(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 September 2017 (14.09.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/155945 Al

(51) International Patent Classification:
C12N 15/10 (2006.01) *CI2P 17/08* (2006.01)
C12N 15/80 (2006.01) *CI2P 17/18* (2006.01)

(21) International Application Number:
PCT/US20 17/02 1087

(22) International Filing Date:
7 March 2017 (07.03.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/305,586 9 March 2016 (09.03.2016) 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, KH, 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).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: METHODS AND SYSTEMS OF CELL-FREE ENZYME DISCOVERY AND OPTIMIZATION

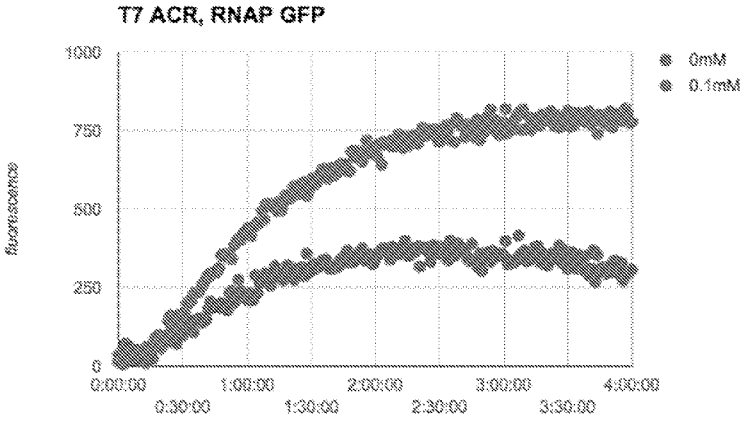

FIG. 1

(57) **Abstract**: Methods and systems of cell-free enzyme discovery and optimization are provided.

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METHODS AND SYSTEMS OF CELL-FREE ENZYME DISCOVERY AND OPTIMIZATION

RELATED APPLICATION DATA

This application claims priority to U.S. Provisional Application No. 62/305,586 filed on March 9, 2016 which is hereby incorporated herein by reference in its entirety for all purposes.

STATEMENT OF GOVERNMENT INTERESTS

This invention was made with government support under DE-FG02-02ER63445 awarded by the U.S. Department of Energy. The government has certain rights in the invention.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 2, 2017, is named 010498_00665US_SL.txt and is 29,599 bytes in size.

FIELD

The present invention relates in general to methods and systems of cell-free enzyme discovery and optimization.

BACKGROUND

Discovery of new enzymatic systems for the production of valuable chemicals is a slow and expensive process. Enzymatic systems are used to turn readily available precursor

compounds into products such as fuels, fine chemicals, pharmaceuticals, agricultural products or even perfumes and fragrances. Enzymatic systems are composed of one or more enzymes that catalyze the necessary series of chemical reactions that take a precursor compound to a product compound. The market for enzyme-catalyzed products currently stands at S150 billion of sales each year and is growing rapidly.

Many times the enzyme necessary for a desired chemical reaction doesn't exist. Other times an enzyme exists but performs sub-optimally. In these situations, it is necessary to make mutations to the enzyme to change its behavior such that it performs at the necessary level. A small enzyme of 300 amino acids can have more variations than there are stars in the universe. If prior knowledge allows an engineer to identify just 5 locations where mutations may be beneficial then the number of possible mutants is reduced to just 100 trillion. Because the number of possible enzyme mutants is so vast and our knowledge of enzyme design so limited the amount of time it takes to find better enzyme variants can be the limiting factor in achieving reasonable product development periods. However, biotechnology firms are strongly motivated to discover new and better enzyme variants because enzyme productivity directly impacts firm profitability.

Because evaluation of enzyme productivity remains the major bottleneck in the discovery of novel and enhanced enzymes this is the most attractive area to innovate in. However, current methods for discovering better enzyme variants rely on low-throughput measurement techniques such as liquid or gas chromatography and mass spectrometry. These primary methods for evaluating enzyme quality require 5-20 minutes of analysis time per sample.

The overall process discovering better enzymes can be summarized as the following steps. First, several mutations are made to the desired enzyme. Next, the enzymes are

produced and reacted with the precursor chemical. Further, each of the individual reactions is measured for product production with a low-throughput technique. Finally, the most successful mutant becomes the starting enzyme for the first step.

Using error prone PCR, degenerate oligonucleotide incorporation, large scale DNA synthesis or other mutation generation strategies allows millions or billions of mutations to be rapidly made to the starting enzyme. However, because the low-throughput measurement techniques require that each enzyme be physically separated from the other enzyme variants it is impossible to evaluate each of these enzymes. At most thousands of enzyme variants can be evaluated in this way per day. While this rate of enzyme evaluation has produced enzymes of astounding value, the possibilities would be staggering if it were feasible to evaluate each of the million to billion enzyme variants generated.

Enzyme evaluation can take place within cells or in cell-free systems. The choice of cellular or cell-free enzyme optimization may depend on enzyme stability, cofactor and precursor availability and product or precursor toxicity, solubility, or transport. When large numbers of cofactors are needed or the enzyme is unstable outside of a cell then the optimization process will be carried out intracellularly. When the product compound is toxic or the enzymes are destined for extracellular use then optimization in a cell-free system is ideal.

When optimization is carried out within the cell, biosensor-based methods exist to enable high-throughput evaluation of enzyme mutants. These biosensors enable millions or billions of cells to be evaluated in a single day. Biosensors are genetically encoded sensors that turn on or off protein production proportional to the amount of product molecule they observe. When the protein they turn on is a fluorescent protein then rapid fluorescence measurement techniques can be used to evaluate a cell for its capacity to produce the product.

When the protein the biosensor turns on is an antidote protein, toxin exposure can be used to find the most productive cells. However no analogous method exists for rapid evaluation of enzyme outside of a cell. There is a great need for a cell-free biosensor-based method that enables high-throughput evaluation of enzyme mutants for enzyme discovery and optimization.

SUMMARY

The present disclosure addresses this need and is based on the discovery that a cell-free biosensor-based method can be used for high-throughput evaluation of enzyme mutants for enzyme discovery and optimization. The present disclosure provides a method of selecting a subset of enzyme variants for the production of a metabolite including providing a plurality of a first nucleotide sequence each encoding a different enzyme variant, providing a precursor molecule wherein the enzyme variants when expressed convert the precursor molecule to the metabolite, providing a second nucleotide sequence encoding a sensor biomolecule, providing a third nucleotide sequence encoding a reporter, wherein the sensor biomolecule when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and screening the enzyme variants by detecting the reporter to identify a subset of enzyme variants. In one aspect, a method of selecting a candidate enzyme variant from a library of enzyme variants for the production of a metabolite is provided. In one embodiment, the method comprises providing a plurality of first nucleotide sequences each encoding a different enzyme variant of the library, providing a precursor molecule wherein the enzyme variant when expressed converts the precursor molecule to the metabolite, providing a second nucleotide sequence encoding a sensor biomolecule, providing a third nucleotide sequence

encoding a reporter, wherein the sensor biomolecule when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and screening the enzyme variants by detecting the reporter to identify the candidate enzyme variant.

The present disclosure provides a method wherein the enzyme variants convert the precursor molecule to the metabolite directly or through one or more intermediate steps. The present disclosure provides a method wherein one or more of the enzyme variants are completely or partially randomized. The present disclosure provides a method wherein the first, second or third nucleotide sequence is DNA or RNA. The present disclosure provides a method wherein the DNA and/or RNA is linear or included on a plasmid. The present disclosure provides a method wherein the nucleotide sequences can be physically separated or attached or any combination thereof. The present disclosure provides a method wherein cofactors are further provided. The present disclosure provides a method wherein the enzyme variants, the sensor biomolecule and the reporter are produced using a cell-free expression system. The present disclosure provides a method wherein the enzyme variants, the sensor biomolecule and the reporter can be produced directly in an evaluation vessel. The present disclosure provides a method wherein the evaluation vessel is in an emulsion or microtiter well format. The present disclosure provides a method wherein the enzyme variants, the sensor biomolecule and the reporter can be produced outside and then combined in an evaluation vessel. The present disclosure provides a method wherein the cell-free expression system comprising commercially available in vitro translation reagents and/or kits. The present disclosure provides a method wherein the subset of enzyme variants are validated by sequencing. The present disclosure provides a method wherein enzyme variants and/or sensor biomolecules are provided. The present disclosure provides a method wherein the

selection process is repeated on the subset of identified enzyme variants for optimization. The present disclosure provides a method wherein the reporter is a fluorescent protein. The present disclosure provides a method wherein the fluorescent protein is GFP. The present disclosure provides a method wherein the reporter is a member selected from the group consisting of mPlum, mCherry, tdTomato, mStrawberry, J-Red, DsRed-monomer, mOrange, mKO, mCitrine, Venus, YPet, EYFP, Emerald, EGFP, CyPet, mCFPm, Cerulean, T-Sapphire, Firefly (FLuc), modified firefly (Ultra-Clo), Click beetle (CBLuc), Sea pansy (RLuc), Copepod crustacean (GLuc), and Ostracod crustacean (CLuc). The present disclosure provides a method wherein the reporter further comprises luciferase for detection by light, pigments for detection by color, surfactants for detection by emulsion breaking, and adhesives for detection by adhesion. The present disclosure provides a method wherein the screening is carried out by fluorescent microscopy, microtiter plate assay, emulsion assay, microfluidic assay, pull-down assay or luciferase high throughput screening. The present disclosure provides a method wherein the sensor biomolecule and the metabolite binding partner is a member pair selected from the group consisting of AcuR/acrylate, cdaR/glucaric acid, ttgR/naringennin, ttgR/phenol, btuB riboswitch/cobalamin, mphR/macrolides, tetR/tetracycline derivates, benM/muconic acid, alkS/medium chain n-alkanes, xylR/xylose, araC/Arabinose, gntR/Gluconate, galS/Galactose, trpR/tryptophan, qacR/Berberine, rmrR/Phytoalexin, cymR/Cumate, melR/Melibiose, rafR/Raffinose, nahR/Salicylate, nocR/Nopaline, clcR/Chlorobenzoate, varR/Virginiamycin, rhaR/Rhamnose, PhoR/Phosphate, MalK/Malate, GlnK/Glutamine, Retinoic acid receptor/Retinoic acid, Estrogen receptor/Estrogen and Ecdysone receptor/Ecdysone. The present disclosure provides a method wherein the sensor biomolecule is a transcription factor, riboswitch, two-component signaling protein, a nuclear hormone receptor, a G-protein coupled receptor, a

periplasmic binding protein, or an engineered protein switch. The present disclosure provides a method wherein the sensor biomolecule is cdaR and the metabolite is a diacid. The present disclosure provides a method wherein the biosensor is an engineered protein switch such as an engineered calmodulin. The present disclosure provides a method wherein the sensor is AcuR and the metabolite is acrylate. The present disclosure provides a method wherein the enzyme is PCS, MIOX, Udh, or INOl. The present disclosure provides a method wherein the precursor molecule is 3-hydroxypropionate. The present disclosure provides a method wherein the reporter protein is an emulsion-breaking protein. The present disclosure provides a method wherein the plurality of the first nucleotide sequences encoding the different enzyme variants are generated by methods comprising gene synthesis, error prone PCR, targeted mutagenesis, or oligonucleotide directed mutagenesis.

The present disclosure further provides a method of identifying a subset of sensor biomolecule variants for a metabolite including providing a plurality of a first nucleotide sequence each encoding a different sensor biomolecule variants, providing a metabolite, providing a second nucleotide sequence encoding a reporter, wherein the sensor biomolecule variant when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and screening the sensor biomolecule variants by detecting the reporter to identify a subset of sensor biomolecule variants. In one aspect, a method of identifying a candidate sensor biomolecule variant from a library of sensor biomolecule variants for a metabolite is provided. The method comprises providing a plurality of first nucleotide sequences each encoding a different sensor biomolecule variant of the library of sensor biomolecule variants, providing a metabolite, providing a second nucleotide sequence encoding a reporter, wherein the sensor biomolecule variant when expressed interacts with the metabolite and induces the

expression of the reporter in a manner dependent on the concentration of the produced metabolite, and screening the sensor biomolecule variants by detecting the reporter to identify the candidate sensor biomolecule variant.

The present disclosure provides a cell-free bio-sensing system for selecting a subset of enzyme variants for the production of a metabolite including a plurality of a first nucleotide sequence each encoding a different enzyme variant, a precursor molecule wherein the enzyme variant when expressed converts the precursor molecule to the metabolite, a second nucleotide sequence encoding a sensor biomolecule, a third nucleotide sequence encoding a reporter,

wherein the sensor biomolecule when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and wherein the enzyme variants are screened by detecting the reporter to identify a subset of enzyme variants. In one aspect, a cell-free bio-sensing system for selecting a candidate enzyme variant from a library of enzyme variants for the production of a metabolite is provided. In one embodiment, the cell-free bio-sensing system comprises a plurality of first nucleotide sequences each encoding a different enzyme variant of the library of enzyme variants, a precursor molecule wherein the enzyme variant when expressed converts the precursor molecule to the metabolite, a second nucleotide sequence encoding a sensor biomolecule, a third nucleotide sequence encoding a reporter, wherein the sensor biomolecule when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and wherein the enzyme variants are screened by detecting the reporter to identify the candidate enzyme variant.

The present disclosure also provides a cell-free bio-sensing system for identifying a subset of sensor biomolecule variants for a metabolite including a plurality of a first

nucleotide sequence each encoding a different sensor biomolecule variant, a metabolite, a second nucleotide sequence encoding a reporter, wherein the sensor biomolecule variant when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and wherein the sensor biomolecule variants are screened by detecting the reporter to identify a subset of sensor biomolecule variants. The present disclosure provides a system wherein the enzyme variants convert the precursor molecule to the metabolite directly or through one or more intermediate steps. The present disclosure provides a system wherein one or more of the enzyme variants are completely or partially randomized. The present disclosure provides a system wherein the first, second or third nucleotide sequence is DNA or RNA. The present disclosure provides a system wherein the DNA and/or RNA is linear or included on a plasmid. The present disclosure provides a system wherein the nucleotide sequences can be physically separated or attached or any combination thereof. The present disclosure provides a system further comprises cofactors. The present disclosure provides a system wherein the enzyme variants, the sensor biomolecule and the reporter are produced using a cell-free expression system. The present disclosure provides a system wherein the enzyme variants, the sensor biomolecule and the reporter can be produced directly in an evaluation vessel. The present disclosure provides a system wherein the evaluation vessel is in an emulsion or microtiter well format. The present disclosure provides a system wherein the enzyme variants, the sensor biomolecule and the reporter can be produced outside and then combined in an evaluation vessel. The present disclosure provides a system wherein the cell-free expression system comprising commercially available in vitro translation reagents and/or kits. The present disclosure provides a system wherein the subset of enzyme variants are validated by sequencing. The present disclosure provides a system wherein enzyme variants and/or sensor

biomolecules are provided. The present disclosure provides a system wherein the selection process is repeated on the subset of identified enzyme variants for optimization. The present disclosure provides a system wherein the reporter is a fluorescent protein. The present disclosure provides a system wherein the fluorescent protein is GFP. The present disclosure provides a system wherein the reporter is a member selected from the group consisting of mPlum, mCherry, tdTomato, mStrawberry, J-Red, DsRed-monomer, mOrange, mKO, mCitrine, Venus, YPet, EYFP, Emerald, EGFP, CyPet, mCFPm, Cerulean, T-Sapphire, Firefly (FLuc), modified firefly (Ultra-Clo), Click beetle (CBLuc), Sea pansy (RLuc), Copepod crustacean (GLuc), and Ostracod crustacean (CLuc). The present disclosure provides a system wherein the reporter further comprises luciferase for detection by light, pigments for detection by color, surfactants for detection by emulsion breaking, and adhesives for detection by adhesion. The present disclosure provides a system wherein the screening is carried out by fluorescent microscopy, microtiter plate assay, emulsion assay, microfluidic assay, pull-down assay or luciferase high throughput screening. The present disclosure provides a system wherein the sensor biomolecule and the metabolite binding partner is a member pair selected from the group consisting of AcuR/acrylate, cdaR/glucaric acid, ttgR/naringennin, ttgR/phenol, btuB riboswitch/cobalamin, mphR/macrolides, tetR/tetracycline derivates, benM/muconic acid, alkS/medium chain n-alkanes, xylR/xylose, araC/Arabinose, gntR/Gluconate, galS/Galactose, trpR/tryptophan, qacR/Berberine, rmrR/Phytoalexin, cymR/Cumate, melR/Melibiose, rafR/Raffinose, nahR/Salicylate, nocR/Nopaline, clcR/Chlorobenzoate, varR/Virginiamycin, rhaR/Rhamnose, PhoR/Phosphate, MalRVMalate, GlnK/Glutamine, Retinoic acid receptor/Retinoic acid, Lacl/allolactose, Estrogen receptor/Estrogen and Ecdysone receptor/Ecdysone. The present disclosure provides a system wherein the sensor biomolecule is a transcription factor,

riboswitch, two-component signaling protein, a nuclear hormone receptor, a G-protein coupled receptor, a periplasmic binding protein, or an engineered protein switch. The present disclosure provides a system wherein the sensor biomolecule is cdaR and the metabolite is a diacid. The present disclosure provides a system wherein the biosensor is an engineered protein switch such as an engineered calmodulin. The present disclosure provides a system wherein the sensor is AcuR and the metabolite is acrylate. The present disclosure provides a system wherein the enzyme is PCS, MIOX, Udh, or INOl. The present disclosure provides a system wherein the precursor molecule is 3-hydroxypropionate. The present disclosure provides a system wherein the reporter protein is an emulsion-breaking protein. The present disclosure provides a system wherein the plurality of the first nucleotide sequences encoding the different enzyme variants are generated by methods comprising gene synthesis, error prone PCR, targeted mutagenesis, or oligonucleotide directed mutagenesis.

In another aspect, a cell-free bio-sensing system for identifying a candidate sensor biomolecule variant from a library of sensor biomolecule variants for a metabolite is provided. In one embodiment, the cell-free bio-sensing system comprises a plurality of first nucleotide sequences each encoding a different sensor biomolecule variant of the library of sensor biomolecule variants, a metabolite, a second nucleotide sequence encoding a reporter, wherein the sensor biomolecule variant when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and wherein the sensor biomolecule variants are screened by detecting the reporter to identify the candidate sensor biomolecule variant.

In some embodiments, the enzyme variants or the first nucleotide sequences encoding the enzyme variants are attached to a solid support for multiplex screening of candidate enzyme variants. In other embodiments, the solid support comprises multiple compartments

in membrane, filter, paper, gel, plate, slide format and the like. In exemplary embodiments, an individual enzyme variant or an individual nucleotide sequence encoding the enzyme variant is trapped in an individual compartment of the multi-compartment solid support. In one embodiment, the enzyme variant is isolated with corresponding precursor molecules and reporter sequences inside an individual compartment. In some embodiments, each individual compartment is immobilized, or temporarily immobilized, within the multi-compartment solid support. In other embodiments, the individual compartment can be sorted by an automated sorting system. In certain embodiments, the individual compartment can be separated from the multi-compartment solid support by manual extraction. In other embodiments, the candidate enzyme variant can be identified based on the known content of each individual compartment, or by targeted sequencing, or in-situ imaging.

It is noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of and "consists essentially of have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

Further features and advantages of certain embodiments of the present invention will become more fully apparent in the following description of embodiments and drawings thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the

Office upon request and payment of the necessary fee. The foregoing and other features and advantages of the present embodiments will be more fully understood from the following detailed description of illustrative embodiments taken in conjunction with the accompanying drawings in which:

FIG. 1 shows fluorescence signal after addition of acrylate.

FIG. 2 shows the result of an in vitro reaction using the addition of purified protein sensor according to an embodiment of the disclosure.

DETAILED DESCRIPTION

The present disclosure provides methods and systems that enable high-throughput enzyme evaluation and discovery to be carried out in cell-free systems. The present disclosure provides a method to make biosensors work without the need for living cells. The biosensors operate in in vitro cell-free systems producing a reporter protein, most commonly a fluorescent protein, in response to the presence of a product compound. The disclosure provides a method that enables enzyme evaluation rates from a thousand up to million enzymes per minute.

Because cells are not involved in the enzyme evaluation process several difficulties in using cell-based systems are obviated. First, problems regarding transport of the precursor molecules into a cell are avoided because there are no membranes in the cell-free system. Second, precursor and product molecule concentrations are not constrained to levels that would be non-toxic to a cell. Many products are highly toxic to cells at the desired production concentration and only by using a cell-free system are these high levels of production attainable. Third, the chemical environment in which the reaction is happening is completely defined allowing the chemical production process to approximate the field of chemistry more than biology.

The process to enable high-throughput cell-free enzyme evaluation can be summarized in the following major steps:

1. Enzyme variants are produced in vitro from DNA or RNA using a mixture of dNTPs, polymerases, translation machinery, cofactors and energy sources.

2. Product precursor and necessary cofactors are introduced to the mixture and incubated for an appropriate period of time.

3. Biosensor protein is added to the mixture with the corresponding reporter DNA.

4. The complete mixture is incubated while reporter is produced at a rate proportional to enzyme productivity.

5. The mixture is evaluated for reporter activity (generally the level fluorescence in the case where the reporter is a fluorescent protein).

At this point the best enzyme variant can be used to generate a series of mutants that can be evaluated in this manner again. The timing of Steps 1 through 3 can be varied to achieve different effects. In Step 3 the biosensor may be added as a purified or crude protein or as genetic material such as DNA or RNA that is then turned into protein by the protein producing system that is already present in the cell-free mixture. The DNA sequences encoding the enzymes, the biosensor and the reporter can be on linear or circular DNA, but must conform to specific design considerations. Cell-free protein expression reagents (polymerases, cofactors, energy sources, etc) can be purchased separately or from any of the commercially available kits.

The cell-free biosensors according to the present disclosure are used to rapidly identify better enzyme variants in micro-titer plates. Three standard 384-well micro-titer plates allow evaluation of more than 1,000 enzyme variants in less than a minute. Each well

of the micro-titer plates contains a different enzyme mutant. The wells that contain enzyme mutants that produce higher amounts of product compound fluorescence more brightly. The micro-titer plate reader readily identifies these wells enabling the scientist to harvest these new and more powerful enzymes. Just one or multiple enzymes can be evolved simultaneously.

The cell-free biosensors according to the present disclosure are also deployed in conjunction with emulsion-based sorting technology to enable enzyme evaluation rates of more than a million enzymes per minute. A master mixture of cell-free translation components, the precursor molecule, reporter DNA and the biosensor is created. The DNA of the enzymes to be evaluated is added to a mixture at a very low concentration. This enzyme DNA is multiplexed such that the individual mutants are mixed together - multiplexing enables a several order magnitude increase in throughput when compared to singleplex analysis. The final evaluation mixture is mixed and allocated to droplet using standard emulsion protocols. Each droplet contains a single enzyme mutant because the concentration of the enzyme DNA is so low in the original mix. The emulsion is incubated for a short period of time resulting in a range of droplet fluorescence intensities. High fluorescence droplets indicate high biosensor activity in those individual droplets. This high biosensor activity is indicative of a high quality enzyme variant that is more productive than other enzyme variants. The droplets can be flowed through an apparatus that evaluates each droplet' s fluorescence and retains only those droplets with predetermined level of fluorescent intensity. These devices are common in the literature. The case above relies on a fluorescent protein as the reporter protein. In the case where the reporter protein is an emulsion-breaking protein the highest quality enzyme variants will be able to break out of the emulsions and can be retained without the need for any droplet sorting.

The cell-free biosensing technique according to the present disclosure also enables the rapid discovery of new biosensors. Biosensor mutants are evaluated for new substrate specificity and response behavior when the variable component of the evaluation mixture is the biosensor itself. In these cases the product molecule is supplied rather than the precursor molecule.

The term "biosensor", as used herein, generally refers to genetically encoded devices that monitor the intracellular concentration of a specific compound. Biosensors produce fluorescence or another readout proportional to the concentration of that compound within the cell.

The term "multiplex", as used herein, generally refers to a process in biology that operates on many distinct elements (e.g., cells, DNA molecules or metabolites) that coexist in space and time. Multiplexing enables a single process to work on millions of elements with the same effort that would be required to carry out the process on a single element.

Sensor/Metabolite Pairs

According to certain aspects, known sensor/metabolite pairs can be used in the fluorescent monitoring methods described herein where the binding of the sensor to the metabolite results in production of fluorescent molecules by the cell-free system. Exemplary known sensor/metabolite pairs include those shown in Table 1 below. Others are known in the art.

Table 1.

Sensor Gene	Molecule	Type of Sensor
cdaR	glucaric acid	Transcriptional activator
ttgR	naringennin (flavanoids)	Transcriptional repressor
btuB riboswitch	cobalamin	Riboswitch
mphR	macrolides	Transcriptional repressor

tetR	tetracycline derivates	Transcriptional repressor
benM	muconic acid	Transcriptional activator
alkS	medium chain n-alkanes	Transcriptional activator
xylR	xylose	Transcriptional activator
araC	Arabinose	Transcriptional activator
gntR	Gluconate	Transcriptional repressor
galS	Galactose	Transcriptional repressor
trpR	tryptophan	Transcriptional repressor
qacR	Berberine	Transcriptional repressor
rmrR	Phytoalexin	Transcriptional repressor
cymR	Cumate	Transcriptional repressor
melR	Melibiose	Transcriptional activator
rafR	Raffinose	Transcriptional activator
nahR	Salicylate	Transcriptional activator
nocR	Nopaline	Transcriptional activator
clcR	Chlorobenzoate	Transcriptional activator
varR	Virginiamycin	Transcriptional repressor
rhaR	Rhamnose	Transcriptional repressor
PhoR	Phosphate	Two-component system
MalK	Malate	Two-component system
GlnK	Glutamine	Two-component system
Retinoic acid receptor	Retinoic acid	Nuclear hormone receptor
Estrogen receptor	Estrogen	Nuclear hormone receptor
Ecdysone receptor	Ecdysone	Nuclear hormone receptor

According to certain aspects described herein, sensor/metabolite pairs can be selected based upon the following considerations: (1) the relationship between stimulus strength and circuit activation; (2) the response time of the biosensor to a stimulus; (3) the heterogeneity of biosensor activation between cells in an isogenic population and/or (4) the cross-reactivity with stimuli of other biosensors. Exemplary biosensors are useful DNA binding proteins having a cognate promoter/operator and that are induced by a target compound such as a

metabolite that can be produced enzymatically through metabolic engineering.

It is to be understood that the examples of sensors and their corresponding metabolite binding partners are exemplary only and that one of skill in the art can readily identify additional sensors and their corresponding metabolite binding partners for use in the present disclosure. The transformed microorganism is intended to express the sensors and the metabolite under suitable conditions.

The biosynthetic pathways for production of any particular metabolite binding partner are known to those of skill in the art. The sensor sequence is known to those of skill in the art, such as being based on a published literature search. For example, nucleic acid and amino acid sequences for the above metabolite binding partners / sensors, or the nucleic acid and amino acid sequences for biosynthetic pathways that produce certain metabolites are fully described in the following: cdaR (Monterrubio et al. 2000 J. Bacteriol 182(9):2672-4), tetR (Lutz and Bujard Nucleic Acids Res. 1997 25(6): 1203-10), alkS (Canosa et al. Mol Micriobiol 2000 35(4):791-9), ttgR (Teran, et al. Antimicrob Agents Chemother. 47(10):3067-72 (2003)), btuB riboswitch (Nahvi, et al. Nucleic Acids Res. 32:143-150 (2004)); glucaric acid (Moon, et al. Appl Env Microbiol. 75:589-595 (2009)), naringenin (Santos, et al. Metabolic Engineering. 13:392-400 (2011)), alkanes (Steen, et al. 463:559-562 (2009)), cobalamin (Raux, et al. Cell Mol Life Sci. 57:1880-1893. (2000)), muconic acid (Niu, et al. Biotechnol Prog. 18:201-211. (2002)) each of which are hereby incorporated by reference in its entirety. Methods described herein can be used to insert the nucleic acids into the genome of the microorganism that are responsible for production of sensors, metabolite binding partners and biosynthetic pathways.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described in Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular*

Cloning: A Laboratory Manual, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., (1989) and by Silhavy, T.J., Bennan, M.L. and Enquist, L.W., *Experiments with Gene Fusions;* Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., (1984); and by Ausubel, F.M. et. al., *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience (1987) each of which are hereby incorporated by reference in its entirety.

Additional useful methods are described in manuals including Advanced Bacterial Genetics (Davis, Roth and Botstein, Cold Spring Harbor Laboratory, 1980), Experiments with Gene Fusions (Silhavy, Berman and Enquist, Cold Spring Harbor Laboratory, 1984), Experiments in Molecular Genetics (Miller, Cold Spring Harbor Laboratory, 1972) Experimental Techniques in Bacterial Genetics (Maloy, in Jones and Bartlett, 1990), and A Short Course in Bacterial Genetics (Miller, Cold Spring Harbor Laboratory 1992) each of which are hereby incorporated by reference in its entirety.

Microorganisms may be genetically modified to delete genes or incorporate genes by methods known to those of skill in the art. Vectors and plasmids useful for transformation of a variety of host cells are common and commercially available from companies such as Invitrogen Corp. (Carlsbad, CA), Stratagene (La Jolla, CA), New England Biolabs, Inc. (Beverly, MA) and Addgene (Cambridge, MA).

Typically, the vector or plasmid contains sequences directing transcription and translation of a relevant gene or genes, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcription termination. Both control regions may be derived from genes homologous to the transformed host cell, although it is to be understood that such control regions may also be derived from genes that are not native to the species chosen as a

production host.

Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of driving these genetic elements is suitable for the present invention including, but not limited to, lac, ara, tet, trp, IPL, IPR, T7, tac, and trc (useful for expression in *Escherichia coli* and *Pseudomonas);* the amy, apr, npr promoters and various phage promoters useful for expression in *Bacillus subtilis,* and *Bacillus licheniformis;* nisA (useful for expression in Gram-positive bacteria, Eichenbaum et al. *Appl. Environ. Microbiol.* 64(8):2763-2769 (1998)); and the synthetic Pll promoter (useful for expression in *Lactobacillus plantarum,* Rud et al., *Microbiology* 152:1011-1019 (2006)). Termination control regions may also be derived from various genes native to the preferred hosts.

Certain vectors are capable of replicating in a broad range of host bacteria and can be transferred by conjugation. The complete and annotated sequence of pRK404 and three related vectors-pRK437, pRK442, and pRK442(H) are available. These derivatives have proven to be valuable tools for genetic manipulation in Gram-negative bacteria (Scott et al., Plasmid 50(l):74-79 (2003)). Several plasmid derivatives of broad-host-range Inc P4 plasmid RSF1010 are also available with promoters that can function in a range of Gram-negative bacteria. Plasmid pAYC36 and pAYC37, have active promoters along with multiple cloning sites to allow for the heterologous gene expression in Gram-negative bacteria.

Chromosomal gene replacement tools are also widely available. For example, a thermosensitive variant of the broad-host-range replicon pWVlOl has been modified to construct a plasmid pVE6002 which can be used to create gene replacement in a range of Gram-positive bacteria (Maguin et al., *J. Bacteriol.* 174(17):5633-5638 (1992)). Additionally,

in vitro transposomes are available to create random mutations in a variety of genomes from commercial sources such as EPICENTRE.RTM. (Madison, Wis.).

Vectors useful for the transformation of *E. coli* are common and commercially available. For example, the desired genes may be isolated from various sources, cloned onto a modified pUC19 vector and transformed into *E. coli* host cells. Alternatively, the genes encoding a desired biosynthetic pathway may be divided into multiple operons, cloned onto expression vectors, and transformed into various *E. coli* strains.

The Lactobacillus genus belongs to the Lactobacillales family and many plasmids and vectors used in the transformation of Bacillus subtilis and Streptococcus may be used for Lactobacillus. Non-limiting examples of suitable vectors include pAM.beta.l and derivatives thereof (Renault et al., *Gene* 183:175-182 (1996); and O'Sullivan et al., *Gene* 137:227-231 (1993)); pMBBl and pHW800, a derivative of pMBBl (Wyckoff et al. *Appl. Environ. Microbiol.* 62:1481-1486 (1996)); pMGl, a conjugative plasmid (Tanimoto et al., *J. Bacteriol.* 184:5800-5804 (2002)); pNZ9520 (Kleerebezem et al., *Appl. Environ. Microbiol.* 63:4581-4584 (1997)); pAM401 (Fujimoto et al., *Appl. Environ. Microbiol.* 67:1262-1267 (2001)); and pAT392 (Arthur et al., *Antimicrob. Agents Chemother.* 38:1899-1903 (1994)). Several plasmids from Lactobacillus plantarum have also been reported (van Kranenburg R, Golic N, Bongers R, Leer R J, de Vos W M, Siezen R J, Kleerebezem M. *Appl. Environ. Microbiol.* 2005 March; 71(3): 1223-1230), which may be used for transformation.

Initiation control regions or promoters, which are useful to drive expression of the relevant pathway coding regions in the desired *Lactobacillus* host cell, may be obtained from *Lactobacillus* or other lactic acid bacteria, or other Gram-positive organisms. A non-limiting example is the nisA promoter from *Lactococcus.* Termination control regions may also be derived from various genes native to the preferred hosts or related bacteria.

The various genes for a desired biosynthetic or other desired pathway may be assembled into any suitable vector or vectors, such as those described above. A single vector need not include all of the genetic material encoding a complete pathway. One or more or a plurality of vectors may be used in any aspect of genetically modifying a cell as described herein. The codons can be optimized for expression based on the codon index deduced from the genome sequences of the host strain, such as for *Lactobacillus plantarum* or *Lactobacillus arizonensis.* The plasmids may be introduced into the host cell using methods known in the art, such as electroporation, as described in any one of the following references: Cruz-Rodz et al. *(Molecular Genetics and Genomics* 224:1252-154 (1990)), Bringel and Hubert *(Appl. Microbiol. Biotechnol.* 33: 664-670 (1990)), and Teresa Alegre, Rodriguez and Mesas *(FEMS Microbiology Letters* 241:73-77 (2004)). Plasmids can also be introduced to *Lactobacillus plantatrum* by conjugation (Shrago, Chassy and Dobrogosz *Appl. Environ. Micro.* 52: 574-576 (1986)). The desired biosynthetic pathway genes can also be integrated into the chromosome of *Lactobacillus* using integration vectors (Hols et al. *Appl. Environ. Micro.* 60:1401-1403 (1990); Jang et al. *Micro. Lett.* 24:191-195 (2003)).

Microorganisms which may serve as host cells and which may be genetically modified to produce recombinant microorganisms as described herein may include one or members of the genera *Clostridium, Escherichia, Rhodococcus, Pseudomonas, Bacillus, Lactobacillus Saccharomyces,* and *Enterococcus.* Particularly suitable microorganisms include *Escherichia coli, Bacillus subtilis,* and *Saccharomyces cerevisiae.*

Methods for Screening Using a Reporter

Methods described herein utilize the detection and measurement of detectable reporters. Exemplary detectable reporters include fluorescent molecules or fluorescent proteins. Exemplary fluorescent reporters include those identified in Shaner et al., Nature

methods, Vol. 2, No. 12, pp. 905-909 (2005) hereby incorporated by reference in its entirety. An exemplary list of fluorescent reporters known to those of skill in the art includes mPlum, mCherry, tdTomato, mStrawberry, J-Red, DsRed-monomer, mOrange, mKO, mCitrine, Venus, YPet, EYFP, Emerald, EGFP, CyPet, mCFPm, Cerulean and T-Sapphire, and the like.

Exemplary non-fluorescent, but light emitting reporters include luciferase and its derivatives such as those disclosed in Thorne et al., Chemistry and Biology, vol. 17, issue 6, pp. 646-657 (2010) hereby incorporated by reference in its entirety. An exemplary list of non-fluorescent reporter known to those of skill in the art include Firefly (FLuc), modified firefly (Ultra-Clo), Click beetle (CBLuc), Sea pansy (RLuc), Copepod crustacean (GLuc), Ostracod crustacean (CLuc) and the like.

The disclosure provides biosensors that link metabolite levels to fluorescent protein expression and enable fluorescence-based screens. The biosensor-based screens according to the present disclosure can provide evaluation rates of up to lxlO^9 designs per day. Fluorescent screening can be evaluated with fluorescent plate readers in 96 or 384-well plates and is useful for prototyping the screening system. These cell-free fluorescent screening can be used for the next round of design or chosen for a commercial production system.

Further methods include microtiter plate assays, for example where screening by fluorescence is done robotically in microtiter plates, such as 1536-well plates or 9600 well plates. Such methods may be combined with robotic handling and advanced plate readers typical of high throughput screening. Further methods include emulsion assays, for example, where the reaction can be trapped within emulsions and assayed using microfluidics, such as described in Wang et al., *Nature Biotechnology,* Volume 32, pp. 473-478 (2014) hereby incorporated by reference in its entirety. Other microfluidic assays can be used to evaluate screening such as those described in Guo et al., *Lab Chip,* 2012,12, 2146-2155 hereby

incorporated by reference in its entirety.

Other methods and assays which do not rely on fluorescent or light emitting reporters can be used to detect reaction rates according to the methods described herein. Such methods include those that use transcription but not necessarily fluorescence or luminescence. Exemplary methods include pull-down assays that can measure high metabolite production capabilities. Further methods include luciferase high-throughput screening such as described in Fan et al, *ASSAY and Drug Development Technologies,* Volume 5, Number 1, pp. 127-136 (2007) hereby incorporated by reference in its entirety.

The above disclosure generally describes the present invention. All references disclosed herein are expressly incorporated by reference. The following examples are set forth as being representative of the present disclosure. These examples are not to be construed as limiting the scope of the present disclosure as these and other equivalent embodiments will be apparent in view of the present disclosure, figures and accompanying claims.

Solid Support

In certain embodiments, the enzyme variants or the nucleotide encoding the enzyme variants, or the sensor biomolecule variants and the like described herein can be immobilized on a support. The support can be simple square grids, checkerboard (e.g., offset) grids, hexagonal arrays and the like. Suitable supports include, but are not limited to, membranes, papers, filters, slides, beads, chips, particles, strands, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, culture dishes, plates (e.g., 96-well, 48-well, 24-well, 12-well, eight-well, six-well, four-well, single-well and the like), and the like. In various embodiments, a solid support may be biological, nonbiological, organic, inorganic, or any combination thereof. The solid support can be made up of multiple individual compartments.

In some embodiments, each of the individual compartment of the multi-compartment solid support can be immobilized or temporarily immobilized to the multi-compartment solid support. The immobilization facilitates sorting of the individual compartments. The immobilized individual compartment can be extracted or removed from the solid support, such as by manual extraction.

In certain embodiments, a support may have functional groups attached to its surface which can be used to bind one or more reagents described herein. One or more reagents can be attached to a support by hybridization, covalent attachment, magnetic attachment, affinity attachment and the like. Supports may also be functionalized using, for example, solid-phase chemistries known in the art (see, e.g., U.S. Pat. No. 5,919,523).

As used herein, the term "attach" refers to both covalent interactions and noncovalent interactions. A covalent interaction is a chemical linkage between two atoms or radicals formed by the sharing of a pair of electrons (i.e., a single bond), two pairs of electrons (i.e., a double bond) or three pairs of electrons (i.e., a triple bond). Covalent interactions are also known in the art as electron pair interactions or electron pair bonds. Noncovalent interactions include, but are not limited to, van der Waals interactions, hydrogen bonds, weak chemical bonds (i.e., via short-range noncovalent forces), hydrophobic interactions, ionic bonds and the like. A review of noncovalent interactions can be found in Alberts et al., in *Molecular Biology of the Cell*, 3d edition, Garland Publishing, 1994.

EXAMPLE I

Cell-free observation of acrylate

DNA encoded biological acrylate sensor was used to detect acrylate. AcuR is a transcription factor whose activity is dependent on the concentration of acrylate. A linear dsDNA encoding the acrylate sensor AcuR is combined with a reporter circular plasmid

which contained a GFP protein controlled by a promoter regulated by AcuR. Thus, GFP fluorescence is related to the amount of acrylate in the sample.

In order to obtain an adequate signal-to-noise ratio (SNR), a T7 promoter was used to drive the expression of the sensor to achieve high expression of the sensor protein and ensure outnumbering of the number of reporter promoters, and well controlled expression. Additionally, an E.coli RNAP promoter was used to drive expression on the reporter, as it reduces the level of background signal, and provides with a more accurate control by AcuR.

Methods

Linear DNA amplification: Linear dsDNA containing AcuR was prepared by PCR amplification using primers which contain a T7 promoter, and ribosome binding site (RBS).

Cell free preparation: In ice, a cell-free reaction was first prepared containing lOuL of component A (NEB, P/N E6800), 7.5 uL of component B (NEB, P/N E6800), 5 U of murine RNAse inhibitor (NEB, P/N M0314S), and 2.5U of *E. coli* RNA polymerase holoenzyme (NEB, P/N M0551S). A combined 250ng of linear dsDNA encoding AcuR, 250ng of circular plasmid containing the reporter GFP was added into the cell-free system reaction. Sequences of the DNA elements are attached below.

Finally, the sample was separated into two different aliquots, and different amounts of sodium acrylate solution was added to achieve concentrations of OmM, and O.lmM, respectively.

The samples were incubated at 37°C, and the fluorescence signal (395nm excitation, 470nm absorption) of the sample was measured every two minutes for 4 hours.

Results

An increase of fluorescence to 750 arbitrary units in the sample containing acrylate was observed, and an increase of fluorescence to 260 arbitrary units in the sample that does not contain acrylate was observed (FIG. 1).

Reporter promoter:

GCTTCACAACCGCACTTGATTTAATAGACCATACCGTCTATTATTTCTGG (SEQ ID NO: 1)

Reporter RBS:

Tttaactttaagaaggagatatacat (SEQ ID NO: 2)

Reporter gene:

Atgcgtaaaggtgaagaactgttcaccggtgttgttccgatcctggttgaactggacggtgacgttaacggtcacaaattctctgttcgt
ggtgaaggtgaaggtgacgctaccaacggtaaactgaccctgaaattcatctgcaccaccggtaaactgccggttccgtggccgacc
ctggttaccaccctgacctacggtgttcagtgcttcgctcgttacccggaccacatgaaacagcacgacttcttcaaatctgctatgccg
gaaggttacgttcaggaacgtaccatctctttcaaagacgacggtacctacaaaacccgtgctgaagttaaattcgaaggtgacaccct
ggttaaccgtatcgaactgaaaggtatcgacttcaaagaagacggtaacatcctgggtcacaaactggaatacaacttcaactctcaca
acgtttacatcaccgctgacaaacagaaaaacggtatcaaagctaacttcaaaatccgtcacaacgttgaagacggttctgttcagctgg
ctgaccactaccagcagaacaccccgatcggtgacggtccggttctgctgccggacaaccactacctgtctacccagtctgttctgtct
aaagacccgaacgaaaaacgtgaccacatggttctgctggaattcgttaccgctgctggtatcacccacggtatggacgaactgtaca
aa (SEQ ID NO: 3)

Promoter and sequence sensor:

Promoter Sequence

Aattaatacgactcactatagggagaccacaac (SEQ ID NO: 4)

Protein Coding Sequence

ATGCCGCTGACCGACACCCCGCCGTCTGTTCCGCAGAAACCGCGTCGTGGTCGTC
CGCGTGGTGCTCCGGACGCTTCTCTGGCTCACCAGTCTCTGATCCGTGCTGGTCT

GGAACACCTGACCGAAAAAGGTTACTCTTCTGTTGGTGTTGACGAAATCCTGAAA

GCTGCTCGTGTTCCGAAAGGTTCTTTCTACCACTACTTCCGTAACAAAGCTGACTT

CGGTCTGGCTCTGATCGAAGCTTACGACACCTACTTCGCTCGTCTCCTCGACCAG

GCGTTCCTGGACGGTTCGCTGGCTCCGCTGGCTCGTCTGCGTCTGTTCACCCGTAT

GGCTGAAGAAGGTATGGCTCGTCACGGTTTCCGTCGTGGTTGCCTGGTTGGTAAC

CTGGGTCAGGAAATGGGTGCTCTGCCGGACGACTTCCGTGCTGCTCTGATCGGTG

TTCTGGAAACCTGGCAGCGTCGTACCGCTCAGCTGTTCCGTGAAGCTCAGGCTTG

CGGTGAACTGTCTGCTGACCACGACCCGGACGCTCTGGCTGAAGCTTTCTGGATC

GGTTGGGAAGGTGCTATCCTGCGTGCTAAACTGGAACTGCGTCCGGACCCGCTGC

ACTCTTTCACCCGTACCTTCGGTCGTCACTTCGTTACCCGTACCCAGGAATAA

(SEQ ID NO: 5)

Terminator Sequence

AAC CCC TTG GGG CCT CTA AAC GGG TCT TGA GGG GTT TTT TG (SEQ ID NO: 6)

Primers used for AcuR amplification:

PI: aattaatacgactcactatagggagaccacaacATG CCG CTG ACC GAC ACC C (SEQ ID NO: 7)

P2: CAA AAA ACC CCT CAA GAC CCG TTT AGA GGC CCC AAG GGG TTT TAT TCC TGG GTA CGG GTA ACG (SEQ ID NO: 8)

EXAMPLE II

Real-time monitoring of acrylate production in a cell-free system

DNA encoded biological acrylate sensor was used to detect acrylate which has been synthesized in real time. A dsDNA linear template encoding the enzyme PCS downstream of a T7 promoter, and the precursor molecule 3-hydroxypropionate was added to the sensor system described in Example 1.

Methods

Linear DNA amplification: Linear dsDNA containing AcuR and PCS was prepared by PCR amplification using primers which contain a T7 promoter, and ribosome binding site (RBS).

Cell free preparation: In ice, a cell-free reaction was first prepared containing 10μL of component A (NEB, P/N E6800), 7.5 μL of component B (NEB, P/N E6800), 5 U of murine RNAse inhibitor (NEB, P/N M0314S), and 2.5U of E. coli RNA polymerase holoenzyme (NEB, P/N M0551S). A combined 250ng of linear dsDNA encoding AcuR, 250ng of linear dsDNA encoding PCS, and 250ng of circular plasmid containing the reporter GFP was added into the cell-free system reaction. Sequences of the DNA elements are attached below.

Finally, the sample was separated into two different aliquots, and different amounts of 3-hydroxypropionate were added to a solution to achieve concentrations of OmM, and O.lmM, respectively. The samples were incubated at 37°C, and the fluorescence signal (395nm excitation, 470nm absorption) of the sample was measured every two minutes for 4 hours.

Promoter and sequence PCS:

Promoter Sequence

Aattaatacgactcactatagggagaccacaac (SEQ ID NO: 4)

Protein Coding Sequence

ATGATCGATACCGCACCGCTGGCACCGCCGCGTGCTCCGCGCAGCAATCCGATTC
GTGATCGCGTGGATTGGGAAGCGCAGCGTGCAGCAGCACTGGCCGATCCGGGTG
CATTTCATGGTGCGATCGCCCGTACCGTTATTCACTGGTATGATCCGCAGCATCA
CTGCTGGATTCGCTTCAACGAAAGCTCTCAGCGTTGGGAAGGTCTGGATGCAGCA
ACGGGTGCTCCGGTTACAGTGGATTATCCTGCCGATTACCAGCCGTGGCAGCAGG

CATTTGATGATAGTGAAGCGCCGTTTTATCGCTGGTTCAGCGGCGGTCTGACGAA

CGCATGTTTTAATGAAGTTGATCGTCACGTGACAATGGGTTACGGCGATGAAGTG

GCGTATTACTTCGAAGGTGATCGCTGGGATAATAGCCTGAACAATGGCCGTGGC

GGTCCGGTGGTTCAGGAAACGATTACCCGTCGCCGTCTGCTGGTTGAAGTGGTTA

AAGCAGCGCAGGTTCTGCGCGATCTGGGCCTGAAAAAAGGTGATCGTATCGCGC

TGAACATGCCGAATATCATGCCGCAGATTTATTACACCGAAGCCGCAAAACGCC

TGGGTATTCTGTATACGCCGGTGTTTGGCGGTTTCAGTGATAAAACCCTGAGCGA

TCGCATCCATAATGCAGGTGCGCGTGTGGTTATTACCTCTGATGGCGCGTATCGT

AACGCCCAGGTGGTTCCGTATAAAGAAGCCTACACGGATCAGGCACTGGATAAA

TACATCCCGGTGGAAACCGCCCAGGCAATTGTTGCACAGACGCTGGCAACCCTG

CCGCTGACCGAAAGTCAGCGCCAGACGATTATCACCGAAGTGGAAGCAGCACTG

GCAGGTGAAATTACGGTTGAACGTTCTGATGTTATGCGCGGTGTGGGCAGTGCGC

TGGCCAAACTGCGCGATCTGGATGCCAGTGTGCAGGCAAAAGTTCGTACCGTGC

TGGCACAGGCGCTGGTTGAAAGCCCGCCGCGCGTGGAAGCAGTGGTTGTGGTTC

GTCATACGGGTCAGGAAATCCTGTGGAATGAAGGCCGTGATCGCTGGAGCCACG

ATCTGCTGGATGCAGCACTGGCGAAAATTCTGGCTAACGCACGCGCCGCAGGTTT

TGATGTTCACTCTGAAAACGATCTGCTGAATCTGCCGGATGATCAGCTGATCCGT

GCTCTGTATGCGAGTATTCCGTGCGAACCAGTTGATGCCGAATATCCGATGTTTA

TTATCTACACGAGCGGTTCTACCGGCAAACCGAAAGGTGTTATTCATGTTCACGG

CGGTTACGTGGCGGGCGTGGTTCATACCCTGCGCGTTAGTTTCGATGCCGAACCG

GGCGATACGATTTATGTGATCGCAGATCCGGGCTGGATCACAGGTCAGAGCTAC

ATGCTGACGGCAACCATGGCAGGTCGTCTGACTGGTGTGATTGCCGAAGGTTCTC

CGCTGTTTCCGAGTGCGGGCCGCTATGCCTCTATTATCGAACGTTACGGTGTTCA

GATTTTTAAAGCGGGCGTTACGTTCCTGAAAACCGTGATGAGTAACCCGCAGAAT

GTTGAAGATGTGCGCCTGTATGATATGCACAGTCTGCGTGTGGCAACCTTTTGTG

CAGAGCCGGTTAGCCCGGCAGTGCAGCAGTTCGGTATGCAGATCATGACGCCGC

AGTATATTAATAGCTACTGGGCGACGGAACATGGCGGTATTGTGTGGACCCACTT

TTATGGCAACCAGGATTTCCCGCTGCGTCCAGATGCACATACGTACCCGCTGCCG

TGGGTTATGGGTGATGTTTGGGTGGCAGAAACCGATGAATCTGGCACCACGCGC

TATCGCGTGGCGGATTTCGATGAAAAAGGTGAAATCGTTATCACCGCACCGTATC

CGTACCTGACGCGAACCCTGTGGGGTGATGTGCCGGGTTTTGAAGCGTATCTGCG

TGGTGAAATCCCGCTGCGTGCATGGAAAGGTGATGCAGAACGTTTCGTTAAAAC

CTACTGGCGTCGTGGTCCGAATGGCGAATGGGGTTATATCCAGGGCGATTTTGCG

ATTAAATACCCGGATGGTAGTTTCACGCTGCATGGCCGCAGCGATGATGTTATTA

ATGTGTCCGGCCACCGTATGGGTACGGAAGAAATCGAAGGTGCCATTCTGCGTG

ATCGCCAGATCACCCCGGATTCTCCGGTGGGTAACTGCATTGTGGTTGGCGCGCC

GCATCGTGAAAAAGGCCTGACCCCGGTTGCATTTATCCAGCCAGCACCGGGTCGT

CACCTGACGGGTGCAGATCGCCGTCGCCTGGATGAACTGGTGCGTACCGAAAAA

GGTGCAGTTAGCGTGCCGGAAGATTATATTGAAGTTAGTGCGTTTCCGGAAACCC

GCAGCGGTAAATACATGCGTCGCTTCCTGCGTAATATGATGCTGGATGAACCGCT

GGGCGATACCACGACCCTGCGCAACCCGGAAGTGCTGGAAGAAATCGCGGCCAA

AATTGCCGAATGGAAACGTCGCCAGCGCATGGCAGAAGAACAGCAGATTATCGA

ACGTTATCGCTACTTTCGTATTGAATATCATCCGCCGACCGCAAGTGCAGGTAAA

CTGGCAGTGGTTACGGTTACCAATCCGCCGGTGAACGCCCTGAATGAACGTGCTC

TGGATGAACTGAACACCATCGTGGATCACCTGGCGCGTCGCCAGGATGTTGCAG

CGATTGTGTTTACGGGTCAGGGTGCTCGCAGCTTCGTGGCCGGTGCGGATATCCG

TCAGCTGCTGGAAGAAATTCATACCGTTGAAGAAGCCATGGCACTGCCGAACAA

TGCGCACCTGGCCTTTCGCAAAATTGAACGTATGAACAAACCGTGCATTGCCGCA

ATCAATGGTGTGGCACTGGGCGGTGGCCTGGAATTTGCGATGGCCTGTCATTATC

GCGTTGCCGATGTGTACGCAGAATTTGGTCAGCCGGAAATCAACCTGCGTCTGCT

GCCGGGTTATGGTGGTACGCAGCGTCTGCCGCGTCTGCTGTACAAACGCAACAAT

GGTACAGGCCTGCTGCGTGCGCTGGAAATGATTCTGGGTGGCCGCAGCGTGCCA

GCAGATGAAGCACTGGAACTGGGTCTGATTGATGCAATCGCGACCGGCGATCAG

GATAGTCTGAGCCTGGCCTGCGCACTGGCGCGTGCGGCAATCGGTGCAGATGGT

CAGCTGATTGAAAGCGCAGCGGTGACCCAGGCCTTTCGTCATCGCCACGAACAG

CTGGATGAATGGCGTAAACCGGACCCGCGCTTCGCGGATGATGAACTGCGCTCT

ATTATCGCCCATCCGCGTATCGAACGCATTATCCGTCAGGCGCATACCGTTGGTC

GTGATGCAGCAGTGCACCGTGCACTGGATGCAATTCGTTATGGCATTATCCATGG

TTTTGAAGCCGGCCTGGAACACGAAGCAAAACTGTTCGCCGAAGCAGTGGTTGA

TCCGAATGGTGGCAAACGCGGCATCCGTGAATTTCTGGATCGTCAGTCTGCACCG

CTGCCGACACGTCGCCCGCTGATTACCCCGGAACAGGAACAGCTGCTGCGTGAT

CAGAAAGAACTGCTGCCGGTGGGTAGTCCGTTTTTCCCTGGCGTTGATCGCATCC

CGAAATGGCAGTATGCGCAGGCCGTGATTCGTGATCCCGATACTGGTGCAGCAG

CACATGGCGATCCGATCGTTGCGGAAAAACAGATTATCGTTCCGGTGGAACGTC

CGCGTGCGAACCAGGCACTGATTTACGTTCTGGCGAGCGAAGTGAACTTTAATG

ATATTTGGGCCATCACAGGTATTCCGGTGAGCCGCTTCGATGAACATGATCGTGA

TTGGCACGTGACGGGTTCTGGTGGCATCGGCCTGATTGTTGCGCTGGGCGAAGAA

GCCCGTCGCGAAGGTCGTCTGAAAGTTGGCGATCTGGTGGCGATCTATAGCGGC

CAGTCTGATCTGCTGAGCCCGCTGATGGGTCTGGACCCGATGGCAGCCGATTTTG

TGATTCAGGGTAATGATACCCCGGATGGCTCTCATCAGCAGTTCATGCTGGCACA

GGCACCGCAGTGCCTGCCGATCCCGACGGATATGAGCATTGAAGCAGCGGGTTC

TTATATCCTGAACCTGGGCACCATTTACCGCGCACTGTTTACGACCCTGCAA (SEQ ID NO: 9)

Terminator Sequence

AAC CCC TTG GGG CCT CTA AAC GGG TCT TGA GGG GTT TTT TG (SEQ ID NO: 6)

EXAMPLE III

Cell-free discovery of enhanced acrylate-producing enzymes

DNA encoded biological acrylate sensor was used to detect acrylate potential PCS mutants with higher activity of acrylate synthesis. A library of PCS enzymes was generated and the system described in Example 2 was used to detect mutants with higher production using the AcuR sensor activated GFP signal.

Methods

Linear DNA amplification of AcuR: Linear dsDNA containing AcuR was prepared by PCR amplification using primers which contain a T7 promoter, and ribosome binding site (RBS).

PCS library generation: We generated a library of PCS enzymes combining error prone PCR, and degenerate primers on the catalytic center. We topo cloned the library, and purified 384 individual clones.

Cell free preparation: In ice, 384 cell-free reactions were prepared in a 384-well plate with V shaped bottom. 10μL of component A (NEB, P/N E6800), 7.5 μL of component B (NEB, P/N E6800), 5 U of murine RNAse inhibitor (NEB, P/N M0314S), 2.5U of E. coli RNA polymerase holoenzyme (NEB, P/N M0551S) were mixed with 3-hydroxypropionate to a concentration of 0.ImM. A combined 250ng of linear dsDNA encoding AcuR, 250ng of plasmid encoding one PCS mutant, and 250ng of circular plasmid containing the reporter

GFP was added into the cell-free system reaction.

Finally, 3-hydroxypropionate was added to a solution to achieve concentrations of O.lmM. The samples were incubated at 37°C, and the fluorescence signal (395nm excitation, 470nm absorption) of the sample was measured every two minutes for 4 hours to each well. Wells showing higher fluorescence were analyzed.

Mutant identification: Wells with higher fluorescence are analyzed by PCR followed by sequence validation.

EXAMPLE IV

Cell-free discovery of enhanced acrylate-producing enzymes with emulsion sorting technology

A large scale DNA encoded biological acrylate sensor was used to detect acrylate potential PCS mutants with higher activity of acrylate synthesis. A library of PCS enzymes was generated and the system described in example 2 was used to detect mutants with higher production using the AcuR sensor activated GFP signal.

Methods

Linear DNA amplification of AcuR: Linear dsDNA containing AcuR was prepared by PCR amplification using primers which contain a T7 promoter, and ribosome binding site (RBS).

PCS library generation: A a library of PCS enzymes was generated combining error prone PCR, and degenerate primers on the catalytic center. Forward primer of PCS contains a 5' biotin.

PCS library was emulsified in mineral oil with presence of beads. Emulsion PCR with biotin primers was performed to amplify the individual members of the library in each of the beads. The beads with successful amplification were recovered (ie

https://www3.appliedbiosystemsxom/cms/groups/mcb_support/documents/generaldocum ents /cms_08 1748.pdf).

Cell free preparation: In ice, 384 cell-free reactions were prepared in a 384-well plate with V shaped bottom. 10 μL of component A (NEB, P/N E6800), 7.5 μL of component B (NEB, P/N E6800), 5 U of murine RNAse inhibitor (NEB, P/N M0314S), 2.5U of E. coli RNA polymerase holoenzyme (NEB, P/N M0551S) were mixed with 3-hydroxypropionate to a concentration of O.lmM. A combined 250ng of linear dsDNA encoding AcuR, 250ng of circular plasmid containing the reporter GFP were added into the cell-free system reaction, and 3-hydroxypropionate was added to a solution to achieve concentrations of O.lmM. This mixture was emulsified with mineral oil, and the beads containing the PCS mutants.

The emulsion was incubated at 37°C for 4 hours.

Mutant identification: Droplet sorter was used to separate droplets with higher fluorescence. Phenol chloroform extraction was used to separate aqueous phase from mineral oil, and PCR followed by sequence was used to identify the PCS mutants with higher activity.

Table 2.

A list of natural and engineered biosensors by molecule sensed is provided. Abbreviated sensor type names refer to the following: allosteric TF, allosteric transcription factor; two-component, two-component systems; FRET, fluorescence resonance energy transfer; GPCR, G-protein coupled receptor.

Molecule(s)	Molecule type	Sensor	Sensor type	Reference
Natural biosensors				
1-butanol	Fatty alcohol, fuel	BmoR	Allosteric TF	[1]
Acrylate	Plastic precursor	AcuR	Allosteric TF	[2**]

Adipate	Dicarboxylic acid	PcaR	Allosteric TF	[1]
B12	Vitamin	BtuB	Riboswitch	[3]
Benzoate, naphthalene	Aromatics	NahR	Allosteric TF	[4]
Erythromycin	Macrolide	MphR	Allosteric TF	[5]
Fatty acids	Fatty acid	FadR	Allosteric TF	[6]
Fatty acids	Fatty acid		GPCR	[7]
Glucarate	Feedstock	CdaR	Allosteric TF	[8]
Lysine	Amino acid	LysR	Allosteric TF	[9]
Muconate	Dicarboxylic acid	BenM	Allosteric TF	[10]
NADPH	Redox	SoxR	Allosteric TF	[11]
Naringenin	Flavonoid	TtgR	Allosteric TF	[12]
Octane	Alkane	AlkS	Allosteric TF	[13]
Succinate	Dicarboxylic acid	DcuR	Two-component	[1]
Tetracyclines	Polyketides	TetR	Allosteric TF	[14]
Engineered biosensors				
3,4-dihydroxybenzoate	Aromatic	PobR	Allosteric TF	[15]
Biphenyl, nitrotoluenes	Aromatics	XylR	Allosteric TF	[16]
Mevalonate	Isoprenoid precursor	AraC	Allosteric TF	[17]
Pyruvate	Alpha-keto acid	*De novo*	FRET	[18]
Theophylline	Alkaloid	*De novo*	Riboswitch	[19]
Thiamine-pp	Vitamin	*De novo*	Riboswitch	[20, 21]
Trehalose-6-p	Sugar	*De novo*	FRET	[22]
Triacetic acid lactones	Feedstock	AraC	Allosteric TF	[23]
Vanillin	Aromatic, flavoring	QacR	Allosteric TF	[24]
Zn^{2+}	Ion	*De novo*	FRET	[25]

Table 3.
Examples of biosensor-mediated high-throughput metabolic engineering

Molecule	Biosensor mode	Titer fold improvement	Throughput	Year	Reference
naringenin	selection	36	10^9	2014	[26]
glucarate	selection	22	10^9	2014	[26]
lysine	screen	37	10^8	2013	[27]
histidine	screen	>40	10^8	2013	[27]
arginine	screen	87	10^8	2013	[27]
triacetate lactone	screen	20	10^4	2013	[28]
mevalonate	screen	3.8	10^5	2011	[29]
butanol	screen	1.4	10^3	2010	[30]

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** This study combines biosensors for four plastic precursors (acrylate, 3-hydroxypropionate, muconate and glucarate) with their respective metabolic pathways to demonstrate fluorescent biosensors can be used to track metabolite production in real-time. A small screen is used to demonstrate biosensor-based culture condition optimization for the production of 3-hydroxypropionate.

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TABLE 4: Sequence of regulator proteins and cognate promoter/operators.

Regulator	Promoter / Operator Sequence	Regulator Sequence
acuR	GCTTCACAACCGCACT TGATTTAATAG ACCAT ACCGTCTATTATTTCTG G (SEQ ID NO: 1)	ATGCCGCTGACCGACACCCCGCCGTCTGTTCCGCAGAAAC CGCGTCGTGGTCGTCCGCGTGGTGCTCCGGACGCTTCTCT GGCTCACCAGTCTCTGATCCGTGCTGGTCTGGAACACCTGA CCGAAAAAGGTTACTCTTCTGTTGGTGTTGACGAAATCCTG AAAGCτGCTCGτGTTCCGAAAGGπ CTTTCTACCACτACTT CCGTAACAAAGCTGACTTCGGTCTGGCTCTGATCGAAGCTT ACGACACCTACTTCGCTCGTCTCCTCGACCAGGCGTTCCTG GACGGTTCGCTGGCTCCGCTGGCTCGTCTGCGTCTGTTCAC CCGTATGGCTGAAGAAGGTATGGCTCGTCACGGTTTCCGT CGTGGTTGCCTGGTTGGTAACCTGGGTCAGGAAATGGGTG CTCTGCCGGACGACTTCCGTGCTGCTCTGATCGGTGTTCTG GAAACCTGGCAGCGTCGTACCGCTCAGCTGTTCCGTGAAG CTCAGGCTTGCGGTGAACTGTCTGCTGACCACGACCCGGA

		CGCTCTGGCTGAAGCTTTCTGGATCGGTTGGGAAGGTGCT ATCCTGCGTGCTAAACTG GAACTG CGTCCGGACCCGCTGC ACTCTTTCACCCGTACCTTCGGTCGTCACTTCGTTACCCGTA CCCAGGAATAA (SEQ ID NO: 5)
araC	AGAAACCAATTGTCCA TATTGCATCAGACATT GCCGTCACTGCGTCTT TTACTGGCTCTTCTCGC TAACCCAACCGGTAAC CCCGCTTATTA AAAGC ATTCTGTAACAAAG CG GGACCAAAGCCATG AC AAAAACGCGTAACAAA AGTGTCTATAATCACG GCAGAAAAGTCC ACAT TGATTATTTG CACGGC GTCACACTTTGCTATGC CATAGCATTTTTATCCA TAAGATTAGCGGATCC TACCTGACGCTTTTTAT CGCAACTCTCTACTGTT TCTCCATACCCGCTTTC ATATCTTTCAC ̈ΓΓΓΓΓΓΓ GGGCTAAC (SEQ ID NO: 10)	ATGGCTGAAGCGCAAAATG ATCCCCTGCTGCCGGGATACT CGTTTAACGCCCATCTGGTGGCGGGTTTAACGCCGATTGA GGCCAACGGTTATCTCGA ̈ΓΓΓΓΓΓΓATCGACCGACCGCTGG GAATGAAAGGTTATATTCTCA ATCTCACCATTCGCGGTCAG GGGGTGGTGAAAAATCAGGGACGAGAATTTGTCTGCCGA CCGGGTGATATTTTG CTGTTCCCGCCAGGAGAG ATTCATCA CTACGGTCGTCATCCGGAGGCTCGCGAATGGTATCACCAG TGGGTTTACTTTCGTCCGCGCGCCTACTGGCATGAATG GCT TAACTGGCCGTCAATATTTG CCAATACGGGTTTCTTTCGCC CGGATGAAG CG CACCAGCCG CATTTCAG CGACCTGTTTGGG GCAAATCATTAACG CCGGGCAAG GGGAAGGGCG CTATTC GGAGCTGCTGGCGATAAATCTGCTTGAGCAATTGTTACTG CGGCGCATGGAAGCGATTAACGAGTCGCTCCATCCACCGA TGGATAATCGGGTACGCGAGG CTTGTCAGTAC ATCAGCGA TCACCTGGCAGACAGCAATTTTG ATATCG CCAGCGTCGCAC AGCATGTTTG CTTGTCGCCGTCGCGTCTGTCACATCTTTTCC GCCAGCAGTTAGGGATTAGCGTCTTAAGCTGGCGCGAGG ACCAACGCATTAGTCAG GCGAAG CTG CTTTTGAGCACTAC CCGGATGCCTATCG CCACCGTCGGTCGCAATGTTG GTTTTG ACGATCAACTCTATTTCTCG CGAGTATTTAAA AAATG CACC GGGGCCAGCCCGAGCGAGTTTCGTGCCGGTTGTGAAGAA AAAGTGAATGATGTAGCCGTCAAGTTGTCAtaa (SEQ ID NO: 11)
cdaR	ATGCTGTTGATTGACG CCAGTGAGAACCCGGA ACCGGAAACGGAATCA AATCCGTGGGTCGAAC AGTGGGGCACGCTGTT GTCCTGATATGTTCAG CGAGCGGTAAATGTCG TTTTAGCGGTGCTGAA TCGAATCTTTTCAG GC AAATG CCAGTAAAAAC TGCTTCATAGCGCGGA TTTTTACTGGCGTTTGC CTGGAGTCAAGCGATC CATTTCATACTCTTCTT TATTTCTTCGTTTTAAC CCTTCCTTTCTTGTTCTT GTTTTCATTTCCGTGAA GTGGATTCCACCGTCC	ATGGCTGGCTGGCATCTTGATACCA AAATG GCG CAG GATA TCGTGGCACGTACCATG CG CATCATCGATACCAATATCAAC GTAATGGATGCCCGTGGGCGAATTATCGGCAGCGGCGATC GTGAGCGTATTGGTGAATTG CACGAAG GTG CATTGCTGGT ACTTTCACAGGGACGAGTCGTCGATATCG ATGACGCGGTA GCACGTCATCTGCACGGTGTG CG GCAGGGGATTAATCTAC CGTTACGGCTG GAAGGTGAAATTGTCG GCGTAATTG GCCT GACAGGTGAACCAGAGAATCTG CGTAAATATG GCGAACT GGTCTGCATGACGG CTGAAATG ATG CTGG AACAGTCG CG GTTGATGCACTTGTTGGCG CAGGATAG CCGTTTGCGGGAA GAACTGGTGATGAACCTGATTCAGG CAGAGGAGAATACTC CCG CACTTACTGAATGG GCG CAACG GCTGGGGATCGATCT CAATCAACCGCGAGTGGTG GCTATTGTTGAGGTCGACAGC GGTCAGCTTGGCGTGGACAG CG CAATG GCG GAGTTAC AA CAACTGCAAAACGCGCTGACTACGCCCGAGCGTAATAATC TGGTGGCGATTGTCTCGCTAACCGAAATG GTGGTGTTGAA ACCGGCGTTGAACTCTTTTG GGCG CTGG GATG CAGAAG AT CATCGTAAGCGAGTTGAACAACTGATTACCCGCATGAAAG

	AGGGCTAATGCCAAAA TCGGGCCTCATTGAAC GCATTAATGTTGTGTT GTTGCACGGTGAGCCG CTATGGCGCGU ˜TT˜N˜A TACTGCTATTGCCAGA TATAAACACG CGCCGT ATTCGGCGAACGACCT ATAAA AACGGCAAAAAA ACACCCTACGTCACCTC TGATTTCCTGGCGATG TCGCAGTCCAGAGTGA GCGTGGCTAACGCGAA ˜ÏÏÏÍCAGGAGTGCAAC A (SEQ ID NO: 12)	AGTACGGCCAGCTGCG ˜ÏÏÏÍCGCGTTTCACTGGGCAACTAT TTTACCGGTCCTGGCAGTATTG CCCGATCCTATCGTACGGC GAAAACG ACGATGGTGGTGGGTAAACAGCGGATGCCAGA AAGTCGCTGCTA ˜Ï˜ÏÏÏÍATCAGGATCTGATGTTACCTGTGT TACTCGACAGTTTG CGTGGCGACTGGCAGGCCAACGAACT GGCGCGACCGCTGGCGCGGCTGAAAACG ATGGACAATAA CGGCTTGCTGCGACGAACGCTGGCGGCGTGGTTTCGCCAC AATGTGCAACCG CTGGCAACGTCAAAG GCGTTGTTTATTC ATCGTAATACCCTG GAGTATCG GCTTAATCGTATATCG GAA CTGACCGGGCTTGATTTGGGCAA ˜ÏÏÏÍGATGACAGGTTGC TGCTGTATGTGGCGTTACAACTGGATGAAGAGCGGtag (SEQ ID NO: 13)
mphR	GGATTGAATATAACCG ACGTGACTGTTACATTT AGGTGGCTAAACCCGT CAA (SEQ ID NO: 14)	ATGCCGCGTCCGAAACTGAAATCTGACGACGAAGTTCTGG AAGCGGCGACCGTTGTTCTGAAACGTTGCGGTCCGATCGA ATTCACCCTGTCTGGTGTTGCGAAAGAAGTTGGTCTGTCTC GTGCGGCGCTGATCCAGCGTTTCACCAACCGTGACACCCT GCTGGTTCGTATGATGGAACGTGGTGTTGAACAGGTTCGT CACTACCTGAACGCGATCCCGATCGGTGCGGGTCCGCAGG GTCTGTGGGAATTCCTGCAGGTTCTGGTTCGTTCTATGAAC ACCCGTAACGACTTCTCTGTTAACTACCTGATCTCTTGGTAC GAACTGCAGGTTCCGGAACTGCGTACCCTGGCGATCCAGC GTAACCGTGCGGTTGTTGAAGGTATCCGTAAACGTCTGCC GCCGGGTGCGCCGGCGGCGGCGGAACTGCTGCTGCACTC TGTTATCGCGGGTGCGACCATGCAGTGGGCGGTTGACCCG GACGGTGAACTGGCGG ACCACGTTCTGGCGCAGATCGCG GCGATCCTGTGCCTGATGTTCCCGGAACACG ACGACTTCCA GCTGCTGCAGGCGCACGCGTAA (SEQ ID NO: 15)
tetR	TCGAGTCCCTATCAGT GATAGAGATTGACATC CCTATCAGTGATAGAG ATACTGAGCACATCAG CAGGACGCACTGACCG AATTCATTAAA (SEQ ID NO: 16)	ATGTCTCGTTTAGATAAAAGTAAAGTGATTAACAG CGCATT AGAGCTGCTTAATGAGGTCGGAATCGAAGGTTTAAC AACC CGTAAACTCGCCCAGAAGCTAGGTGTAGAGCAGCCTACAT TGTATTGGCATGTAAAAAATAAG CGGGC˜ÏÏÍGCTCGACGC CTTAGCCATTGAGATGTTAGATAGGCACCATACTCAL˜ÏÏÍÍ GCCC˜Ï˜ÏÍAGAAGGGGAAAGC ˜ÍGGCAAGA˜ÏÏÏÏÍACGTAA TAACGCTAAAAG ˜ÏÏÏÍAGA˜ÍG˜ÍGC˜Ï˜ÍACTAAGTCATCGCG ATGGAGCAAAAGTACATTTAG GTACACGGCCTACAGAAAA ACAGTATGAAACTCTCGAAAATCAATTAGCC ˜ÏÏÏÏÍATGCC AACAAGG ˜Ï˜ÏÏÏÍCACTAGAGAATGCATTATATGCACTCAGC GCAGTGGGGCA˜Ï˜ÏÏÍAC˜Ï˜ÍAGGTTGCGTATTGGAAGATC AAGAGCATCAAGTCGCTAAAGAAGAAAGGGAAACACCTA CTACTGATAGTATGCCGCCATTATTACGACAAGCTATCGAA TTATTTGATCACCAAGGTGCAGAGCCAGCCTTCTTATTCGG CCTTGAATTGATCATATGCGGATTAGAAAAACAACTTAAAT

		GTGAAAGTGGGTCTTAA (SEQ ID NO: 17)
ttgR	CACCCAGCAGTATTTA CAAACAACCATGAATG TAAGTATATTCCTTAG CAA (SEQ ID NO: 18)	ATGGTGCGTCGCACCAAAGAAGAAGCACAGGAAACGCGT GCG CAGATTATCGAAGCGGCCGAACGCG CGTTTTATAAAC GTG GTGTG GCACGTACCACG CTG GCAGATATTG CAGAACT GGCAG GTGTTACCCGCG GTG CA ATCTACTGGCATTTCAAC AATAA AGCCG AACTG GTTCAG GCACTG CTG GATTCTCTGC ACG AAACG CATG ATCACCTGG CCCGTG CAAG CG AATCTG A AGATG AACTGG ACCCG CTG GGCTG CATG CG CA AACTG CTG CTG CAG GTGTTTAACG AACTG GTTCTG GATG CACGTACCC GTCGCATTAATGAAATCCTGCATCACAAATGCGAATTTACG GATG ATATGTGTG AAATTCGTCAG CAGCGCCAGAGCGCCG TG CTG GATTGTCATA AAG GTATCACCCTG GCACTG GCAAA CG CAGTTCGTCG CG GTCAG CTG CCG GGTG AACTG GATGTG GAACG CG CAG CG GTTG CG ATGTTTG CCTATGTGGATG GCC TGATTG GTCGTTG GCTG CTG CTG CCG GATAGTGTTG ATCT GCTGGGCGATGTGGAAAAATGGGTTGATACCGGTCTGGA TATG CTG CGTCTG AGCCCGGCG CTG CG CAAATAA (SEQ ID NO: 19)

TABLE 5: Sequence of MIOX orthologs

MIOX Variant	Sequence
Candida albicans	ATGGTAAACAAGGTCGGTAAATCTACTCTCGATAAGAGCACAAACCTAG ATAAATCCAAAGGGAATATATTAGAGAAACTAGATGATGATATACTTCAT GTCAATAGAATTCGAGGCTCTTTAACTAACAAAACTCCAATCACCAAAAC CCATTCGATAG ATGATG AGCTTAAACTAG AAG AAC AATCA GAAACTG CC GCCG ATGAAA ATTGGCAAATAG CATCGGAATATTATAAAA ACATAG ACA CG AAGGCTTTCCG CCAATATG AATTAG CTTGTG ATAG AGTCAAACAGTTT TATG AAG AACA ACATG AAAAAC AAACCGTGGCGTATAATATTCAAG CAA **G**AATTAATTTCAA AACTAAA ACAAG AGCAAG **A**ATG ACAGTTTG **GG**AAG **G** ACTAG AGAAATTAA ACAAATTGTTAG ATGATTCTGATCCCGACACCGAAT TGTCACAAATAG ATCATG CATTACAGACG GCAG AAG CTATACGG CG AGA TGGGAAACCACGATGGTTTCAATTAGTTGGGTTGATTCATGATTTAGGGA AATTACTATA ΤΤΤΤΤΤΤGATTCTCGTGGTCAATG GGATGTAGTG GGTGATA CTTTCCCTGTTGGTTGTAAATTCCrGAAACGGATTATTTTCCCTGATAGTTT TAAAAATAATCCAGATTTCCTAAATCCATTGTATAATACCAAATATGGCAT ATATTCA AAACATTGTG GATTAG ATAA AGTCATGTTG AGTTGG GGTCATG ATG AGTATATGTATCATGTTG CG AAA AAG AATTCGACATTACCACCGGAA GCATTGGCAATG ATAAG GTATCATTCATTTTATCCTTGGCATCAAG AATTG **G**CATATAGTTATTTAATG **G**ATG AGCATGATA AAG AGATGTTG **A**AAG CAG TCA AAG CTTTCAATTCCTATGATTTATATTCCA AGATAG ATCAACAGTATG ATGTTGAAGAGTTGAAACCATATTACCTAGAGTTGATTGATGAGTTTTTC

	CCAAATAAAGTAATTGA [illegible]AA (SEQ ID NO: 20)
Francisella sp. TX077308	ATGAGTCAGACCGTGGAAAACACGTTTGGCGAATTTCGTAACTACACCG ATAG CAAATTCCAG GATCGTGTG GAACG CACGTACA A A GATATG CACATT AACCAG AATCTG GAATACGTTACCCAG ATG A A A GATAA ATACTTCA AACT GGATCTGGGTAAAATGGATGTGTACGAAG [illegible]CAAACTGCTGGAAAAC GTTCATGATG A AAG CGATCCGGATA ATG ATCTG CCG CAG ATCGA A CACG CATATCA GACCG CG GA A GCCTG CCAGAAC AAATTCCTG AAATCTG ATACG GAACTGCGCGAAAATGCGCTGATTCGTAGTATL [illegible]CGCGATCATGAATG GCAGAGCATTCCGAAAATCTGGCAGGATTTCTATACCAAAAAACAGAGTC TG GGCA ATCTGTACA GCCATATTA AAG ATTG GTCTTG GTTTCCG CTG GTT GGCTTCGTTCACG ATCTG GGTAAA ATCATG ACCCTG CCG GAATATG GTCA GCTG CCGCAGTGG AGCACCGTG GGTG ATACGTACCCG ATTGCCTG CCCG TTTGCAAGCGCGAACGTG [illegible]CACCGTGAATTTGTTAAAGATTCTAAA GATTACA ACA ATT A CAATACCG A A AGTG A A A CG CATTATG GCA A ATACG A G A A A A A ATGTGGTTTCGATA ACGTGGATATGAGCTTCGGTCACGATGA A TACATCTACAAAG [illegible]CGA ACAGGGCAGCGATATCCCGTATGAAGGTCT GTACCTGCTGCGCTATCA[illegible]ACCCGTGGCACACCCCGCAGACGG G CG GTCATG CGTATCAGG A ACTG GCCAACG A A A A A G ATTG GCTG CTGCT GCCGCTG CTG A A A GCCTTTCA G A A A G CG GATCTGTATTCTAA ACTG CCG G A ACTG CCG CCG A A AG A AGTG CTG GA G A A A A A ATACA A A AGTCTG CTG GA TAAATGGGTTCCGAACAAGAAAATTAACTGGTAA (SEQ ID NO: 21)
Flavobacterium johnsoniae	ATGA A A A A G CATATAGACACAGACAATCCGTTGA A A A A TTTAGATGAGT GGGA A GATG ATTTGTTA ATG CG ATATCCTG ACCCTTCTG A AGTA A ATG A A AGTTTA A A AG A A A A G CAG A A A G A A G A ATTTAG A A ATTATGTCG ATTCTG AAAGAGTAGA A A CGGTA A A A G A A [illegible]ACAGGATA A A CCATACCTACCA AACTTATGAC [illegible]GTATG CAGTA A AG A A CA A G A ATTTCTG CA ATTTA ATA GA A A A G A A ATGTCA ATCTGGGA AGCTGTCGA G [illegible]A A A CACGCTTGTA GACGACAGTGACCCAGATATTGACTTAGACCAGACACAGCACU [illegible]ACA GACTTCAGA AGCCATTCGTGCTGATGGTCATCCGGATTGGTTTGTACTGA CAGGTTTCATTCACGATTTGGGTA A AG [illegible]ATGCTTATTTGGAGA ACCGC AATGGGCAGTCGTTGGCGATAC [illegible]CCGGTTGGCTGTGCGTATTCGGAT A A A A TTGTGTATTCAGA A [illegible]A A AG A A A ATCCGGATTATACAGATGA GAGATTCA ATACTA A A CTAG GA ATCTAC ACTG A A A ACTG CG GATTAG ATA ACGTA A A A A TG A GCTG GGGTCATGACG A A TATTTGTATC A GATTATG A A A GATTATTTACCG GATCCTG[illegible]A[illegible]ACA[illegible]GA[illegible]CG[illegible]A[illegible]CAC[illegible]C[illegible]AT TCG CAG CATA A A G A A A ATG CGTATGCACATTTA ATG A ATG A A A A A G ACA

	TCGAAATGTTTG ACTGGGTTCGAAAATTCAATCCGTACG ATTTGTATACA AAG GCTCCTGTAA AACCA GATGTTCAG GCATTACTTCCTTATTATAA AGA ATTAGTTGCTAAATATTTGCCTGAAAAATTGAAG TTTTAA (SEQ ID NO: 22)
Mus musculus	ATG AAAGTG GATGTTG GCCCGGACCCGAG CCTG GTTTACCG CCCG GATG TGGACCCG GAAATG GCAAA AAG CAAAG ATTCGTTTCGTAACTAC ACCA G TGGCCCGCTGCTGGATCGTG TTTTTACCACGTATAAACTGATGCATACCC ACCAGACGGTTGAL TTTGTCAGCCGTAAACGCATTCAATATGGCGGTTTC TCTTACAAG AAAATG ACCATCATG GAAGCGGTGGG CATG CTG GATG ACC TGGTTGATGAATCAGATCCGGACGTCGA TTTTCCGAATTCGTTTCATGCG TTCCAGACGGCCGAAGGTATTCGCAAAGCCCACCCGGACAAAGATTGGT TCCATCTGGTCGG CCTGCTG CACG ATCTG GGTAA AATCATG GCACTGTG G GGTG AACCG CAGTGG GCTGTG GTTG GTG ATACL TTTCCGGTGGGTTGCC GTCCGCAAGCAAGTGTCGTG TTTTGTGACTCCACCTTCCAGGACAACCCG GATCTG CAAG ACCCG CG CTATTCAACG GAACTG GGCATGTACC AGCCG C ATTGCGGTCTGGAAAACGTGCTGATGTCGTGGGGTCACGATGAATACCT GTACCAG ATG ATG AAATTCAACAA ATTCA GCCTG CCGTCTG AAG CCTTCT ACATG ATCCGTTTCCATAGTTTCTACCCGTG GCACACCGG CG GTG ATTATC GCCAGCTGTGCTCCCAGCAAGACCTGGATATGCTGCCGTGGGTGCAAGA ATTCAACAA ATTCG ATCTGTACACG AAATGTCCG GATCTG CCG GACGTTG AATCTCTGCGTCCGTACTACCAAGGTCTGATTGATAAATACTGTCCGGGC ACCCTGTCGTGGTAA (SEQ ID NO: 23)

Example V

In vitro screening using addition of purified sensor protein

A plasmid containing a T7 promoter, lac operator/binding site, RBS, and GFP coding region was constructed (pET-minus_lacI) and prepared for in vitro transcription and translation reactions. The addition of purified LacI represses the constitutive plasmid production of GFP at 100pg/μL and lng/μL levels. Fluorescent measurement of GFP production after induction of the sensor by the addition of IPTG (40 mM) shows increased GFP production. The addition of purified sensor allows for fine-tuning of repression levels

present in each reaction well and prevents cofounding effects of library variants impacting in vitro sensor production. (Fig. 2)

Methods:

A lOOng/μL dilution of purified LacI protein (Novoprotein Cat No. CG57) was created in a buffer containing 20mM Tris, 300mM NaCl, and 5mM DTT. Serial dilutions were performed to obtain concentrations of lng/μL and O.lng^L. The cell-free reactions used 4μL of S30 Premix Plus (Promega kit Cat No. LI110). Then, RNase inhibitor (ThermoFisher Cat No. N8080119) was added to a final reaction concentration of 0.05% to each of the reaction tubes. Then, 3.6μL of T7-S30 Circular Extract (Promega kit Cat No. LI110) was added to all tubes, followed by 2μL of purified pET-GFP plasmid DNA at 99.3 μg/μL and 0.4μL of water.

For induction and response measurement, 1μL of 400μM IPTG was added to half of the tubes, for a final IPTG concentration of 40μM. Finally, IμL of the LacI dilutions at each of the indicated concentrations was added.

Reactions were assembled on ice, then vortexed briefly, spun down, and incubated at 37°C overnight. After incubation, IOμL of each reaction was put into a well of a black, clear/flat-bottom, 384-well-plate, and fluorescence was measured on the Biotek Synergy Neo using 485nm excitation and 528nm emission wavelengths.

Mutant identification: Each well of a reaction plate contains a library member with genetic variation affecting biosynthesis of a target molecule. Exogenous addition of purified sensor allows for fine-tuning of repression levels present in each reaction well, permitting selection of library members showing exceptional sensor response across multiple, controlled sensor concentration levels.

LOCUS pET_minus_lacI 4813 bp ds-DNA linear 15-JUL-2016

DEFINITION .

FEATURES Location/Qualifiers

misc_feature 856..858

/label="split2"

protein_binding 412..432

/label="lacO"

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/label="split5"

misc_feature 1184..1189

/label="PstI"

miscjeature 478..1221

/label="stGFP"

misc_feature 658..660

/label="splitl"

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/label="AvaI"

CDS complement(4051..4242)

/label="ROP"

proteinJinding 1249..1293

/label="S-Tag"

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/label="NdeI"

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/label="ApaLI"

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/label="NcoI"

misc_feature 1231..1236

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misc_feature 976..978

/label="split4"

terminator 1345..1392

/label="T7 terminator"

CDS complement(1999..2859)

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/label="EcoRI"

misc_feature 3363..3368

/label="ApaLI"

misc_feature 965..970

/label="EagI"

misc_feature 2739.-2744

/label="ApaLI"

misc_feature 391..407

/label="T7 promoter"

misc_feature 1090..1092

/label="split6"

misc_feature 369..374

/label="ClaI"

misc_feature 508..513

/label="NheI"

RBS 465..470

/label="RBS-l"

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/label="ColEl, pBR322 ori"

misc_feature 1237..1242

/label="XhoI"

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121 agcagcccag tagtaggttg aggccgttga gcaccgccgc cgcaaggaat ggtgcatgca

181 aggagatggc gcccaacagt cccccggcca cggggcctgc caccataccc acgccgaaac

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421 cggataacaa ttcccctcta gaaataattt tgtttaactt taagaaggag atataccatg

481 ggtcatcacc accaccatca cggtggcgct agcaaaggtg aagagctgtt tacgggtgta

541 gtaccgatct tagtggaatt agacggcgac gtgaacggtc acaaatttag cgtgcgcggc

601 gaaggcgaag gtgacgctac caatggtaaa ttgaccctga agtttatttg cacaacaggc

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721 agtcgttacc cagatcatat gaaacaacac gatttttca aatcagccat gcctgaagga

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1321 gctgctgcca ccgctgagca ataactagca taacccccttg gggcctctaa acgggtcttg

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1561 ttccccgtca agctctaaat cgggggctcc ctttagggtt ccgatttagt gctttacggc

1621 acctcgaccc caaaaaactt gattagggtg atggttcacg tagtgggcca tcgccctgat

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1861 acaaaatatt aacgtttaca atttctggcg gcacgatggc atgagattat caaaaaggat

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2101 gggcttacca tctggcccca gtgctgcaat gataccgcga gacccacgct caccggctcc

2161 agatttatca gcaataaacc agccagccgg aagggccgag cgcagaagtg gtcctgcaac

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3121 ctaccaactc tttttccgaa ggtaactggc ttcagcagag cgcagatacc aaatactgtc

3181 cttctagtgt agccgtagtt aggccaccac ttcaagaact ctgtagcacc gcctacatac

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4741 gaaggctctc aagggcatcg gtcgagatcc cggtgcctaa tgagtgagct aacttacatt

4801 aattgcgttg cgc

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Claims:

1. A method of selecting a candidate enzyme variant from a library of enzyme variants for the production of a metabolite comprising

providing a plurality of first nucleotide sequences each encoding a different enzyme variant of the library,

providing a precursor molecule wherein the enzyme variant when expressed converts the precursor molecule to the metabolite,

providing a second nucleotide sequence encoding a sensor biomolecule,

providing a third nucleotide sequence encoding a reporter,

wherein the sensor biomolecule when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and

screening the enzyme variants by detecting the reporter to identify the candidate enzyme variant.

2. The method of claim 1 wherein the enzyme variant converts the precursor molecule to the metabolite directly or through one or more intermediate steps.

3. The method of claim 2 wherein one or more of the intermediate steps are completely or partially randomized.

4. The method of claim 1 wherein the first, second or third nucleotide sequence is DNA or RNA.

5. The method of claim 4 wherein the DNA and/or RNA is linear or included on a plasmid.

6. The method of claim 1 wherein the nucleotide sequences can be physically separated or attached or any combination thereof.

7. The method of claim 1 wherein cofactors are further provided.

8. The method of claim 1 wherein the enzyme variants, the sensor biomolecule and the reporter are produced using a cell-free expression system.

9. The method of claim 1 wherein the enzyme variants, the sensor biomolecule and the reporter can be produced directly in an evaluation vessel.

10. The method of claim 9 wherein the evaluation vessel is in an emulsion or microliter well format.

11. The method of claim 1 wherein the enzyme variants, the sensor biomolecule and the reporter can be produced outside and then combined in an evaluation vessel.

12. The method of claim 8 wherein the cell-free expression system comprising commercially available in vitro translation reagents and/or kits.

13. The method of claim 1 wherein the candidate enzyme variant is validated by sequencing the nucleotide encoding the enzyme variant.

14. The method of claim 1 wherein enzyme variants and/or sensor biomolecules are provided.

15. The method of claim 1 wherein the selection process is repeated on a subset of identified candidate enzyme variants for optimization.

16. The method of claim 1 wherein the reporter is a fluorescent protein.

17. The method of claim 16 wherein the fluorescent protein is GFP.

18. The method of claim 1 wherein the reporter is a member selected from the group consisting of mPlum, mCherry, tdTomato, mStrawberry, J-Red, DsRed-monomer, mOrange, mKO, mCitrine, Venus, YPet, EYFP, Emerald, EGFP, CyPet, mCFPm, Cerulean, T-Sapphire, Firefly (FLuc), modified firefly (Ultra-Clo), Click beetle (CBLuc), Sea pansy (RLuc), Copepod crustacean (GLuc), and Ostracod crustacean (CLuc).

19. The method of claim 1 wherein the reporter further comprises luciferase for detection by light, pigments for detection by color, surfactants for detection by emulsion breaking, and adhesives for detection by adhesion.

20. The method of claim 1 wherein the screening is carried out by fluorescent microscopy, microtiter plate assay, emulsion assay, microfluidic assay, pull-down assay or luciferase high throughput screening.

21. The method of claim 1 wherein the sensor biomolecule and the metabolite binding partner is a member pair selected from the group consisting of AcuR/acrylate, cdaR/glucaric acid, ttgR/naringennin, ttgR/phenol, btuB riboswitch/cobalamin, mphR/macrolides, tetR/tetracycline derivates, benM/muconic acid, alkS/medium chain n-alkanes, xylR/xylose, araC/Arabinose, gntR/Gluconate, galS/Galactose, trpR/tryptophan, qacR/Berberine, rmrR/Phytoalexin, cymR/Cumate, melR/Melibiose, rafR/Raffinose, nahR/Salicylate, nocR/Nopaline, clcR/Chlorobenzoate, varR/Virginiamycin, rhaR/Rhamnose, PhoR/Phosphate, MalK/Malate, GlnK/Glutamine, Retinoic acid receptor/Retinoic acid, Lacl/allolactose, Estrogen receptor/Estrogen and Ecdysone receptor/Ecdysone.

22. The method of claim 1 wherein the sensor biomolecule is a transcription factor, riboswitch, two-component signaling protein, a nuclear hormone receptor, a G-protein coupled receptor, a periplasmic binding protein, or an engineered protein switch.

23. The method of claim 1 wherein the sensor biomolecule is cdaR and the metabolite is a diacid.

24. The method of claim 22, wherein the biosensor is an engineered protein switch such as an engineered calmodulin.

25. The method of claim 1 wherein the sensor is AcuR and the metabolite is acrylate.

26. The method of claim 1 wherein the enzyme is PCS, MIOX, Udh, or INOl.

27. The method of claim 1 wherein the precursor molecule is 3-hydroxypropionate.

28. The method of claim 1 wherein the reporter protein is an emulsion-breaking protein.

29. The method of claim 1 wherein the plurality of first nucleotide sequences encoding the different enzyme variants are generated by methods comprising gene synthesis, error prone PCR, targeted mutagenesis, or oligonucleotide directed mutagenesis.

30. A method of identifying a candidate sensor biomolecule variant from a library of sensor biomolecule variants for a metabolite comprising

providing a plurality of first nucleotide sequences each encoding a different sensor biomolecule variant of the library of sensor biomolecule variants,

providing a metabolite,

providing a second nucleotide sequence encoding a reporter,

wherein the sensor biomolecule variant when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and

screening the sensor biomolecule variants by detecting the reporter to identify the candidate sensor biomolecule variant.

31. A cell-free bio-sensing system for selecting a candidate enzyme variant from a library of enzyme variants for the production of a metabolite comprising:

a plurality of first nucleotide sequences each encoding a different enzyme variant of the library of enzyme variants,

a precursor molecule wherein the enzyme variant when expressed converts the precursor molecule to the metabolite,

a second nucleotide sequence encoding a sensor biomolecule,

a third nucleotide sequence encoding a reporter,

wherein the sensor biomolecule when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and

wherein the enzyme variants are screened by detecting the reporter to identify the candidate enzyme variant.

32. The system of claim 31 wherein the enzyme variants convert the precursor molecule to the metabolite directly or through one or more intermediate steps.

33. The system of claim 32 wherein one or more of the one or more intermediate steps are completely or partially randomized.

34. The system of claim 31 wherein the first, second or third nucleotide sequence is DNA or RNA.

35. The system of claim 34 wherein the DNA and/or RNA is linear or included on a plasmid.

36. The system of claim 31 wherein the nucleotide sequences can be physically separated or attached or any combination thereof.

37. The system of claim 31 further comprises cofactors.

38. The system of claim 31 wherein the enzyme variants, the sensor biomolecule and the reporter are produced using a cell-free expression system.

39. The system of claim 31 wherein the enzyme variants, the sensor biomolecule and the reporter can be produced directly in an evaluation vessel.

40. The system of claim 39 wherein the evaluation vessel is in an emulsion or microtiter well format.

41. The system of claim 31 wherein the enzyme variants, the sensor biomolecule and the reporter can be produced outside and then combined in an evaluation vessel.

42. The system of claim 38 wherein the cell-free expression system comprising commercially available in vitro translation reagents and/or kits.

43. The system of claim 31 wherein the candidate enzyme variant is validated by sequencing the nucleotide encoding the enzyme variant.

44. The system of claim 31 wherein enzyme variants and/or sensor biomolecules are provided.

45. The system of claim 31 wherein the selection process is repeated on a subset of identified candidate enzyme variants for optimization.

46. The system of claim 31 wherein the reporter is a fluorescent protein.

47. The system of claim 46 wherein the fluorescent protein is GFP.

48. The system of claim 31 wherein the reporter is a member selected from the group consisting of mPlum, mCherry, tdTomato, mStrawberry, J-Red, DsRed-monomer, mOrange, mKO, mCitrine, Venus, YPet, EYFP, Emerald, EGFP, CyPet, mCFPm, Cerulean, T-Sapphire, Firefly (FLuc), modified firefly (Ultra-Clo), Click beetle (CBLuc), Sea pansy (RLuc), Copepod crustacean (GLuc), and Ostracod crustacean (CLuc).

49. The system of claim 31 wherein the reporter further comprises luciferase for detection by light, pigments for detection by color, surfactants for detection by emulsion breaking, and adhesives for detection by adhesion.

50. The system of claim 31 wherein the screening is carried out by fluorescent microscopy, microtiter plate assay, emulsion assay, microfluidic assay, pull-down assay or luciferase high throughput screening.

51. The system of claim 31 wherein the sensor biomolecule and the metabolite binding partner is a member pair selected from the group consisting of AcuR/acrylate, cdaR/glucaric acid, ttgR/naringennin, ttgR/phenol, btuB riboswitch/cobalamin, mphR/macrolides,

tetR/tetracycline derivates, benM/muconic acid, alkS/medium chain n-alkanes, xylR/xylose, araC/Arabinose, gntR/Gluconate, galS/Galactose, trpR/tryptophan, qacR/Berberine, rmrR/Phytoalexin, cymR/Cumate, melR/Melibiose, rafR/Raffinose, nahR/Salicylate, nocR/Nopaline, clcR/Chlorobenzoate, varR/Virginiamycin, rhaR/Rhamnose, PhoR/Phosphate, MalK/Malate, GlnK/Glutamine, Retinoic acid receptor/Retinoic acid, LacI/allolactose, Estrogen receptor/Estrogen and Ecdysone receptor/Ecdysone.

52. The system of claim 31 wherein the sensor biomolecule is a transcription factor, riboswitch, two-component signaling protein, a nuclear hormone receptor, a G-protein coupled receptor, a periplasmic binding protein, or an engineered protein switch.

53. The system of claim 31 wherein the sensor biomolecule is cdaR and the metabolite is a diacid.

54. The system of claim 53, wherein the biosensor is an engineered protein switch such as an engineered calmodulin.

55. The system of claim 31 wherein the sensor is AcuR and the metabolite is acrylate.

56. The system of claim 31 wherein the enzyme is PCS, MIOX, Udh, or INOl.

57. The system of claim 31 wherein the precursor molecule is 3-hydroxypropionate.

58. The system of claim 31 wherein the reporter protein is an emulsion-breaking protein.

59. The system of claim 31 wherein the plurality of the first nucleotide sequences encoding the different enzyme variants are generated by methods comprising gene synthesis, error prone PCR, targeted mutagenesis, or oligonucleotide directed mutagenesis.

60. A cell-free bio-sensing system for identifying a candidate sensor biomolecule variant from a library of sensor biomolecule variants for a metabolite comprising

a plurality of first nucleotide sequences each encoding a different sensor biomolecule variant of the library of sensor biomolecule variants,

a metabolite,

a second nucleotide sequence encoding a reporter,

wherein the sensor biomolecule variant when expressed interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and

wherein the sensor biomolecule variants are screened by detecting the reporter to identify the candidate sensor biomolecule variant.

61. The method of claim 1 wherein the enzyme variants or the first nucleotide sequences encoding the enzyme variants are attached to a solid support for multiplex screening of candidate enzyme variants.

62. The method of claim 61 wherein the solid support comprises multiple compartments in membrane, filter, paper, gel, plate, slide format and the like.

63. The method of claim 62 wherein an individual enzyme variant or an individual nucleotide sequence encoding the enzyme variant is trapped in an individual compartment of the multi-compartment solid support.

64. The method of claim 63 wherein the enzyme variant is isolated with corresponding precursor molecules and reporter sequences inside an individual compartment.

65. The method of claim 63 wherein each individual compartment is immobilized, or temporarily immobilized, within the multi-compartment solid support.

66. The method of claim 63 wherein the individual compartment can be sorted by an automated sorting system.

67. The method of claim 63 wherein the individual compartment can be separated from the multi-compartment solid support by manual extraction.

68. The method of claim 63 wherein the candidate enzyme variant can be identified based on the known content of each individual compartment, or by targeted sequencing, or in-situ imaging.

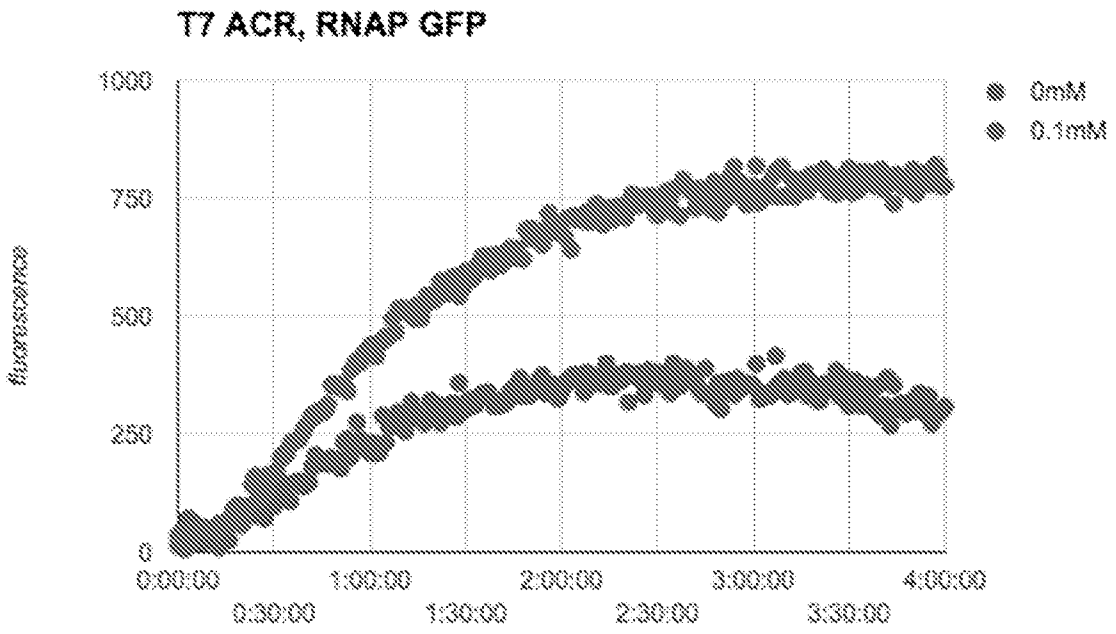
T7 ACR, RNAP GFP
fluorescence
1000
750
500
250
0
0:00:00
0:30:00
1:00:00
1:30:00
2:00:00
2:30:00
3:00:00
3:30:00
4:00:00
0mM
0.1mM

FIG. 1

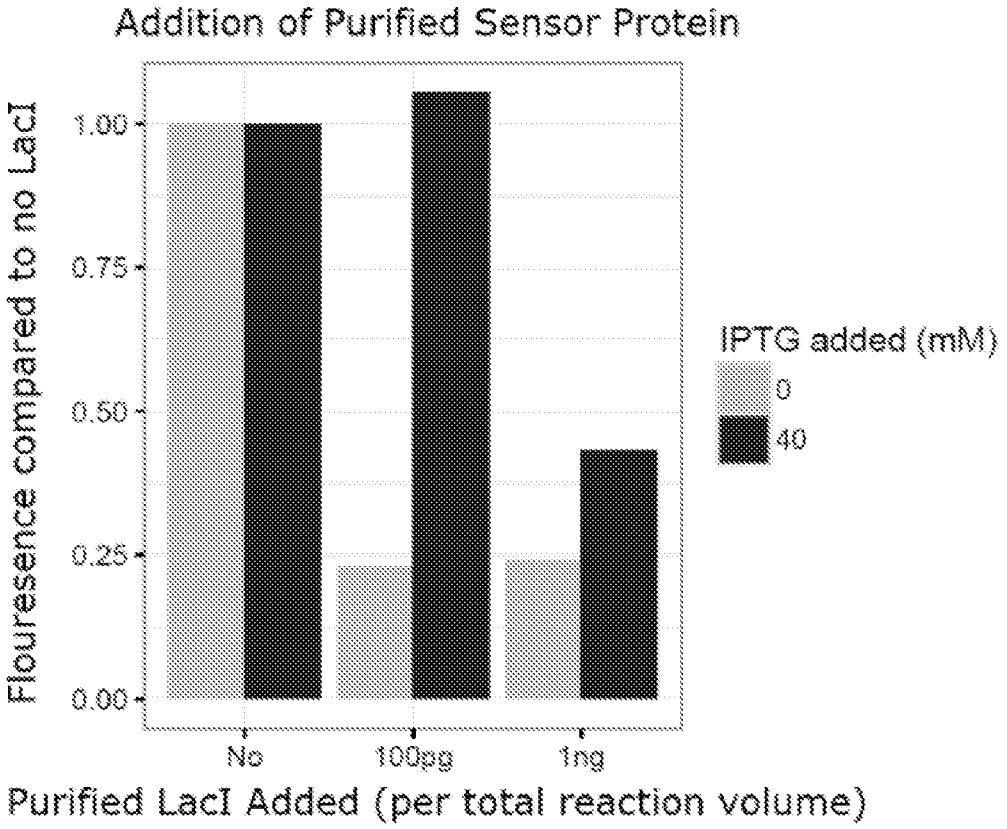
Addition of Purified Sensor Protein
Flouresence compared to no LacI
1.00
0.75
0.50
0.25
0.00
No
100pg
1ng
Purified LacI Added (per total reaction volume)
IPTG added (mM)
0
40

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/21087

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/10, 15/80; C12P 17/08, 17/18 (2017.01)
CPC - C12N 15/1079, 1/38, 15/80, 15/1058; G01N 33/5005, 33/54386, 33/5304; C12P 7/58, 13/04, 13/14

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2002/0127623 A1 (MINSHULL, J et al.) September 12, 2002; paragraphs [0002], [0010], [0041], [0060], [0061], [0063], [0070], [0074], [0082], [0088], [0108], [0110], [0138], [0141], [0159], [0188], [0237], [0256], [0332], [0335]	30, 60 --------- 1-29, 31-59, 61-68
Y	US 2015/0133307 A1 (CODEXIS, INC.) May 14, 2015; abstract; paragraphs [0004]-[0006], [0008], [0010], [0011], [0013]-[0015], [0038], [0047], [0086], [0102], [0119], [0137], [0189], [0191], [0192], [0248], [0250], [0254], [0257]	1-29, 31-59, 61-68
Y	US 2016/0017317 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) January 21, 2016; paragraphs [0010], [0046], [0061], [0072]; figure 13	3, 21, 23, 33, 51, 53
Y	US 2009/0227470 A1 (WITHOLT, B et al.) September 10, 2009; abstract; paragraphs [0011], [0092]	18, 48
Y	(CURSON, ARJ et al.) Screening of Metagenomic and Genomic Libraries Reveals Three Classes of Bacterial Enzymes That Overcome the Toxicity of Acrylate. PLOS One. 21 May 2014, Vol. 9, No. 5; pages 1-13; abstract; page 2, first column, third paragraph; DOI; 10.1371/journal.pone.0097660	25, 55
Y	US 2015/0037853 A1 (GINKGO BIOWORKS, INC.) February 5, 2015; paragraphs [0015], [0019], [0022], [0029], [0041], [0293]	26, 27, 56, 57

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

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent but published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 June 2017 (28.06.2017)	Date of mailing of the international search report 19 JUL 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

Group I, Claims 1-29, 31-59 and 61-68 are directed toward a cell-free bio-sensing system for selecting a candidate enzyme variant from a library of enzyme variants for the production of a metabolite, and method associated therewith.
Group II, claims 30 and 60 are directed toward a cell-free bio-sensing system for identifying a candidate sensor biomolecule variant from a library of sensor biomolecule variants, and method associated therewith.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include enzyme variants, not present in Group II; the special technical features of Group II include sensor biomolecule variants, not present in Group I.
-""-Continued Within the Next Supplemental Box-***-

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US1 7/21 087

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2015/0361418 A1 (LIFE TECHNOLOGIES CORPORATION) December 17, 2015; abstract; paragraphs [0049], [0051], [0073], [0079]	28, 58
Y	US 2004/0142486 A1 (WESELAK, MR et al.) July 22, 2004; paragraphs [0013], [0015], [0016]	66

INTERNATIONAL SEARCH REPORT

International application No.
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-""-Continued from Box No. III: Lack of Unity of Invention-*"-

Groups I and II share the technical features including: a cell-free bio-sensing system for identifying a candidate variant from a library of variants, comprising a plurality of first nucleotide sequences each encoding a different variant of the library of variants, a metabolite, a second nucleotide sequence encoding a reporter, wherein a sensor biomolecule interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and wherein the variants are screened by detecting the reporter to identify the candidate variant; and a method of identifying a candidate variant from a library of variants comprising providing a plurality of first nucleotide sequences each encoding a different variant of the library of variants, providing a metabolite, providing a second nucleotide sequence encoding a reporter, wherein a sensor biomolecule interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and screening the variants by detecting the reporter to identify the candidate variant.

However, these shared technical features are previously disclosed by the article 'Characterizing and prototyping genetic networks with cell-free transcription-translation reactions' by Takahashi et al. (hereinafter 'Takahashi') in view of US 2016/0060304 A1 to E I DuPont De Nemours and Company et al. (hereinafter 'DuPont').

Takahashi discloses a cell-free bio-sensing system (a cell-free analyte detection genetic network (bio-sensing system); page 2, first column, fifth paragraph), comprising first nucleotide sequence encoding a ligand-modulated transcriptional modulator (comprising first nucleotide sequence encoding tetR (a ligand-modulated transcriptional modulator); page 4, second column, third paragraph), a metabolite (a product of metabolic or biosynthetic engineering (a metabolite); page 11, first column, first paragraph; second column, second paragraph), a second nucleotide sequence encoding a reporter (and a sequence encoding a green fluorescent protein (a second nucleotide sequence encoding a reporter); page 4, second column, third paragraph). Takahashi further discloses wherein protein transcriptional repressors and activators, which allow for inducible transcriptional repression or activation also include LacI, AraC, and lambda repressors CI and Cro (wherein protein transcriptional repressors and activators, which allow for inducible transcriptional repression or activation also include LacI, AraC, and lambda repressors CI and Cro; page 10, second column, second paragraph).

Takahashi does not disclose: for identifying a candidate variant from a library of variants; a plurality of first nucleotide sequences each encoding a different variant of the library of variants; wherein a sensor biomolecule interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and wherein the variants are screened by detecting the reporter to identify the candidate variant; and a method of identifying a candidate variant from a library of variants comprising providing a plurality of first nucleotide sequences each encoding a different variant of the library of variants, providing a metabolite, providing a second nucleotide sequence encoding a reporter, wherein a sensor biomolecule interacts with the metabolite and induces the expression of the reporter in a manner dependent on the concentration of the produced metabolite, and screening the variants by detecting the reporter to identify the candidate variant.

DuPont discloses identifying a candidate variant from a library of variants (identifying a candidate variant sulfonylurea repressor from a library of tetR variants; paragraphs [0022], [0166]); a plurality of first nucleotide sequences each encoding a different variant of the library of variants (a plurality of first nucleotide sequences each encoding a different variant of the library of variants; paragraphs [0022], [0166], [0167]); wherein a sensor biomolecule interacts with a compound and induces the expression of the reporter in a manner dependent on the concentration of the compound (wherein a sensor biomolecule interacts with a compound and induces the expression of the reporter in a manner dependent on the concentration of the compound; paragraphs [0168], [0169], [0174]), and wherein the variants are screened by detecting the reporter to identify the candidate variant (wherein the variants are screened by detecting the reporter to identify the candidate variant; paragraphs [0022], [0166], [0167], [0174]); and a method of identifying a candidate variant from a library of variants (a method of identifying a candidate variant from a library of variants; paragraphs [0022], [0166], [0167]) comprising providing a plurality of first nucleotide sequences each encoding a different variant of the library of variants (comprising providing a plurality of first nucleotide sequences each encoding a different variant of the library of variants; paragraphs [0022], [0166], [0167]), providing a compound that binds to one or more variants (providing a compound that binds to one or more variants; paragraph [0022]), providing a second nucleotide sequence encoding a reporter (providing a second nucleotide sequence encoding a reporter; paragraph [0174]), wherein a sensor biomolecule interacts with the compound and induces the expression of the reporter in a manner dependent on the concentration of the compound (wherein a sensor biomolecule interacts with the compound and induces the expression of the reporter in a manner dependent on the concentration of the compound; paragraphs [0022], [0174]), and screening the variants by detecting the reporter to identify the candidate variant (and screening the variants by detecting the reporter to identify the candidate variant; paragraphs [0022], [0166], [0174]).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Takahashi to have used the system disclosed by Takahashi to perform the screening disclosed by DuPont in order to enable the more rapid and direct determination of the effects of the screened compounds on the screened variants without the additional complications of cell growth, and potentially different absorption or metabolism rates of the different compounds by the cells. It further would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have exchanged the tetR system of Takahashi and DuPont for an alternative detection and transcription modulation system, such as LacI, based on the particular compounds tested or metabolites produced, such as metabolites capable of binding to the LacI protein or variants thereof, in order to enable the screening of alternative sensor variant proteins, as disclosed by DuPont, using different transcription control systems, such as LacI, as disclosed.by Takahashi.

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