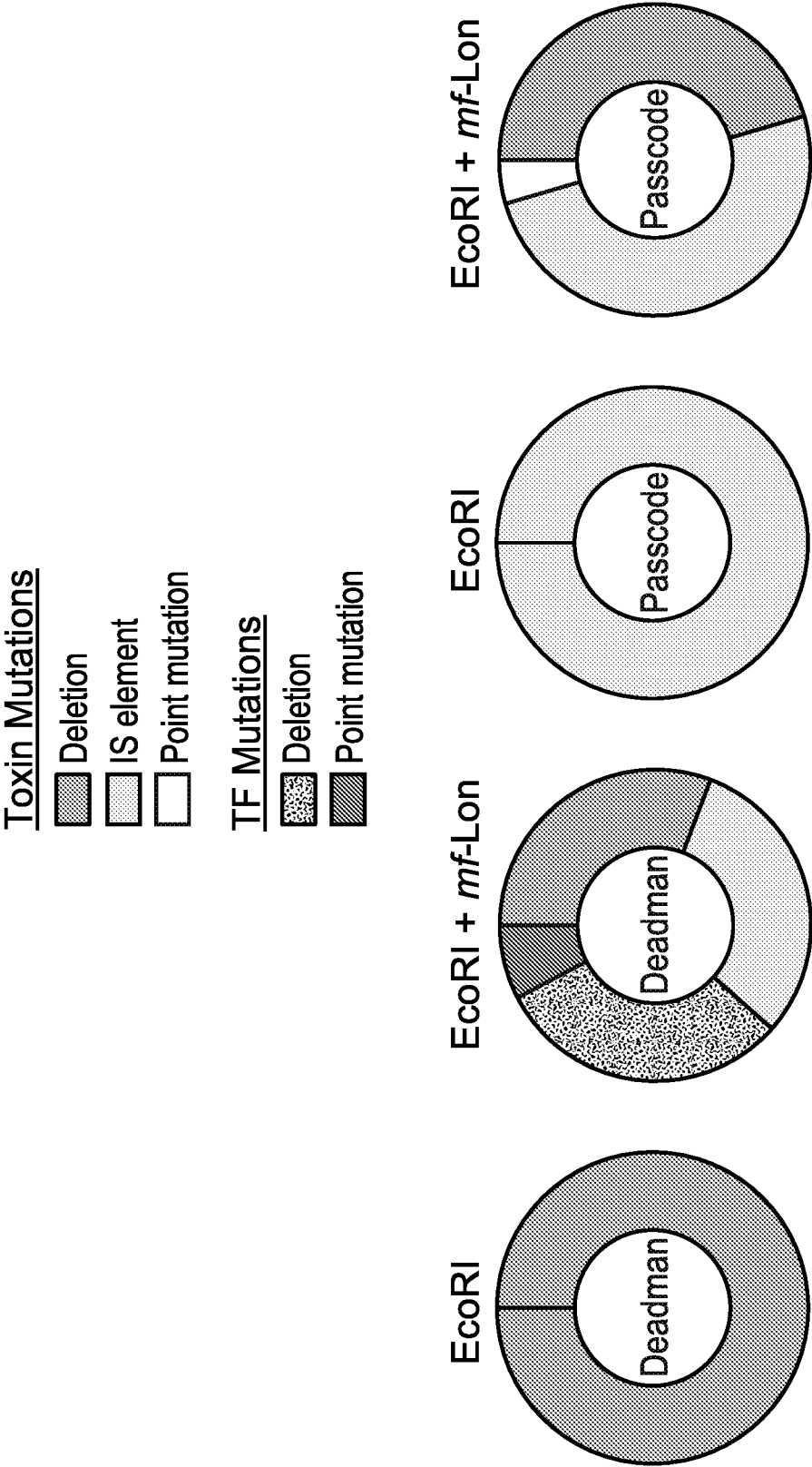


FIG. 5C

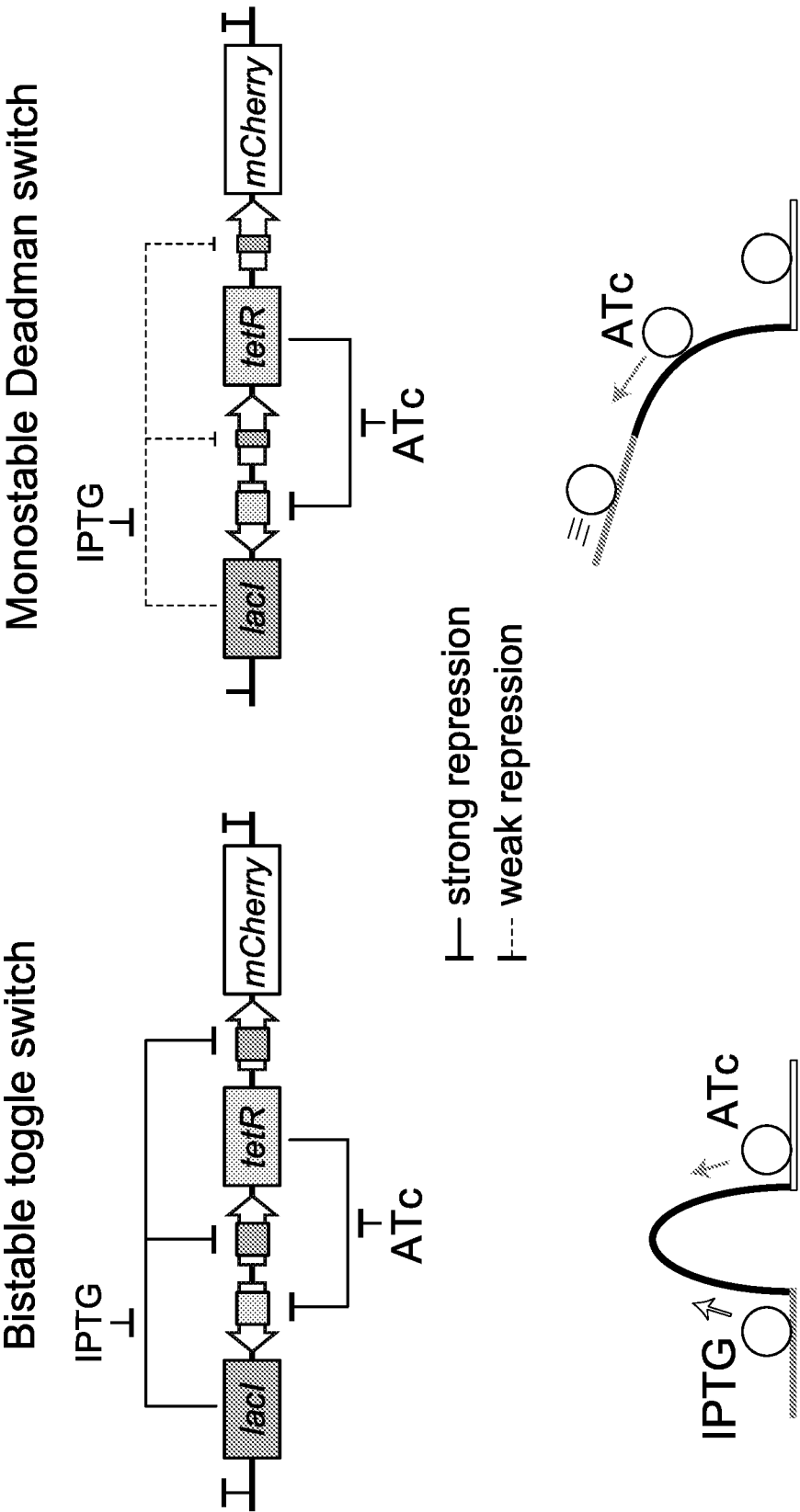
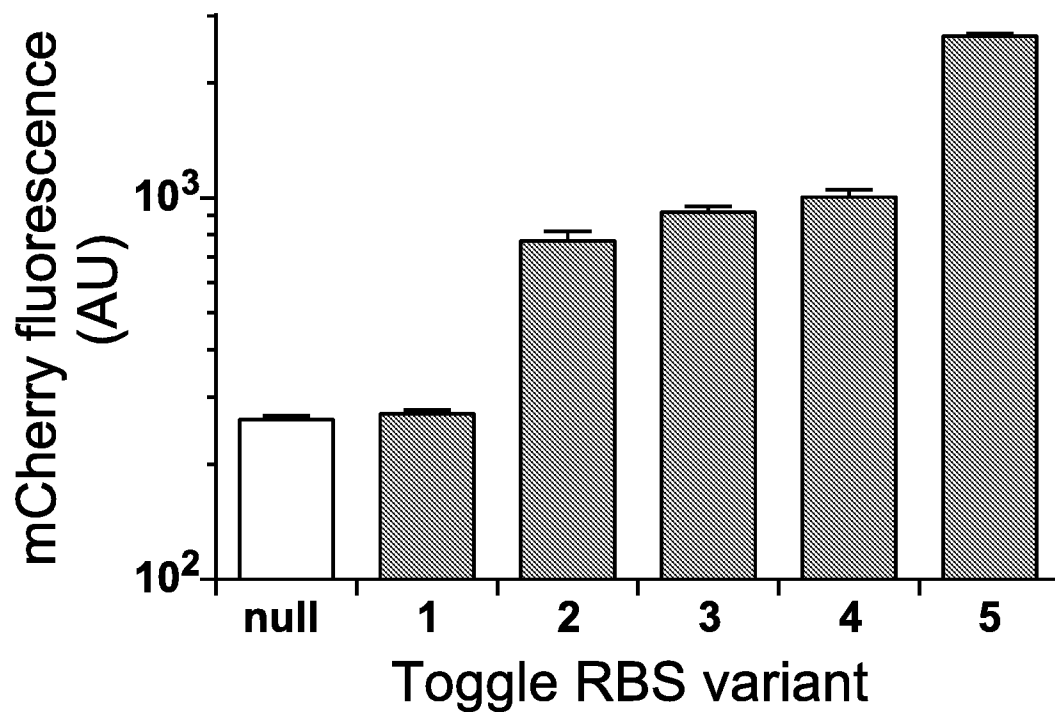


FIG. 6

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Toggle	RBS	LacI	TetR	TetR / LacI
1	L2-T1	42.84 ± 0.76	4.60 ± 0.10	0.11
2	L2-T2	42.84 ± 0.76	145.04 ± 4.53	3.38
3	L3-T3	68.9 ± 2.67	268.82 ± 4.76	3.90
4	L2-T3	42.84 ± 0.76	268.82 ± 4.76	6.27
5	L1-T3	5.96 ± 0.44	268.82 ± 4.76	45.10

FIG. 7A**FIG. 7B**

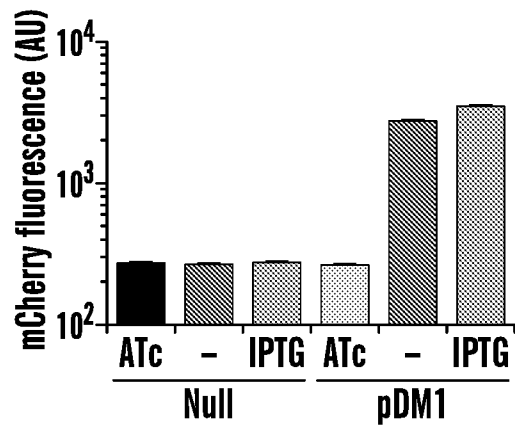


FIG. 8A

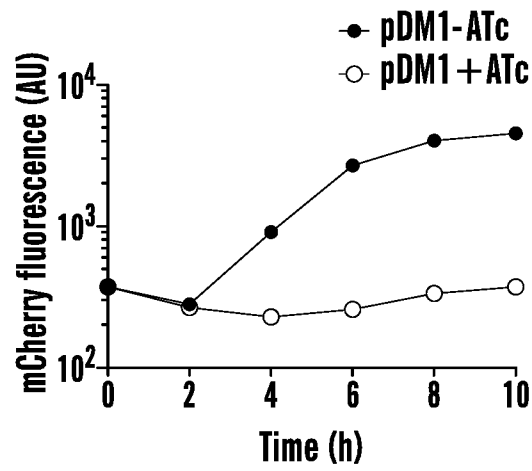


FIG. 8B

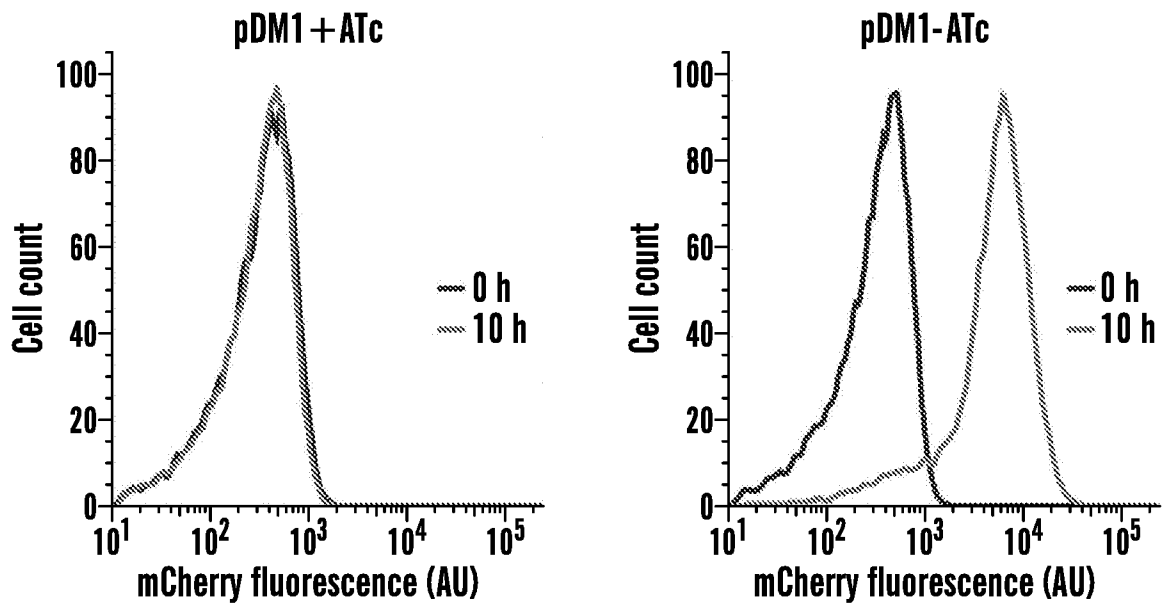


FIG. 8C

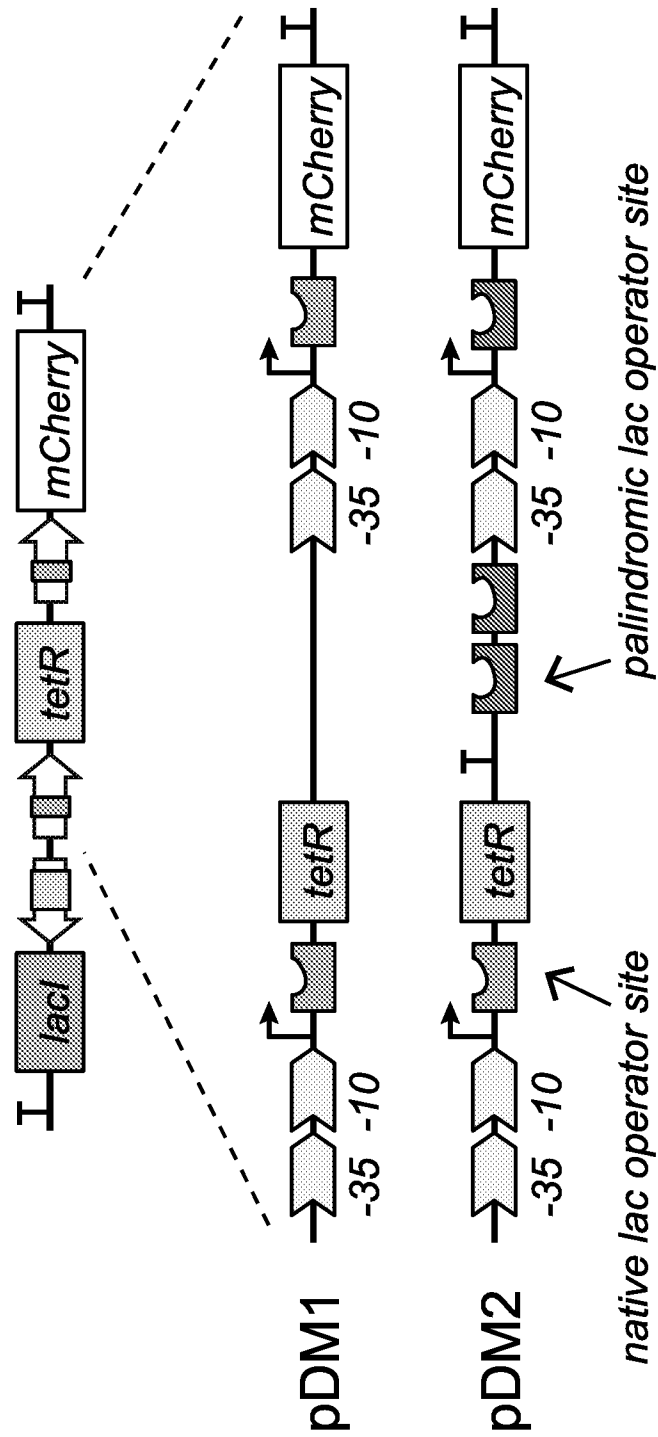


FIG. 9A

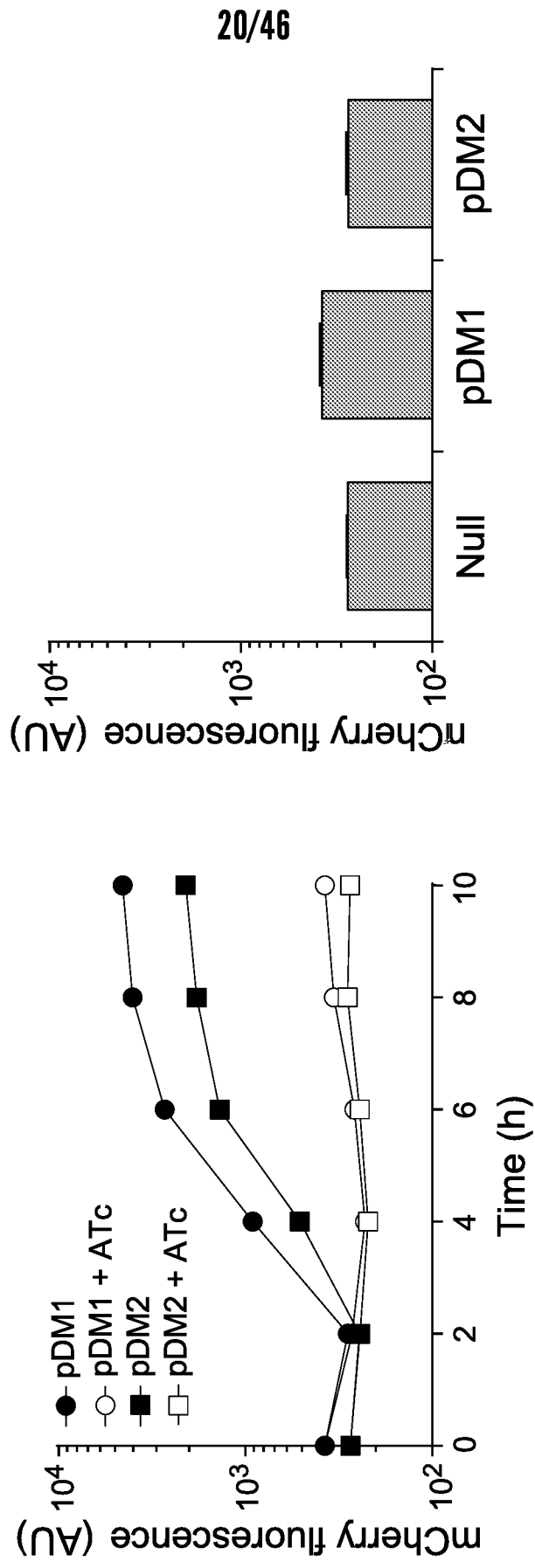


FIG. 9C

FIG. 9B

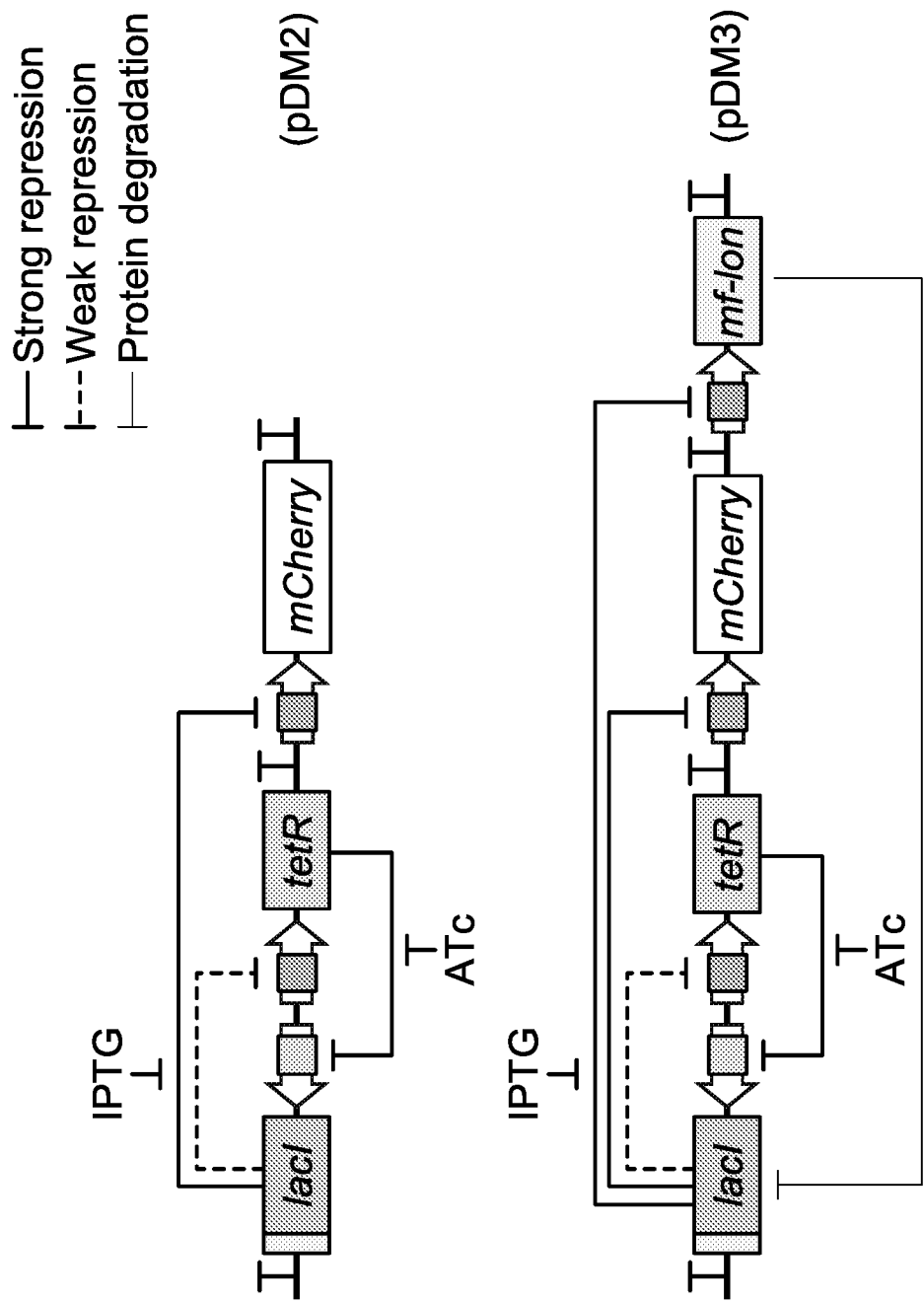


FIG. 10A

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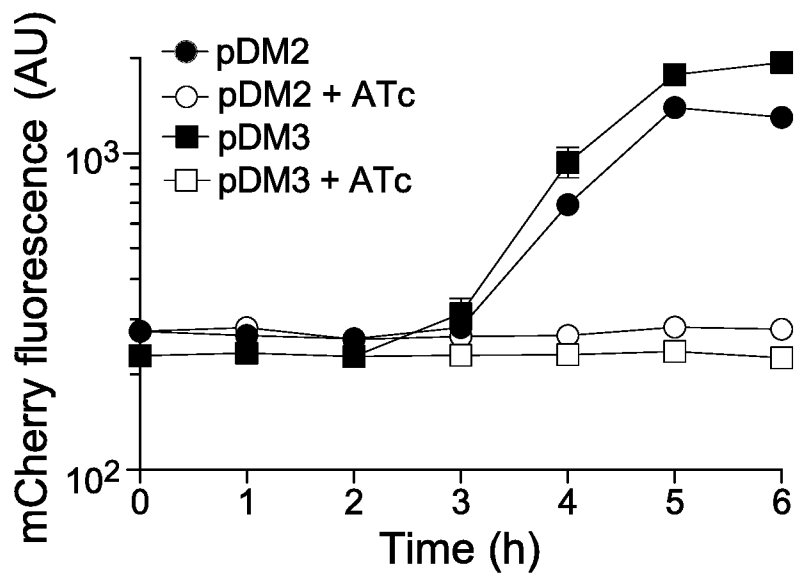


FIG. 10B

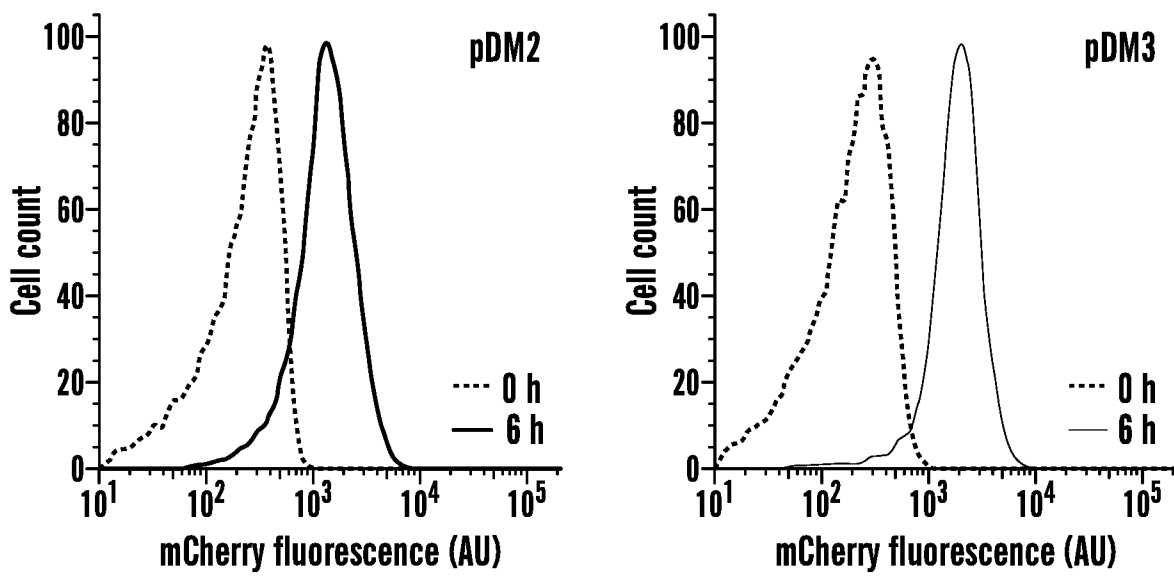


FIG. 10C

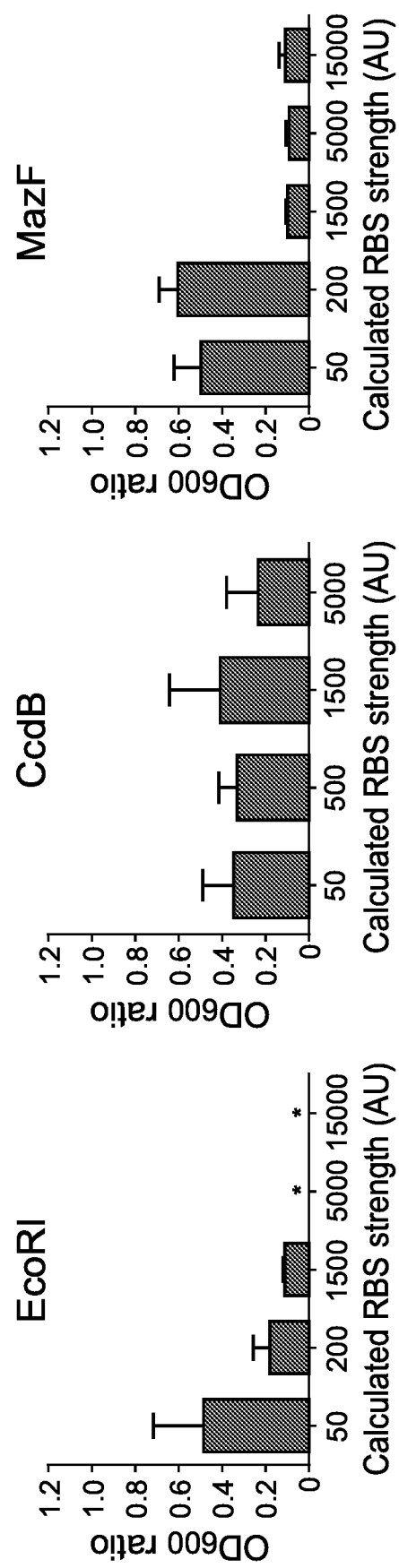


FIG. 11A

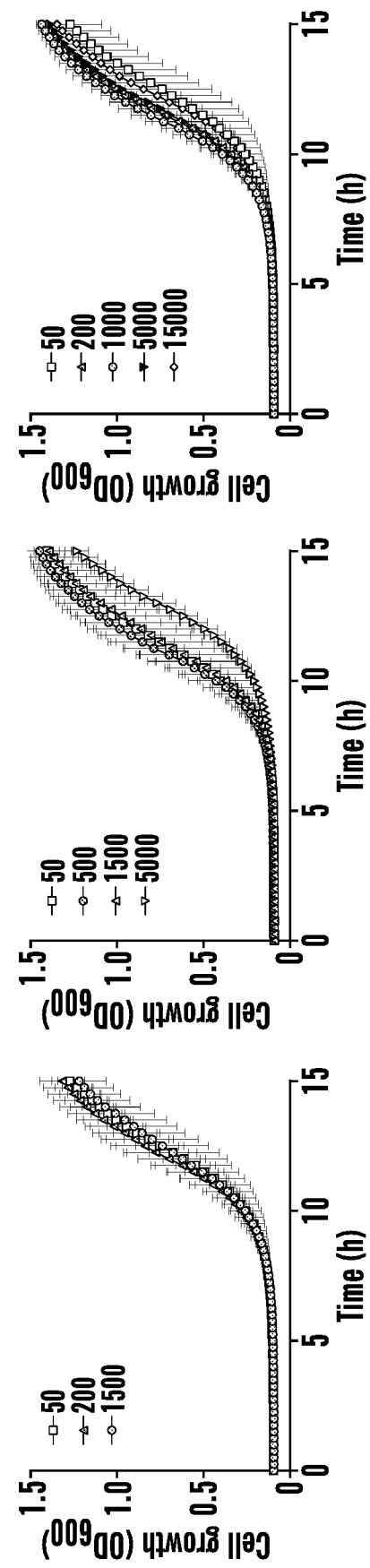


FIG. 11B

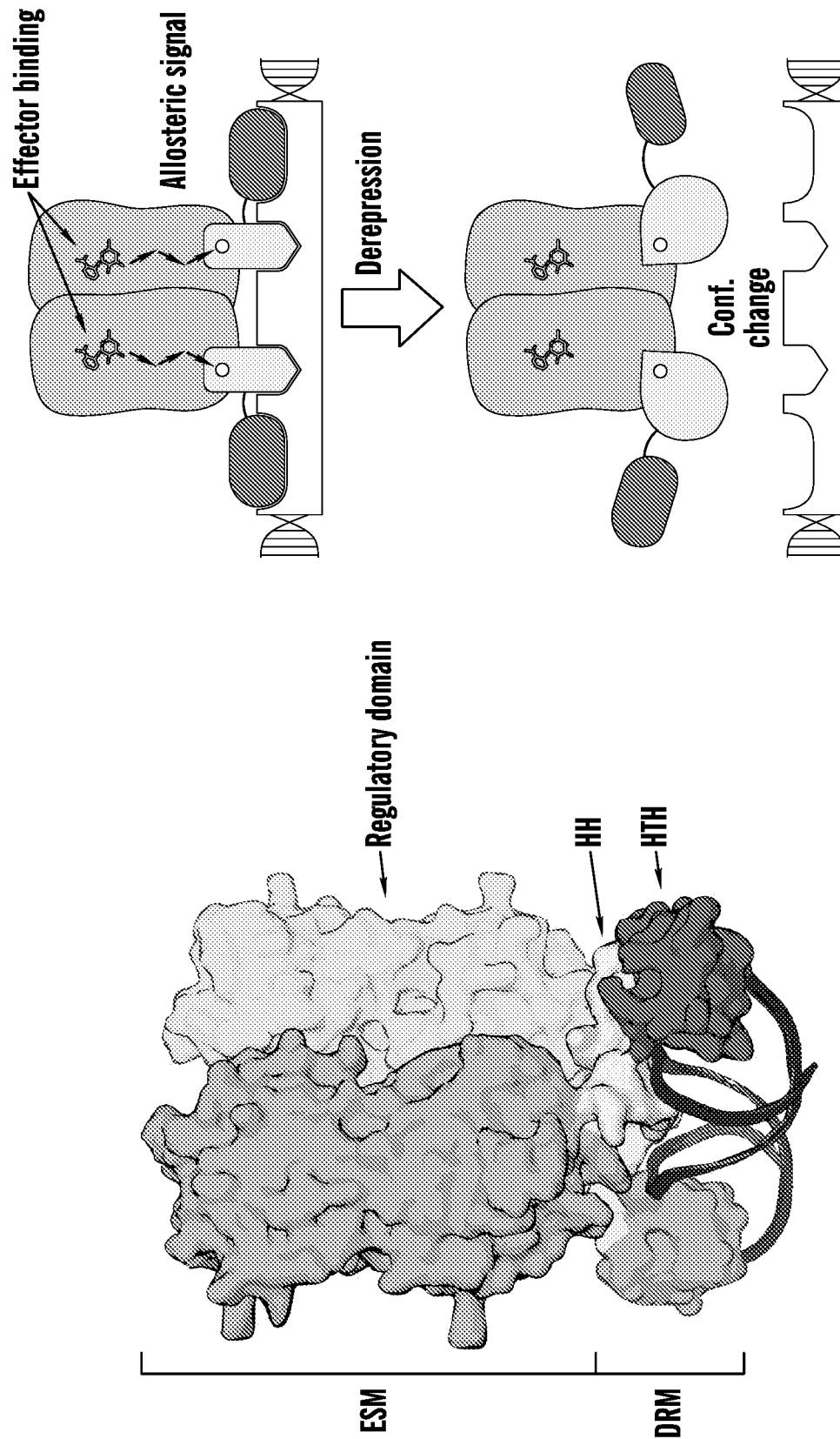


FIG. 12

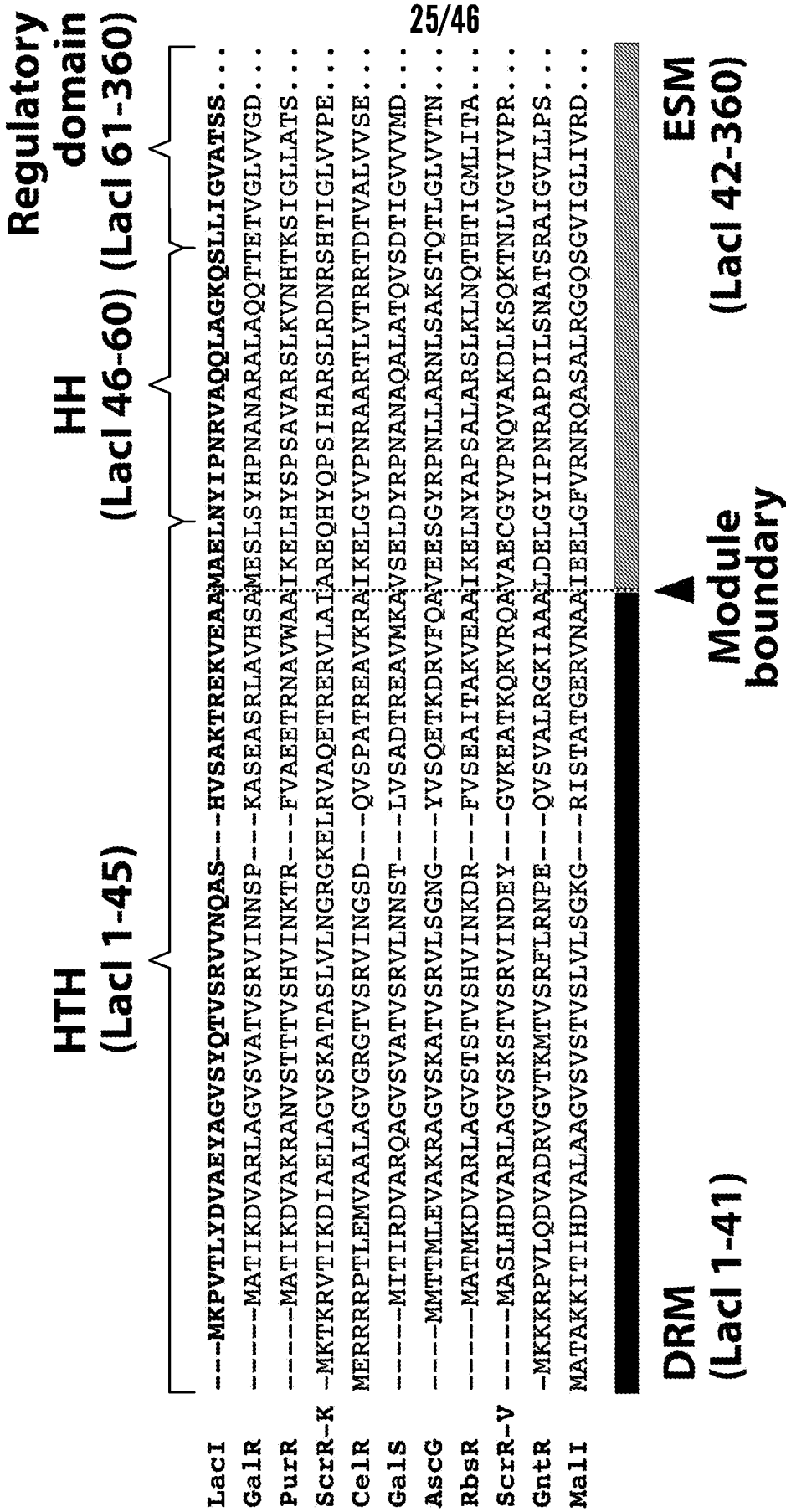


FIG. 13

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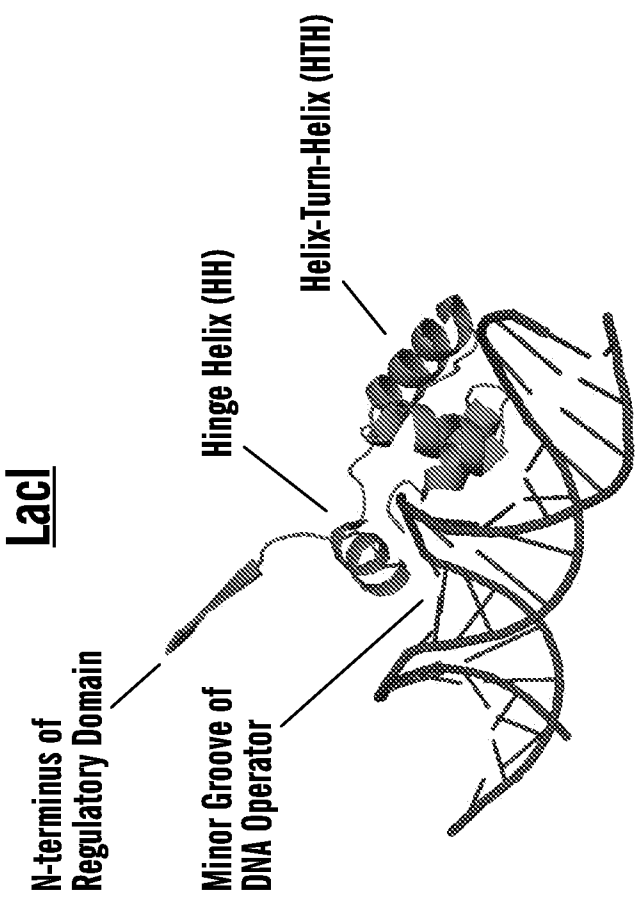
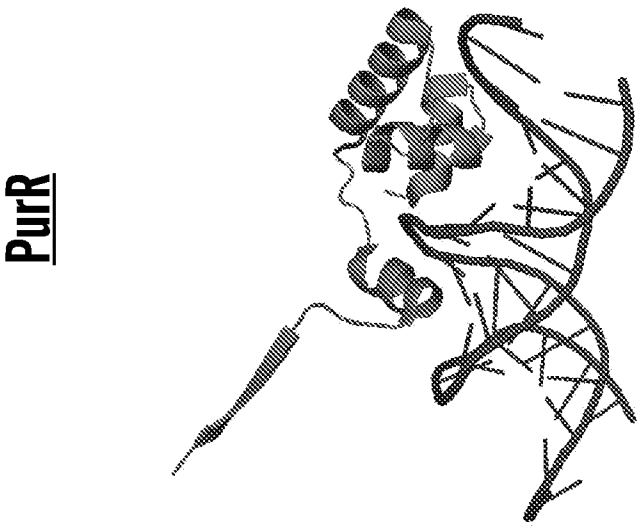


FIG. 14

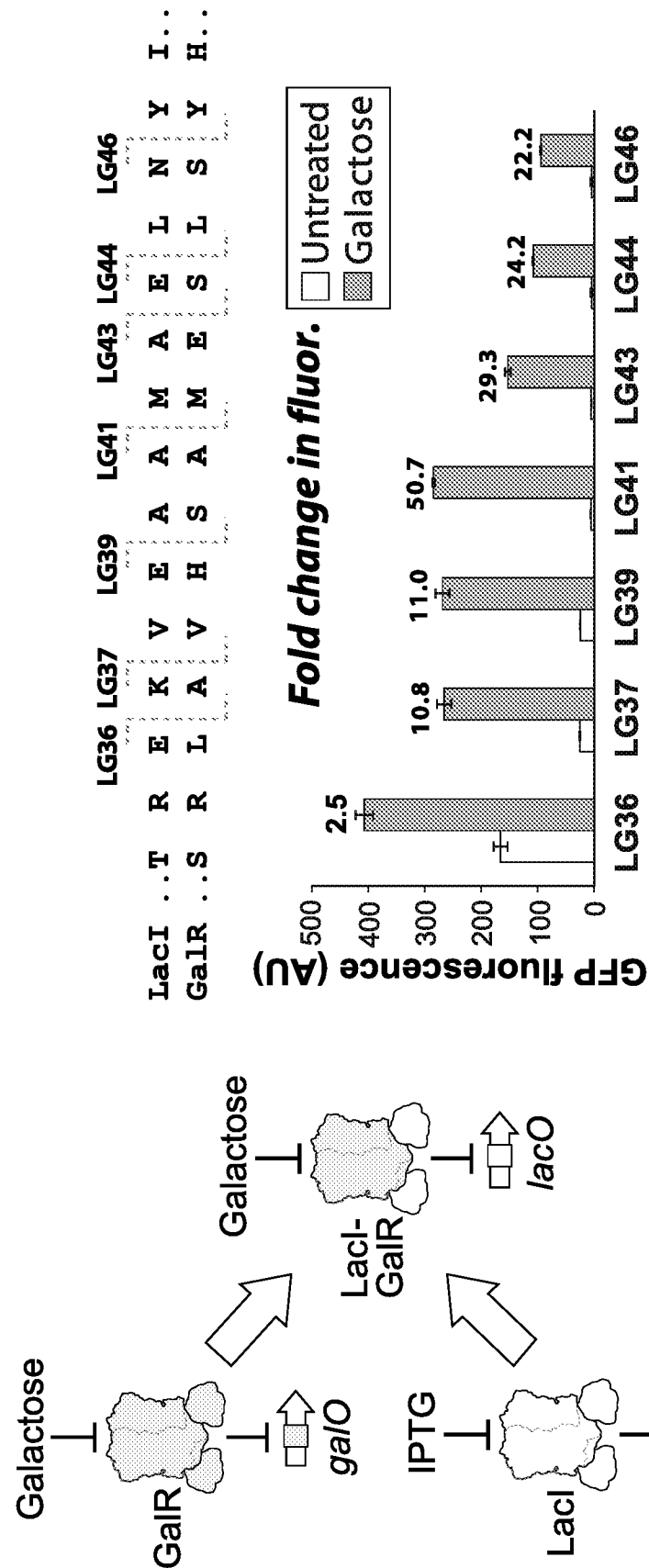
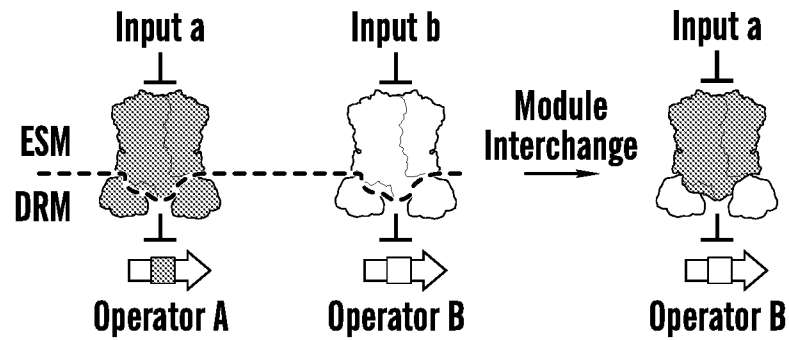
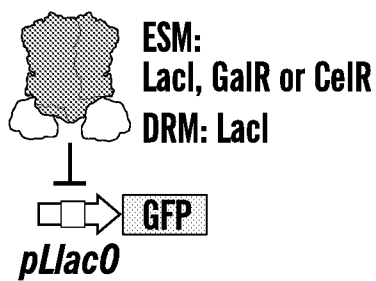
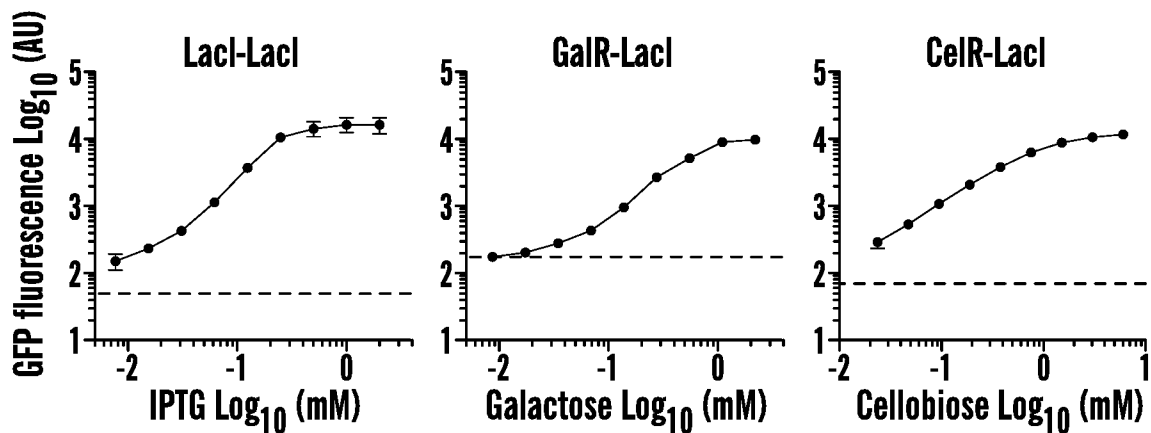


FIG. 15

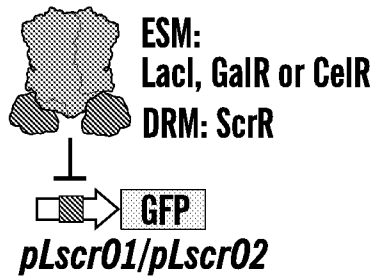
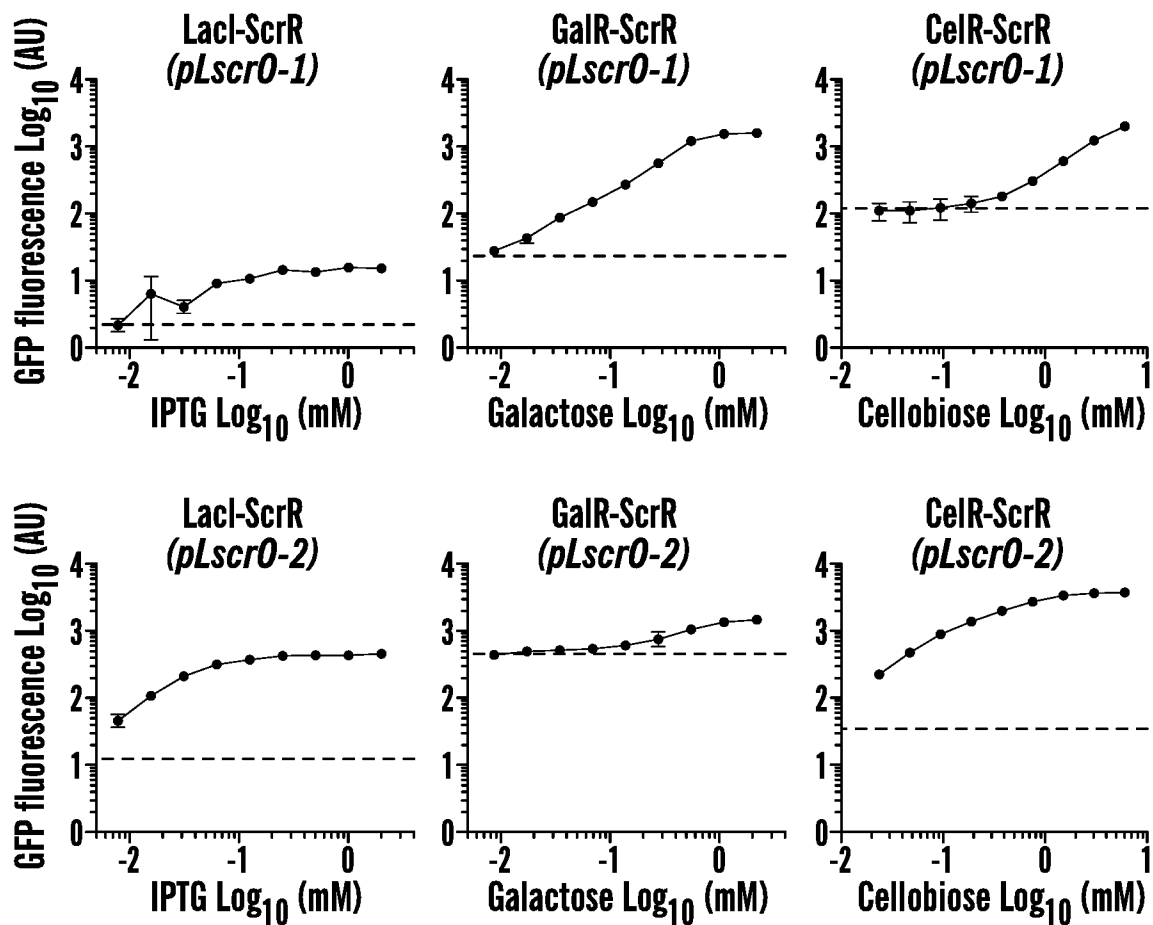
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**FIG. 16A***pLlac0*:

5' -TTGACAATTGTGAGCGCTCACAAGATACT- 3'
 -35 box *lac0* operator -10 box

**FIG. 16B**

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*pLscr0-1:*5' -TTATTAAACCGGTTTAGCAT**TTGACA**ATTAAACCGGTTTAGCAGATACT - 3'*pLscr0-2:*5' -TTATTAAACCGGTTTA---**TTGACA**---TAAACCGGTTTAGCAGATACT - 3'*scr0 operator**-35 box**scr0 operator**-10 box***FIG. 16C**

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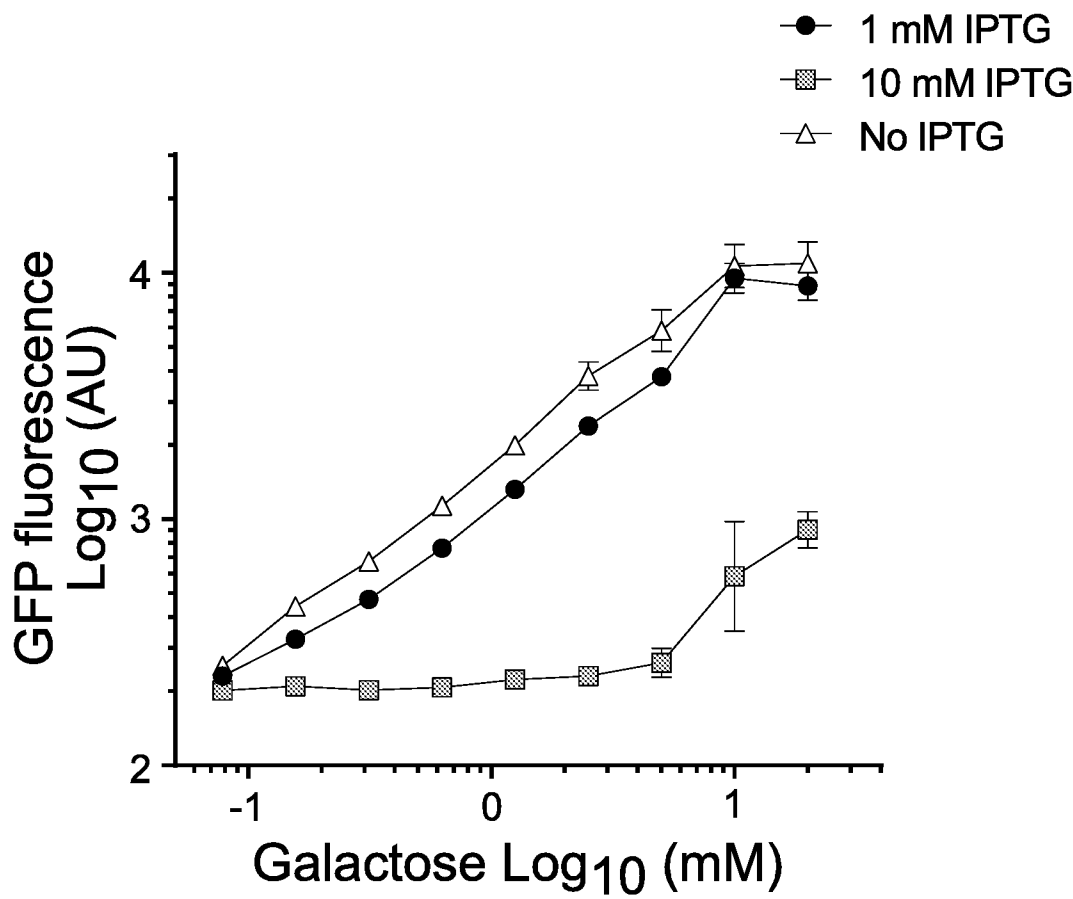
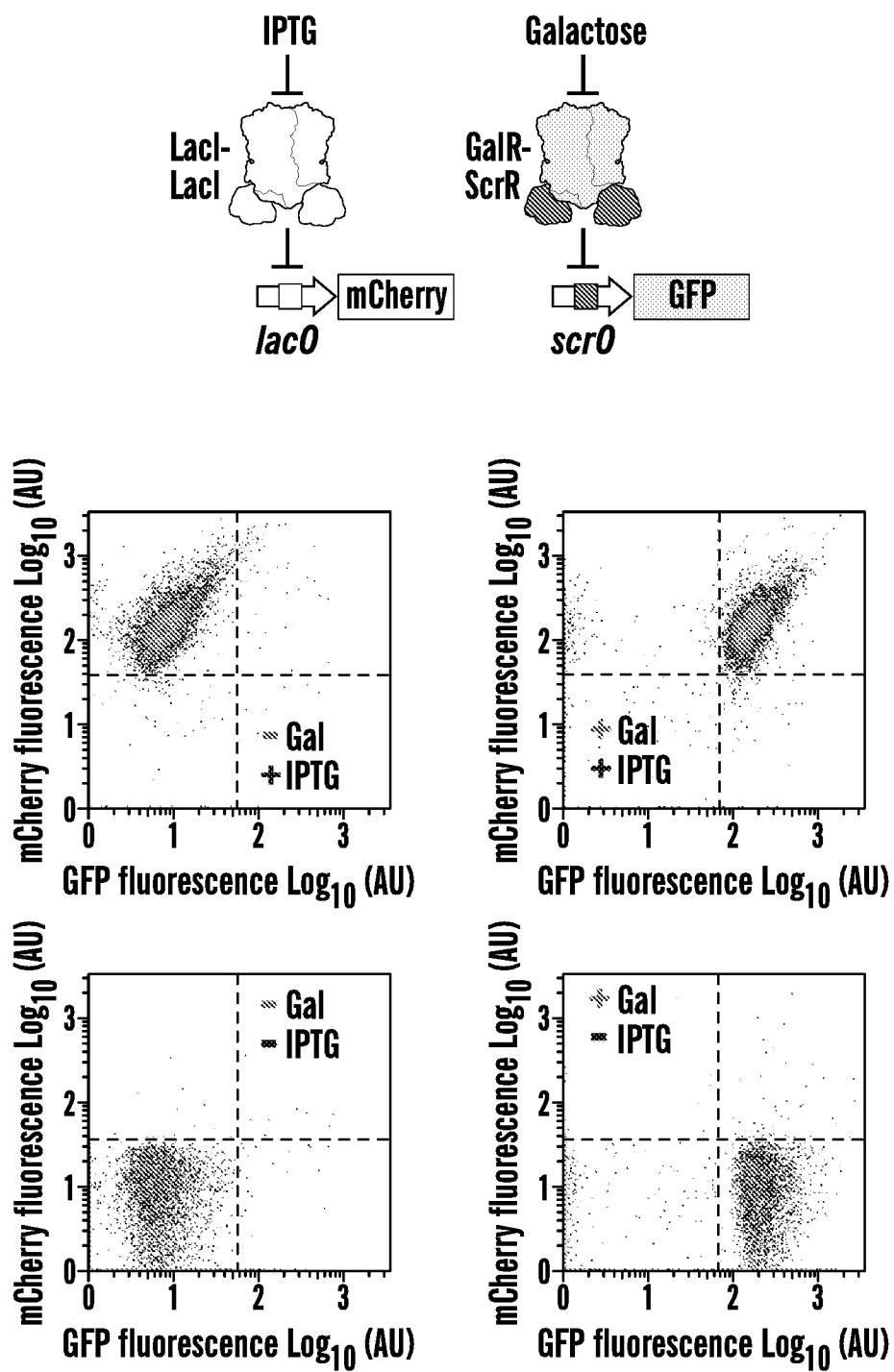


FIG. 17

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**FIG. 18**

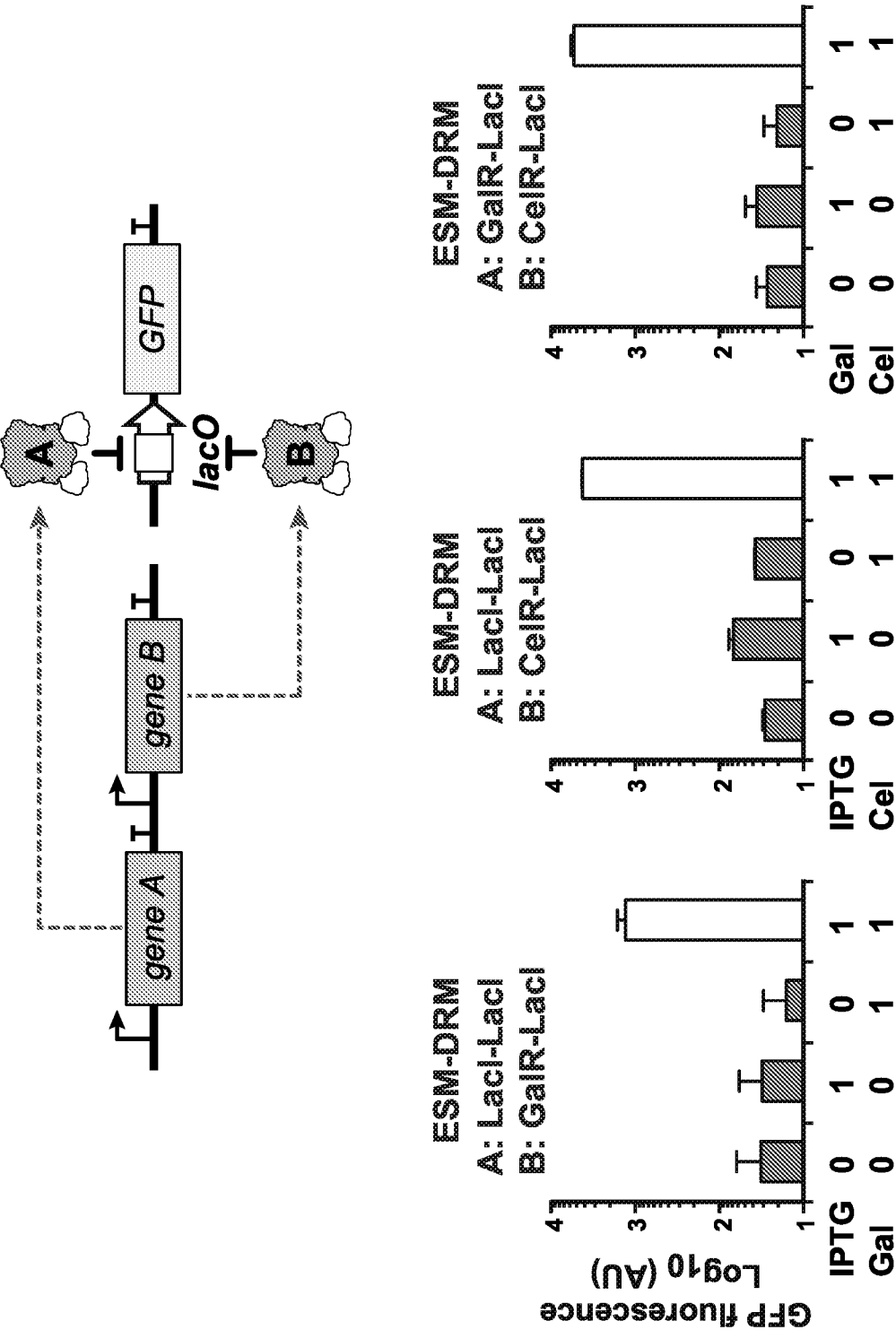


FIG. 19

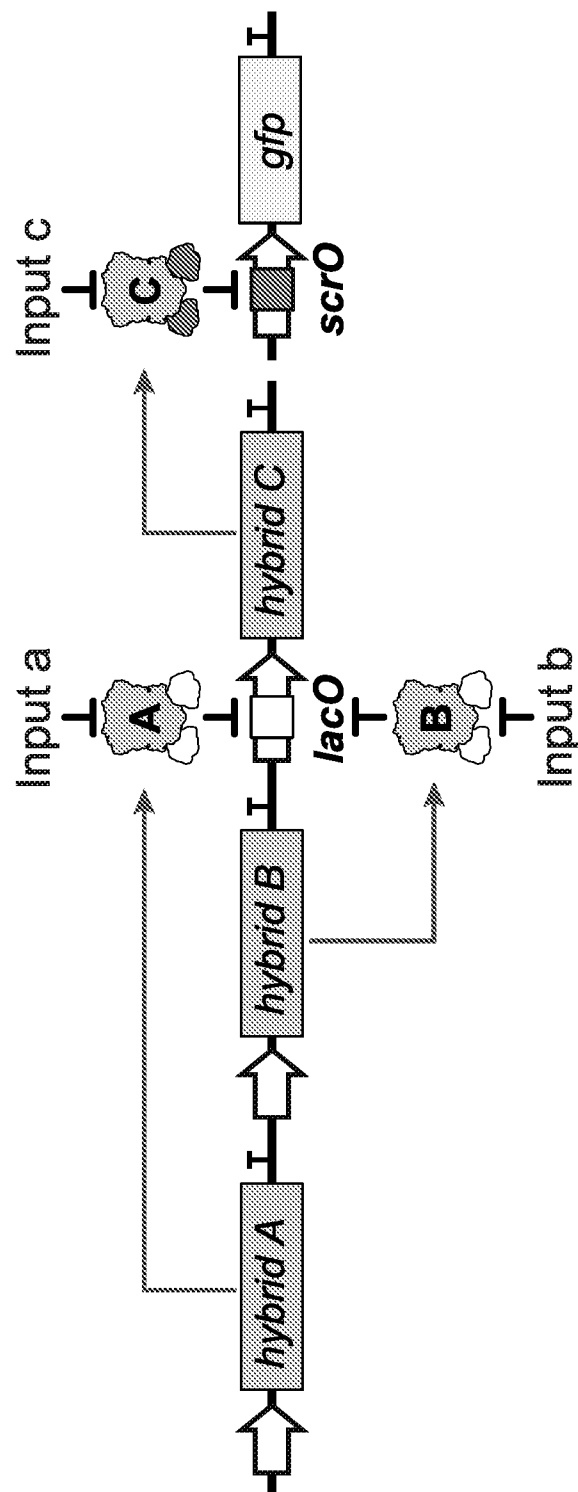


FIG. 20A

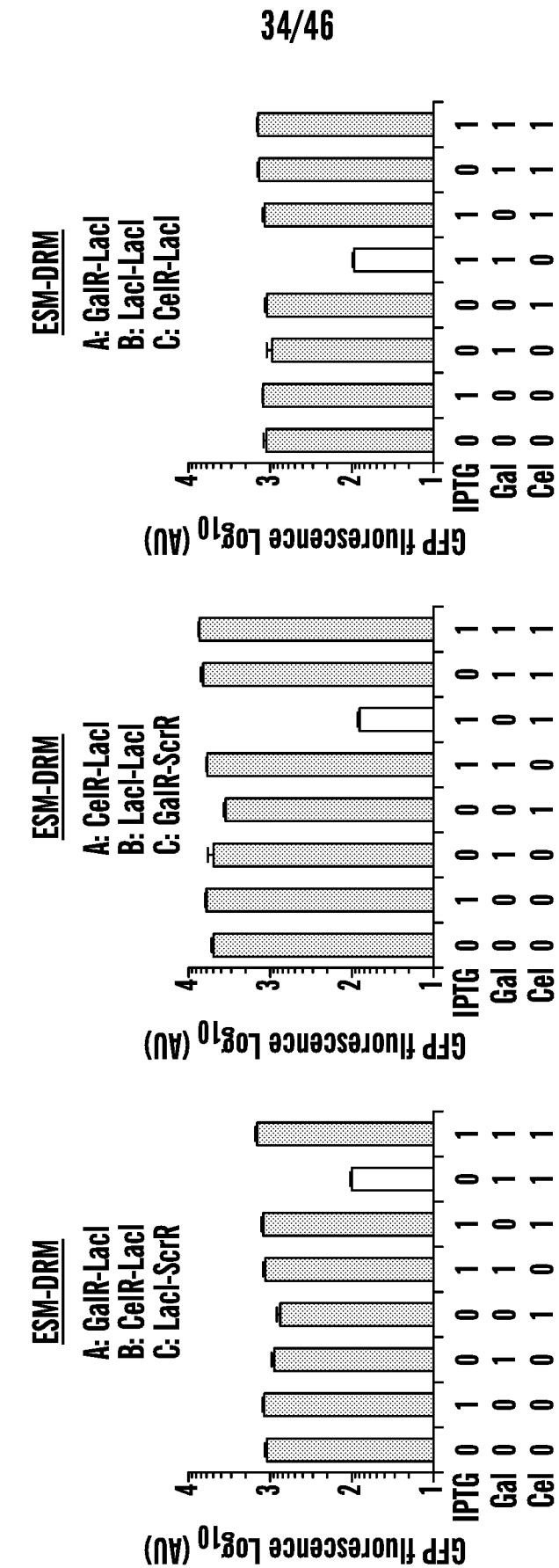


FIG. 20B

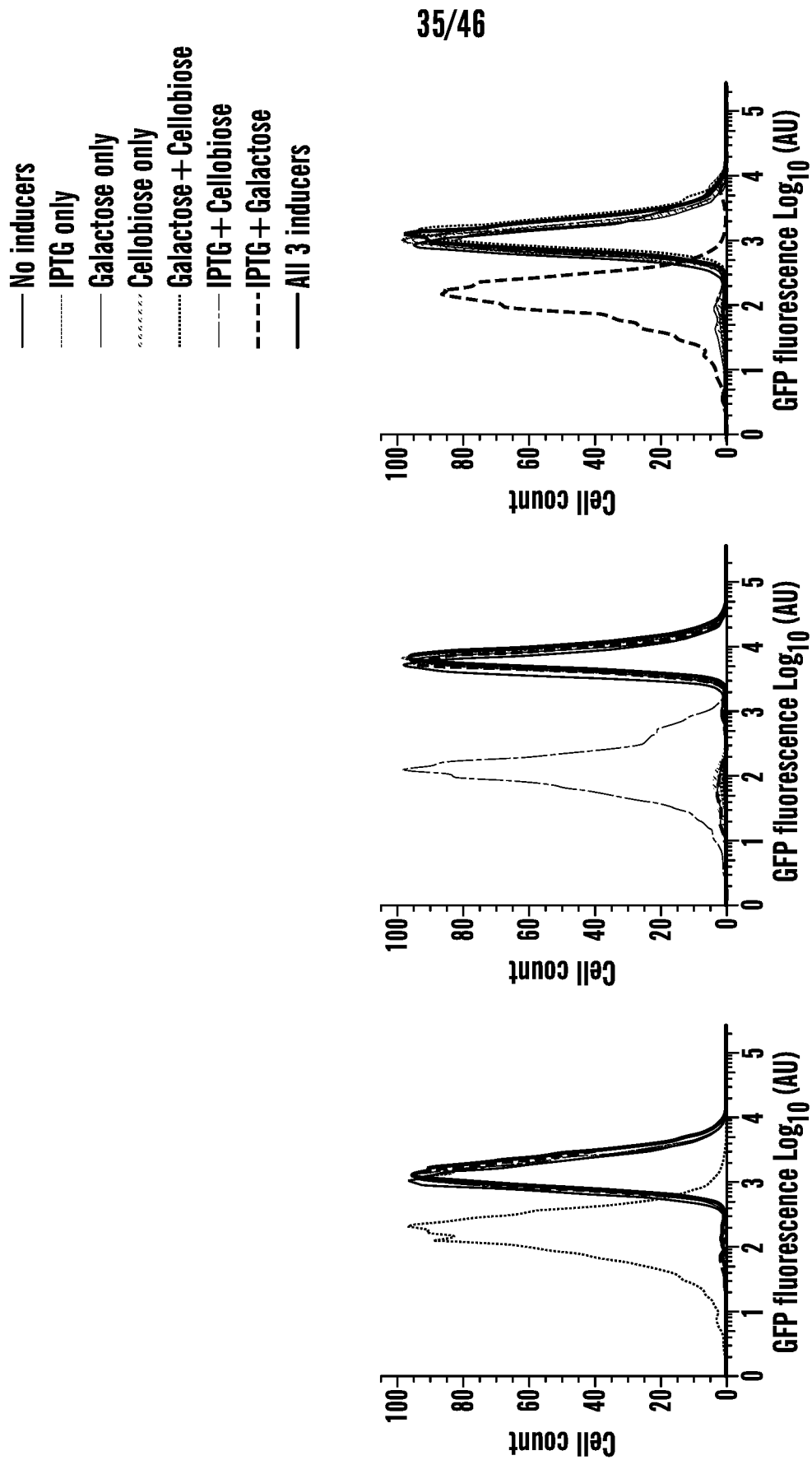


FIG. 20C

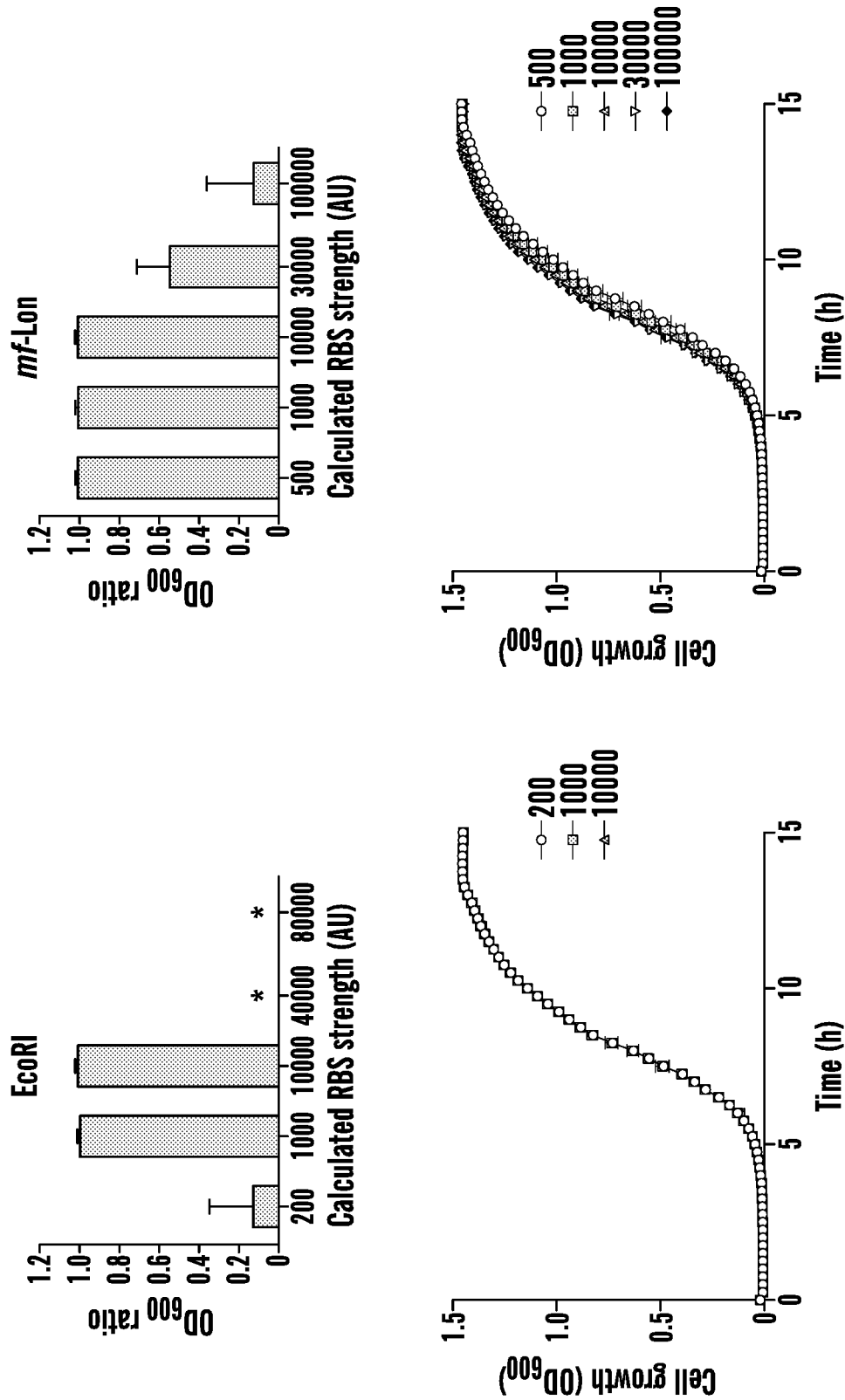


FIG. 21

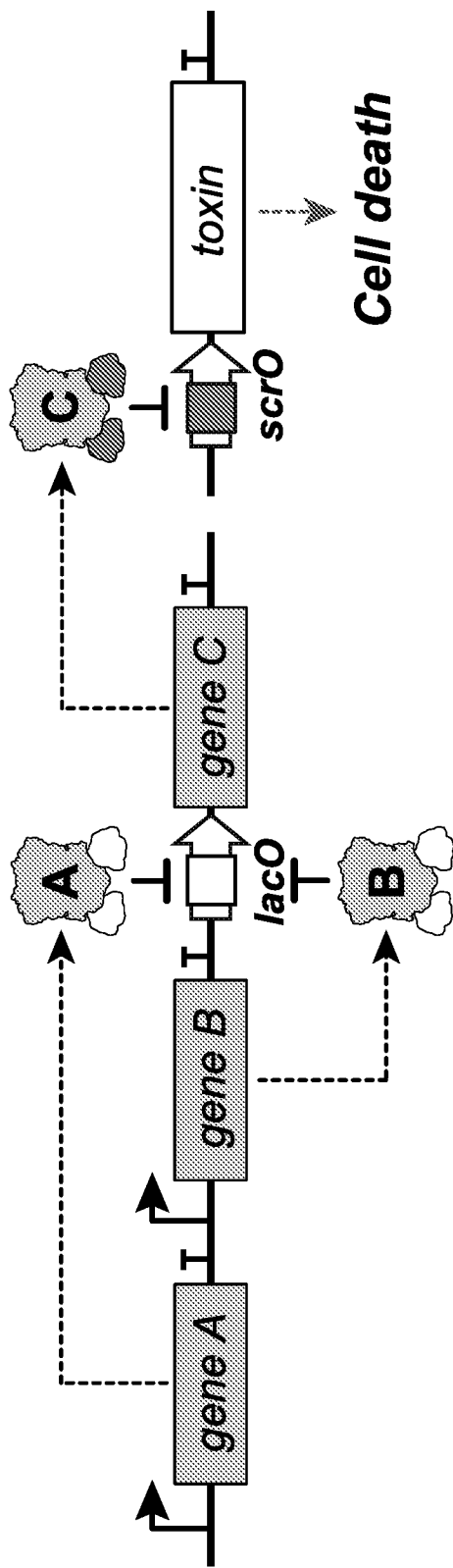


FIG. 22

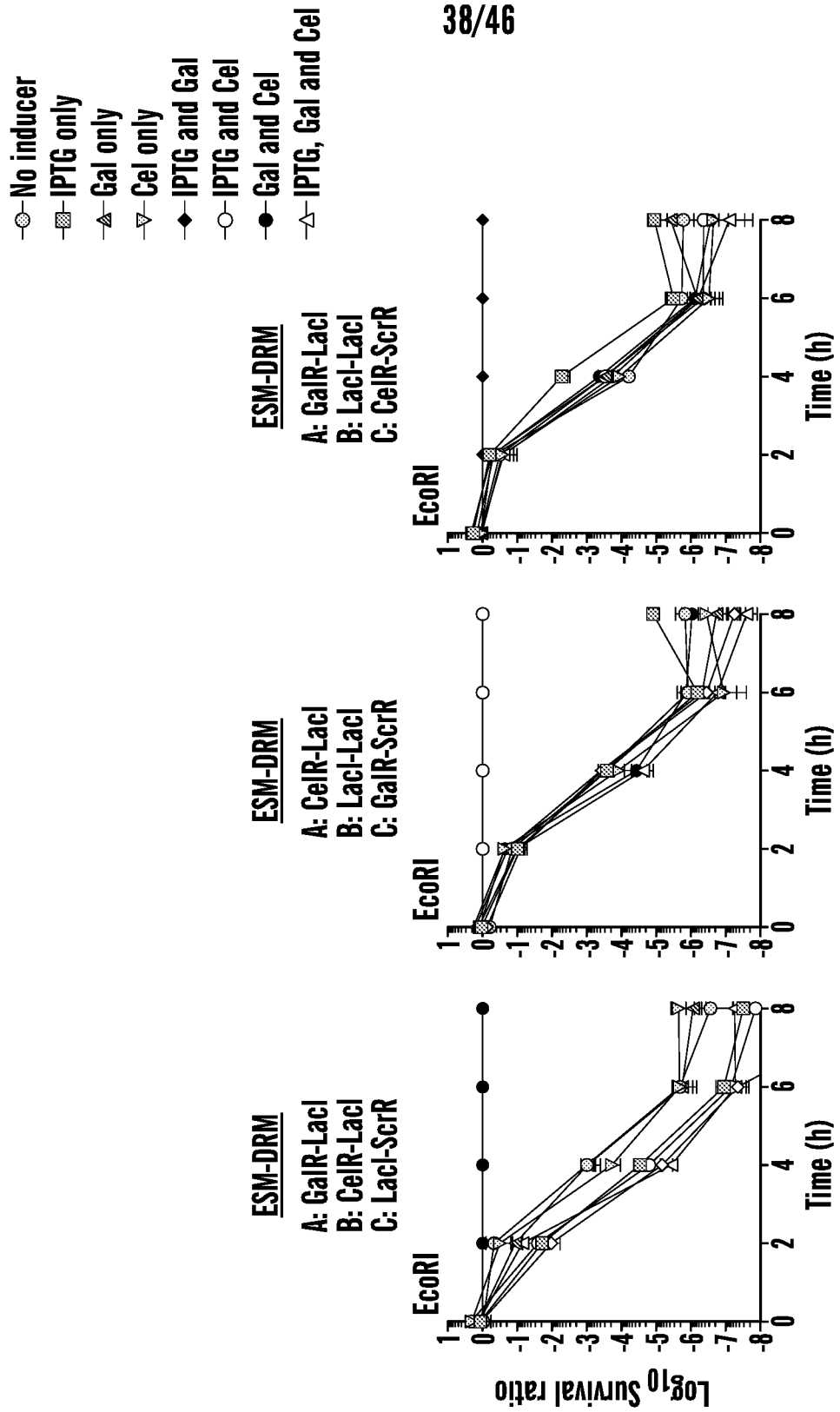


FIG. 22 (cont.)

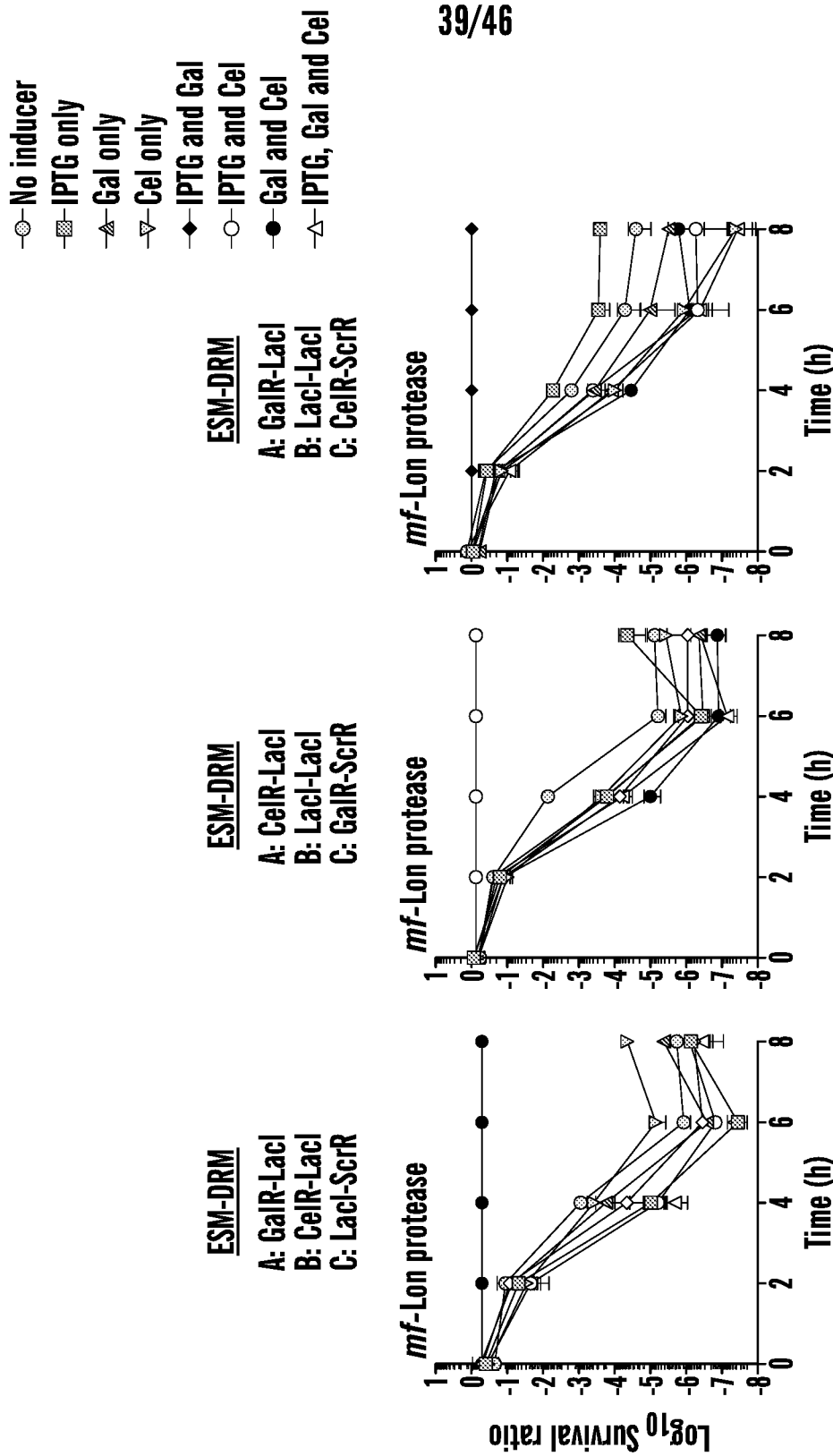


FIG. 22 (cont.)

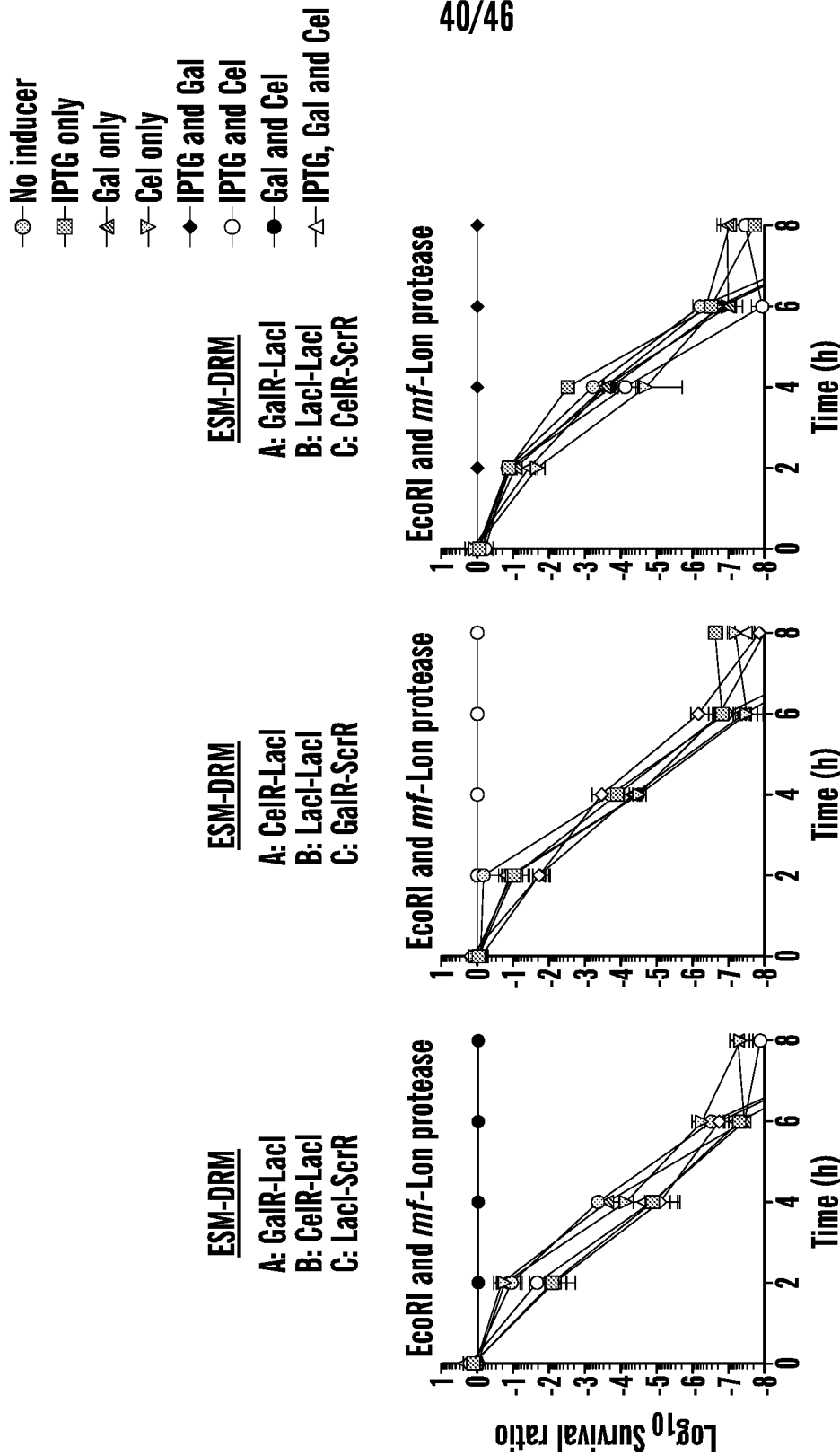


FIG. 22 (cont.)

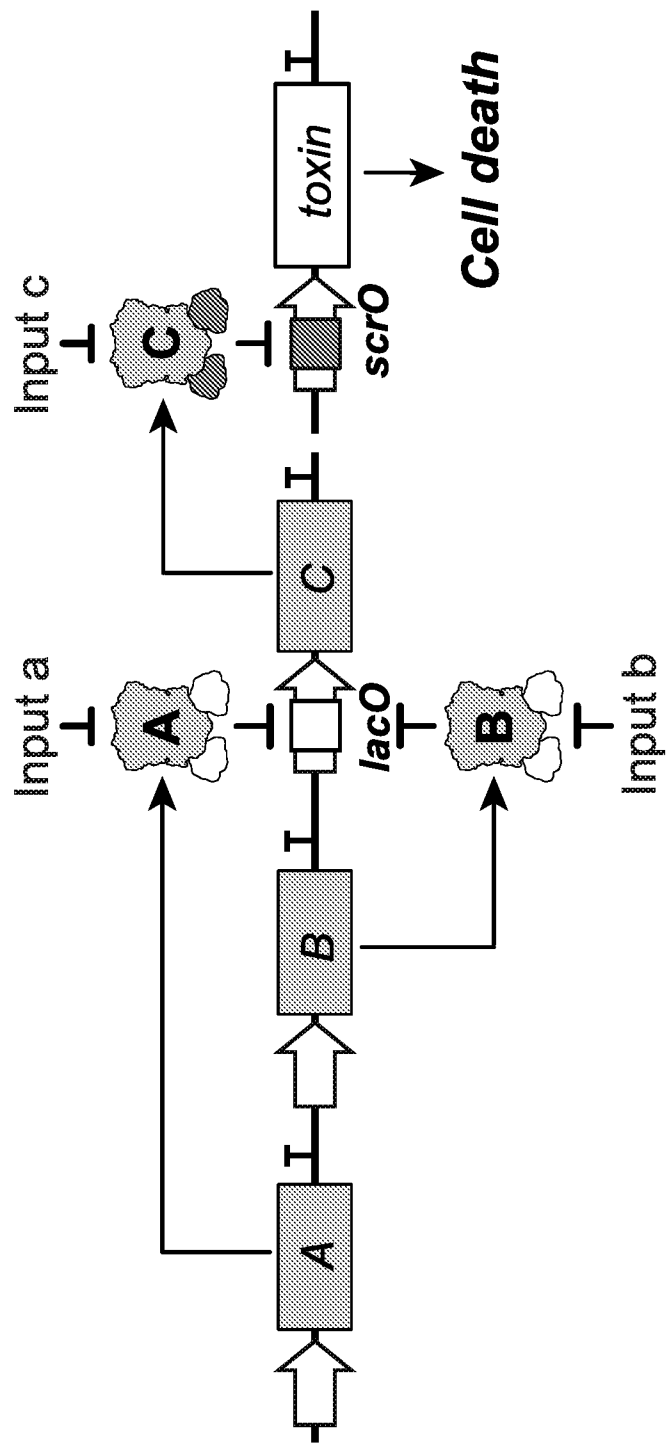


FIG. 23

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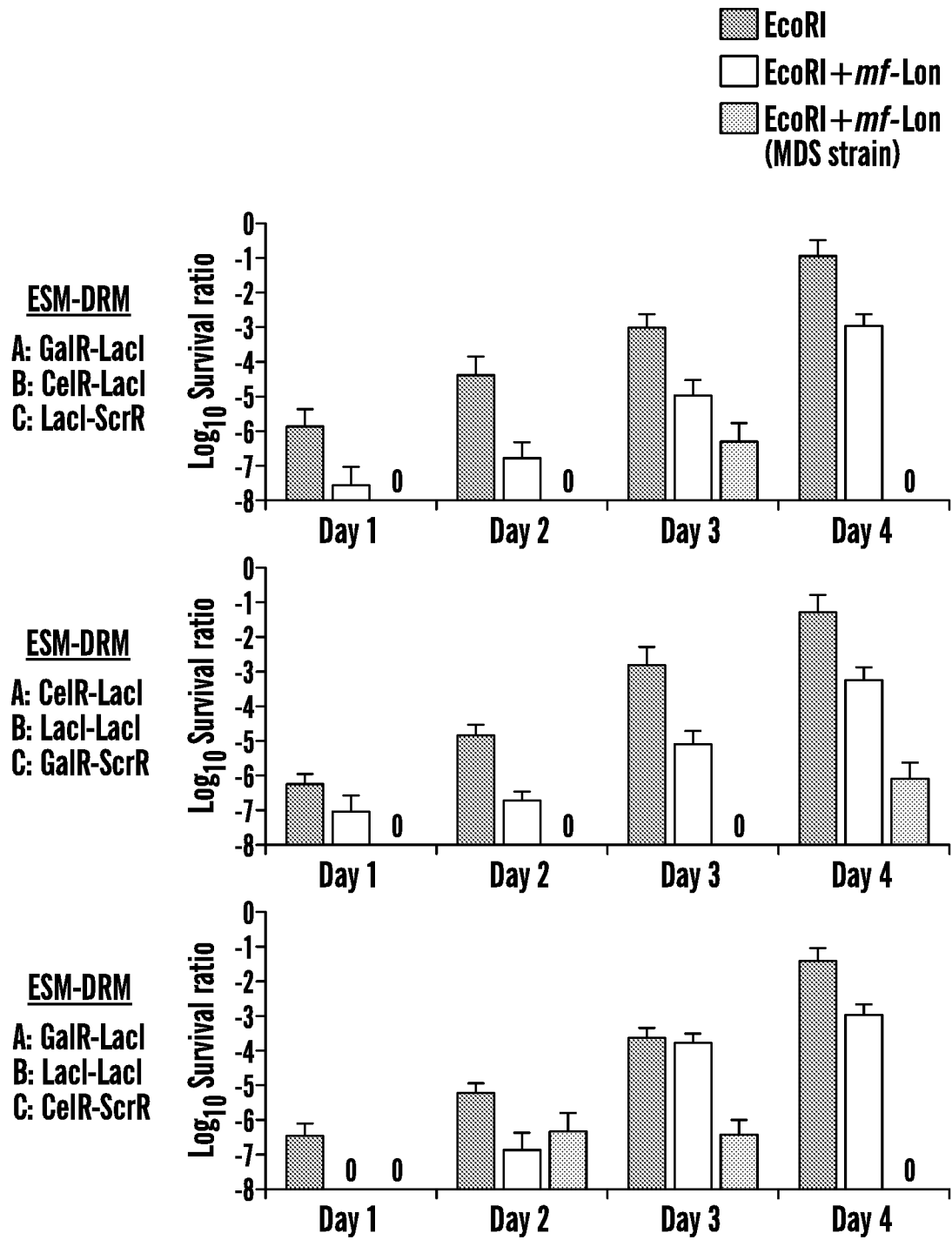


FIG. 23 (cont.)

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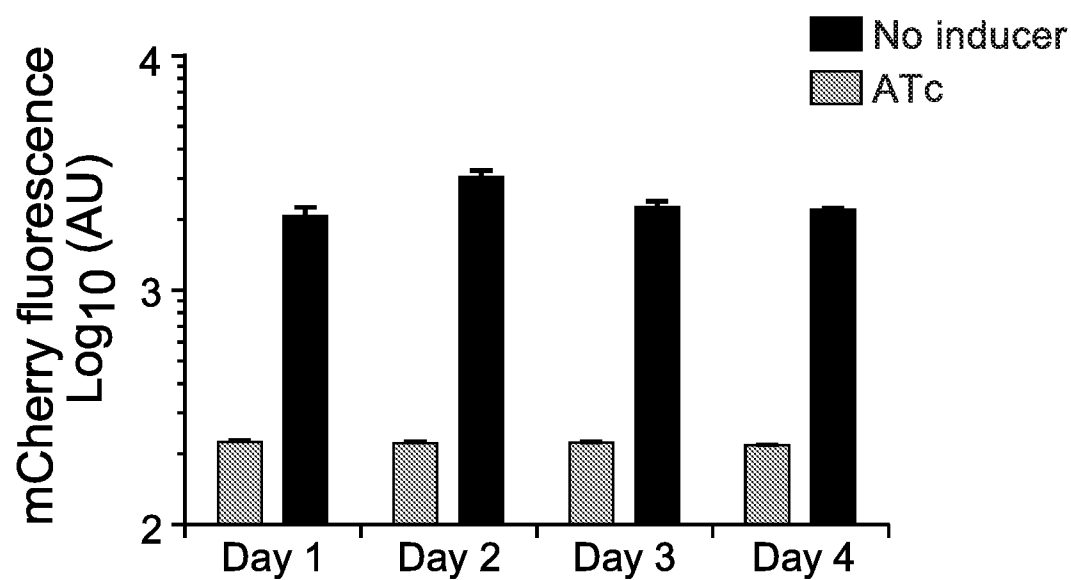


FIG. 24A

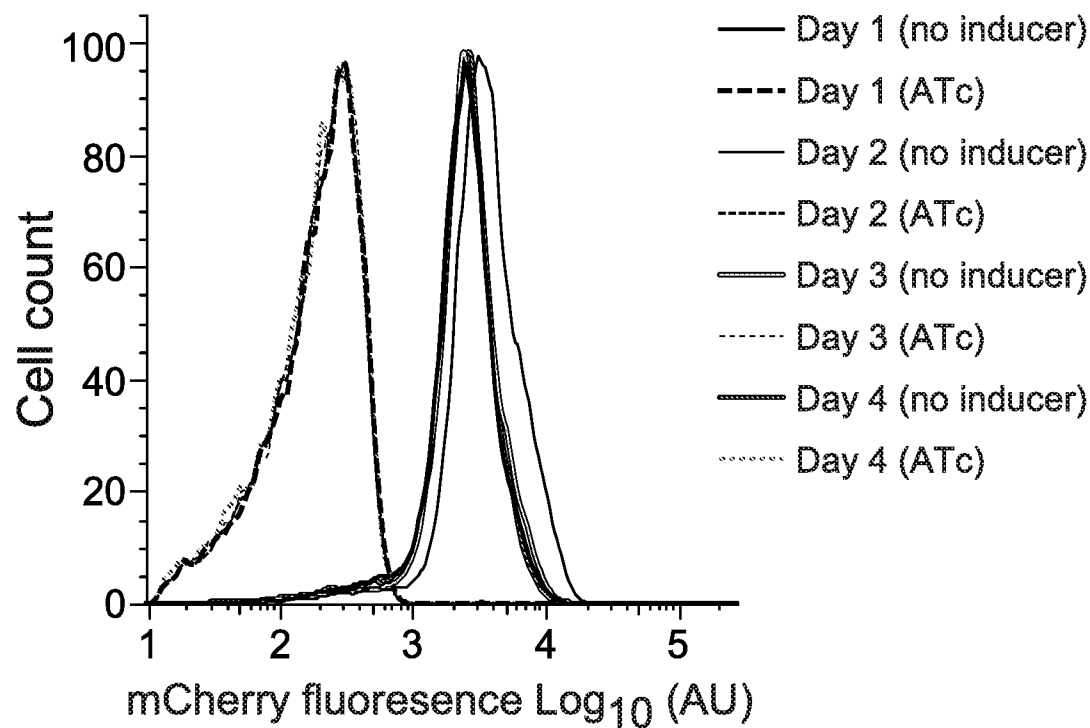


FIG. 24B

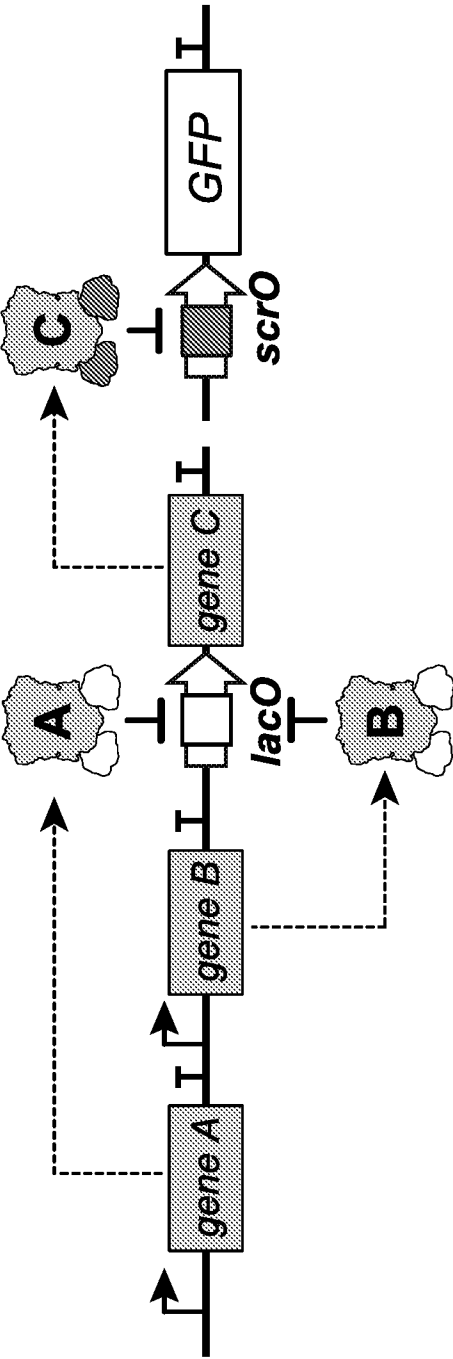


FIG. 25

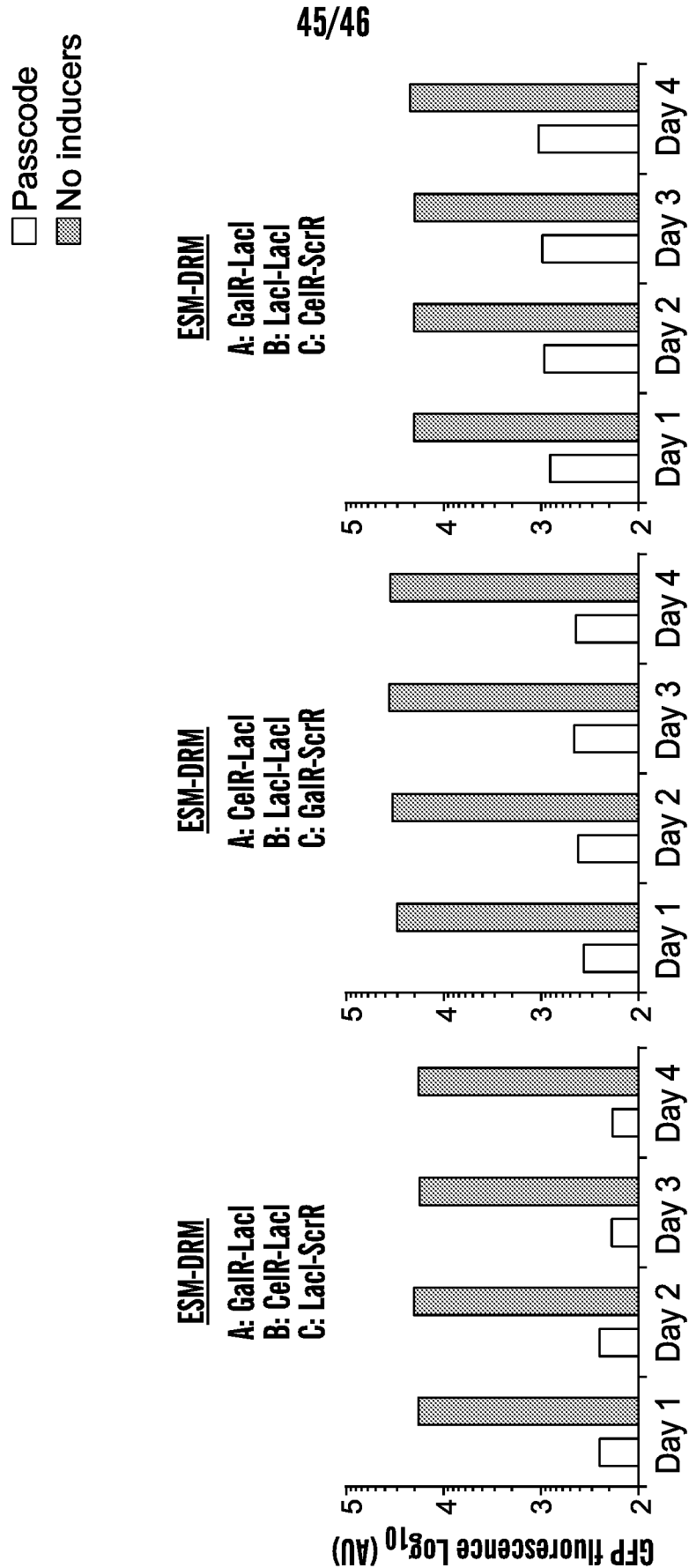


FIG. 25 (cont.)

- Day 1 (Passcode)
- Day 1 (no inducers)
- Day 2 (Passcode)
- Day 2 (no inducers)
- Day 3 (Passcode)
- Day 3 (no inducers)
- Day 4 (Passcode)
- Day 4 (no inducers)

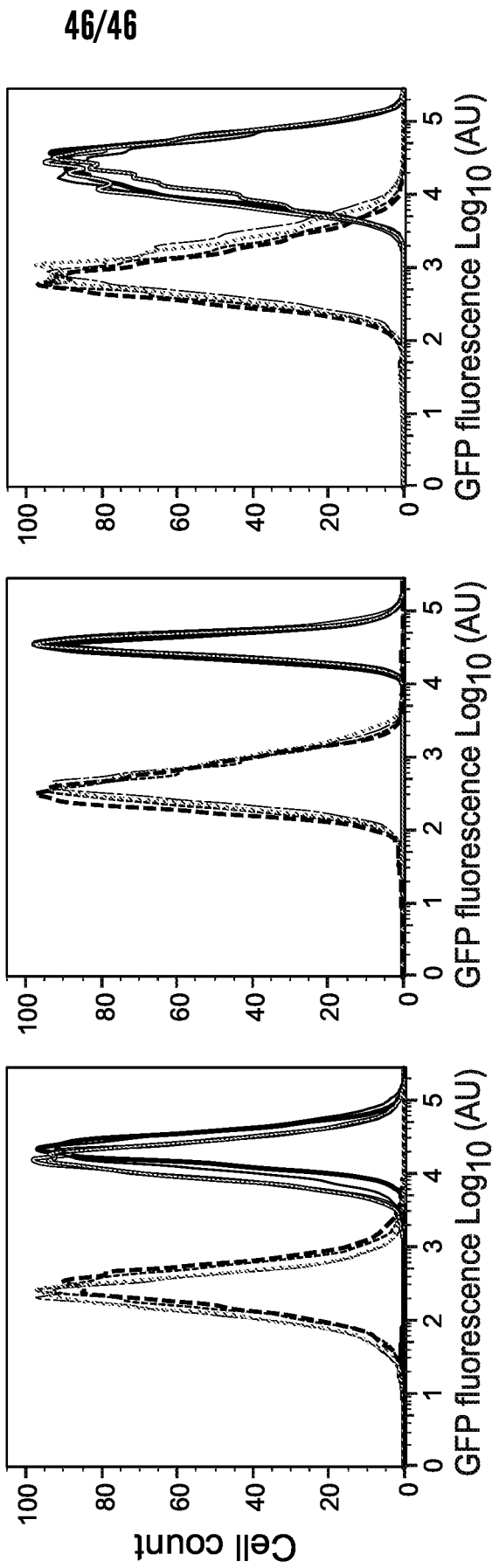


FIG. 25 (cont.)