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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2018 (26.04.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/075486 A1

(51) International Patent Classification:
C12Q 1/6897 (2018.01) *G01N 33/566* (2006.01)
G01N 33/53 (2006.01) *C07K 14/00* (2006.01)

(21) International Application Number:
PCT/US2017/056933

(22) International Filing Date:
17 October 2017 (17.10.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/409,127 17 October 2016 (17.10.2016) 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, JO, 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))*

WO 2018/075486 A1

(54) Title: GENERATION OF NOVEL METABOLITE-RESPONSIVE TRANSCRIPTION REGULATOR BIOSENSORS

(57) Abstract: Disclosed are systems, components, and methods for sensing a ligand in a cell or a reaction mixture. The disclosed systems, components, and methods may include and/or utilize a fusion protein comprising a ligand-binding protein and a DNA-binding protein. The fusion protein binds the ligand of the ligand-binding protein and modulates expression of a reporter gene operably linked to a promoter that is engineered to include specific binding sites for the DNA-binding protein. The difference in expression of the reporter gene in the presence of the ligand versus expression of the reporter gene in the absence of the ligand can be correlated to the concentration of the ligand in a reaction mixture.

GENERATION OF NOVEL METABOLITE-RESPONSIVE TRANSCRIPTION REGULATOR BIOSENSORS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] This invention was made with government support under MCB1341414 awarded by the National Science Foundation, FP-91761101-0 awarded by the Environmental Protection Agency (EPA), and MH103910 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0002] This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 62/409,127, filed on October 17, 2016, the content of which is incorporated by reference in its entirety.

BACKGROUND

[0003] The field of the invention relates to biosensors comprising recombinant proteins and reporter systems. In particular, the field of the invention relates to biosensors comprising recombinant proteins that bind to a ligand, such as a cellular metabolite, and then modulate transcription of a reporter based on binding to the ligand.

[0004] Efforts to engineer microbial factories have benefitted from mining biological diversity and high throughput synthesis of novel enzymatic ensembles, yet screening and optimizing metabolic pathways remain rate-limiting steps. Metabolite-responsive biosensors may help to address these persistent challenges by enabling the monitoring of metabolite levels in individual cells and the implementation of metabolite-responsive feedback control. We are currently limited to naturally-evolved biosensors, which are insufficient for monitoring many metabolites of interest. Thus, a method for engineering novel biosensors would be powerful, yet we lack a generalizable approach that enables the construction of a

wide range of biosensors. As a step towards this goal, we developed a bottom-up strategy for converting metabolite-binding proteins into metabolite-responsive transcriptional regulators. By pairing a modular protein design approach with a library of synthetic promoters and applying robust statistical analyses, we identified quantitative design principles for engineering biosensor-regulated promoters and for achieving design-driven improvements of biosensor performance. We demonstrated the feasibility of this strategy by fusing a programmable DNA binding motif (zinc finger module) with a model ligand binding protein (maltose binding protein), to generate a novel biosensor conferring maltose-regulated gene expression. This technology enables the design of novel biosensors for diverse synthetic biology applications.

SUMMARY

[0005] Disclosed are systems, components, and methods for sensing a ligand in a cell or a reaction mixture. The disclosed systems, components, and methods may include and/or utilize a fusion protein comprising a ligand-binding protein and a DNA-binding protein that otherwise may be referred to as a "biosensor." The fusion protein, or biosensor, binds the ligand of the ligand-binding protein and modulates expression of a reporter gene operably linked to a promoter that is engineered to include specific binding sites for the DNA-binding protein. The difference in expression of the reporter gene in the presence of the ligand versus expression of the reporter gene in the absence of the ligand can be correlated to the concentration of the ligand in the system. Also disclosed are recombinant methods for preparing and selecting fusion proteins that function as biosensors in the disclosed systems and methods.

BRIEF DESCRIPTION OF THE FIGURES

[0006] Figure 1. Developing novel zinc finger protein-regulated constitutive promoters. (A) Crystal structure of a Cys_2-His_2 class of zinc finger binding to its cognate 9 bp DNA sequence (PDB #4R2A) (B) Repression of the constitutive promoter library by BCR-ABL1 (normalized to No Sites control, which lacks BCR-ABL1 binding sites) (C) Relative

expression of the top 5 most repressible promoters was evaluated during both exponential growth (as in panel B) and after reaching stationary phase. (D) Select promoter constructs were evaluated by flow cytometry to assess variation in expression and repression across the population; one plot representative of three biological replicates is shown for each condition. A concentration of 1% arabinose was used to induce the expression of the BCR-ABL1 zinc finger. Relative expression is defined as the ratio of GFP/OD600 (for any given promoter) of the induced case relative to that of the uninduced case, divided by this same ratio for the No Sites promoter (a full description and rationale can be found in the Materials and Methods section). Relative expression values calculated from these data are explicitly compared to comparable microplate assay-based metrics in Figure 9. Microplate data were collected over 7 sequential time points, spanning ~1.5 h of mid-exponential phase growth, and averaged. All data represent mean values calculated from three independent experiments, and error bars represent one standard deviation.

[0007] Figure 2. Inspection-based evaluation of promoter design rules. Promoters were manually grouped to represent exploration of design features including (A) presence and location of ZFP binding sites between the -10 box and -35 region, (B) combinatorial effects of having a pair of binding sites between the -10 box and -35 region, (C) variations in the locations of individual ZFP binding sites within the core, (D, E) contributions of additional BCR-ABL1 binding sites either downstream or upstream of the -10 box and -35 region, (F) spacing between the -10 box and the downstream ZFP binding sites, (G) combinatorial effects of directly flanking the -10 box and -35 region with ZFP binding sites. All data are re-plotted from Figure 1B. Abbreviations and conventions: ZF is the 9 bp BCR-ABL1 binding site; 1, 2, and 3 represent the first, second, or third, 3 bp finger binding sites within the BCR-ABL1 binding site; boxes represent spacer sequences of the indicated length (1, 2, 3, 4, 5, or 6 bp). Microplate data were collected over 7 sequential time points, spanning ~1.5 h of mid-exponential phase growth, and averaged. All data represent mean values calculated from three independent experiments, and error bars represent one standard deviation.

[0008] Figure 3. Computational identification of promoter design features conferring ZFP-mediated repressibility (A) Shorthand names and descriptions of the 17 features chosen to describe the promoter library. One binding site is defined as the nine base pair ZFP binding site. One pair is defined as two adjacent binding site sequences. (B) PLSR analysis of the degree to which promoter features explain variance in the relative expression data (BCR-ABL-mediated repressibility) reported in Figure 1. Each series evaluates the explanatory power achieved using an increasing number of features or principle components, each of which is added to the set in ranked order from most to least important. (C) Lasso regression analysis of the degree to which promoter features explain variance in the relative expression data reported in Figure 1. This plot displays the number of features with non-zero coefficients (and the resulting mean squared error) as λ is increased, causing less important features to be eliminated from the regression analysis. The mean squared error was obtained through 10-fold cross validation, which also produced a standard error for the mean squared error, which is shown as error bars. (D) Relative importance of each feature, vis-à-vis explaining BCR-ABL1-mediated repressibility as determined by PLSR, Random Forest, and Lasso regression, with the overall order listed here determined by average rank across the three feature selection methods. (+) - positive coefficients (large feature values confer more repressibility); and (-) - negative coefficients (small feature values confer more repressibility). Detailed regression coefficients and importance values are provided in Figure 10.

[0009] Figure 4. Engineering novel biosensors using the split zinc finger (SZF) and Split Protein (SP) strategies. (A) This cartoon illustrates the proposed mechanism of action of an SZF biosensor. (B) SZF biosensor performance, when paired with the reporter plasmids indicated, was evaluated by inducing biosensor expression (30 μM IPTG) and evaluating alleviation of repression upon addition of maltose (100 mM). Five graph bars from left to right: Reporter Only; Biosensor; Biosensor + Maltose; Biosensor + IPTG; and Biosensor + IPTG + Maltose. (C) This cartoon illustrates the proposed mechanism of action of an SP biosensor. (D) SP biosensor performance was evaluated as in panel B. Five graph bars from left to right: Reporter Only; Biosensor; Biosensor + Maltose; Biosensor + IPTG; and

Biosensor + IPTG + Maltose. (E) Comparison of the repression (i.e., reduction of relative expression) of reporter output upon expression of the SZF and SP biosensors compared to that mediated by inducing expression of the BCR-ABL1 ZFP alone. Three top points are values from SZF biosensors. Three bottom points are values from SP biosensors. (F) Tradeoff between level of expression of the SP biosensor (IPTG dose) with both repression (-maltose, bottom curve) and alleviation (+100 mM maltose, top curve) of expression from the Go66 reporter. (G) Response of reporter output to various *extracellular* concentrations of maltose, under two levels of biosensor expression (IPTG doses). Top horizontal bars indicate the 0 mM maltose case for 10 μM IPTG. Bottom horizontal boars indicated the 0 mM maltose case for 30 μM IPTG, with the width of each bar indicating one standard deviation. Top curves indicate 10 μM IPTG for increasing concentration of maltose: 0.1, 1, 10, or 100 mM. Bottom curves indicate 30 μM IPTG for increasing concentration of maltose: 0.1, 1, 10, or 100 mM. (H) The impact of the W340A mutation (SP: top curve: SP(W340A): bottom curve), which is reported to diminish maltose binding [72], on biosensor performance was evaluated using the Go92 reporter and analyses paralleling those used in panel G. Horizontal bars correspond to the indicated biosensor with 0 mM maltose, with the width of each bar indicating one standard deviation. Microplate data were collected over 7 sequential time points, spanning ~1.5 h of mid-exponential phase growth, and averaged. Relative expression was utilized in order to implicitly correct for any minor effects that IPTG or maltose many confer on GFP/ OD_{600} in a manner that is unrelated to expression of the ZFP or biosensor (see Materials and Methods for details). All data points represent mean values calculated from two independent experiments, each run in biological triplicate, and error bars represent one standard deviation (**$p \leq 0.01$, ***$p \leq 0.001$).

[0010] Figure 5. Contributions of biosensor biophysical properties to biosensor performance. At top, the illustration summarizes the biosensor design space explorations described in this figure, using the SP biosensor as a reference case. SP-Zif268 incorporates the tighter binding Zif268 ZFP in place of BCR-ABL1. In SP-mC and mC-SP, mCherry was fused to the C-terminus or N-terminus of the SP biosensor, respectively. Below, biosensor

performance, when paired with the Go92 reporter, was evaluated by inducing biosensor expression (30 μM IPTG) and evaluating alleviation of repression upon addition of maltose (100 mM). Microplate data were collected over 7 sequential time points, spanning ~1.5 h of mid-exponential phase growth, and averaged. All data represent mean values calculated from two independent experiments, each run in biological triplicate, and error bars represent one standard deviation (*$p \leq 0.05$, *** $p \leq 0.001$).

[0011] Figure 6. Plasmid maps of representative plasmids used in this study. pAY242 is the pBAD (arabinose-inducible) vector expressing the BCR-ABL1 zinc finger and mCherry (co-cistronically). This is a high copy plasmid (ColE1 origin) and contains the KanR resistance marker. pAY268 is a representative reporter plasmid with the Go3 promoter, driving the expression of EGFP. This is a medium copy plasmid (pA15 origin) and contains the Amp^R resistance marker. pAY419 drives expression of the SP biosensor (and mCherry, co-cistronically) from the pTrc2 promoter (which is IPTG-inducible). The site at which MBP is split via the BCR1 insertion is indicated on this map. This is a high copy plasmid (ColE1 origin) and contains the Kan^R resistance marker.

[0012] Figure 7. Engineered promoter library details. Each BCR-ABL1-based promoter used in this study is listed and annotated as per the key at top. All Zif268 promoters (not listed) are identical in every way to their BCR-ABL1 counterparts except that the Zif268 binding site (GCGTGGGCG) replaces each instance of the BCR-ABL1 binding site.

[0013] Figure 8. Specific fluorescence variation across the promoter library. (A) The impact of BCR-ABL1 expression on GFP reporter output was evaluated using a library of promoters bearing BCR-ABL1 sites at various locations in the promoter. The specific fluorescence (GFP fluorescence per OD_{600}) was measured for each promoter in the library in the absence of BCR-ABL1 and normalized to that of the No Sites control promoter. (B) Comparison of specific fluorescence without BCR-ABL1 to BCR-ABL1-mediated repressibility (relative expression); note that each quantity was normalized to value associated with the No Sites control promoter. Relative expression is defined as the ratio of GFP/OD_{600}

(for any given promoter) of the induced case relative to that of the uninduced case, divided by this same ratio for the No Sites promoter (a full description and rationale can be found in the Materials and Methods section of the main manuscript). All experiments were run in biological triplicate, and error bars indicate one standard deviation.

[0014] Figure 9. Comparison of flow cytometry and microplate assay-based quantification of BCR-ABL1-mediated repressibility. Select promoter constructs were analyzed by both methods. Close association of each point with the diagonal line (y = x) drawn as a visual guide indicates agreement between the two methods of quantifying relative expression, with the possible exception of Go19, which was the least repressible promoter analyzed. All samples were normalized to the No Sites control promoter. Experiments were conducted in biological triplicate, and error bars indicate one standard deviation.

[0015] Figure 10. Feature selection for BCR-ABL1-mediated repression. (A) Partial least squares repression (PLSR) coefficients associated with each feature (bars) are plotted along with corresponding standard deviations (error bars, which indicate the error around the mean coefficient value obtained by iterative permutation; this mean value was 0 in all cases, and thus error bars are plotted as deviations from 0). (B) PLSR coefficients associated with each feature were normalized by dividing each coefficient by its associated standard deviation, as obtained by iterative permutation (see Materials and Methods). Features with a large normalized coefficient value are most important. (C) Average mean squared errors obtained when each feature was permuted during the Random Forest analysis (see Materials and Methods). Features associated with a high mean squared error (when permuted) are more important. Feature numbers correspond to those listed in Figure 3A in the main text.

[0016] Figure 11. Analysis of SP biosensor performance at the individual cell level. Cells containing the SP biosensor and the Go92 reporter were grown and induced in the same manner as was used for microplate reader analysis, and then these cells were analyzed by flow cytometry. Cells were gated using forward and side scatter to exclude debris. The histogram represents cells that contain neither the biosensor nor the reporter plasmids. The

remaining histograms represent cells expressing the SP biosensor and the Go92 reporter, cultured under the medium conditions indicated.

[0017] Figure 12. The effect of maltose on the BCR-ABL1 zinc finger's repressibility over a range of IPTG induction levels. The BCR-ABL1 zinc finger was expressed with the pTrc2 promoter in combination with the Go66 reporter. Compare to biosensor performance in Figure 4F. Note that the differences in repressibility between pTrc-BCR-ABL1 (shown here) and pBAD-BCR-ABL1 (shown in Figure 1, Figure 2, 8, and 9) may be attributed to different levels of expression of this ZFP from the aforementioned promoters. Given the potential for catabolite repression of the pBAD promoter in the presence of maltose, pTrc-BCR-ABL1 was constructed to enable testing the effect of maltose on BCR-ABL1-mediated repression of the reporters. All data represent mean values calculated from two independent experiments, each run in biological triplicate, and error bars represent one standard deviation.

[0018] Figure 13. Impact of 100mM maltose and IPTG on cell growth. (A, B) Growth curves were collected for cells transformed with either the SP biosensor + the No Sites reporter (A) or the SP biosensor + the Go66 reporter (B). Experiments were conducted in biological triplicate, and error bars indicate one standard deviation.

[0019] Figure 14. Fold induction and alleviation calculated using metrics previously applied to natural biosensors. Using the fold induction methods described by Rogers *et al.* (2015), fold repression was calculated by dividing the (maximum, uninduced background subtracted GFP/OD_{600}) by the (induced, background subtracted GFP/OD_{600}, repressed fluorescence). Fold alleviation was similarly calculated by dividing the (maximum, background subtracted GFP/OD_{600}, maltose-induced fluorescence), by the (induced, background subtracted GFP/OD_{600}, repressed fluorescence). GFP/OD_{600} values were calculated 10 hours after induction. Each range indicated is one standard deviation, in which error was propagated according to the division rule.

[0020] Figure 15. Overview of the BERDI method for generating novel metabolite-responsive transcription factor biosensors. (A) Library generation - The donor plasmid

containing the gene of interest, the transposon, and the transposase enzymes create a library of random insertions. (B) Cloning of the transposed gene. Gene containing the inserted transposon is isolated, and cloned into a similarly digested expression plasmid. (C) Exchanging the transposon for the ZFP. The transposon is replaced with ZFP and are transformed into cells containing the reporter GFP plasmid. (D) Cartoon of potential enrichment strategy for metabolite responsive biosensors using FACS. See materials and methods for experimental details.

[0021] Figure 16. Analysis of naïve library diversity. (A) Initial evaluation of distribution of biosensor diversity via gel electrophoresis comparing the naïve library of biosensors, to the same sample now treated with a restriction enzyme unique to the ZFP (BamHI). Lane 1, full length biosensors (1455bp). Lane 2, digested biosensors. Lane 3, DNA ladder with the corresponding bp values listed. (B) Intensity trace of both lanes from panel A created in ImageJ. Position zero maps to the space in the gel below any visible DNA, taken to be the background intensity. (C) Experimentally observed ZFP insertion positions into MBP. Insertions found via NGS and/or via colony (Sanger) sequencing are displayed.

[0022] Figure 17. Isolation of functional biosensors by screening. (A) Cartoon depicting the overall biosensor enrichment strategy applied. Briefly, three rounds of FACS were performed, each time sorting the induced population using a gate encompassing no more than 1% of the "ON" (uninduced) population. One subsequent round of sorting was done to isolate only repressors that were reversible. Cells were then plated, and clonally evaluated for maltose responsiveness. Successful biosensors were sequenced to determine the insertions position. (B) Crystal structure of MBP is shown (PDB# 1ANF)(Quiocho *et al.*, 1997), with the insertional positions (in amino acid number) of each biosensor labeled. A cluster of spheres represents the ligand, maltose (space-filling model). (C) Flow cytometry of the reference biosensor compared to the three new biosensors. Biosensor production was induced with 30 μM IPTG and maltose was added at 100 mM. The insertional position (in amino acid number), and whether the ZFP is a single, or double insertion, is listed in the top left corner of

each plot. Plots represent a minimum of 10,000 cells in each condition and are representative of multiple independent experiments.

[0023] Figure 18. Impact of biosensor expression level on performance. Response of reporter output to the addition of IPTG and IPTG along with maltose measured by flow cytometry. The maltose concentration used here was 100mM. The vertical line at 10^3 GFP fluorescence units is a visual aid to facilitate comparison across the conditions. Plots represent multiple independent experiments.

[0024] Figure 19. Impact of linker tuning on biosensor performance. (A) Comparison of the effect of amino acid linkers between reference biosensor and its transposon-created counterpart on repressibility and alleviation with maltose. (B) Impact of varying linker lengths for the 335P biosensor. Biosensors were induced with 30 μM IPTG and maltose was added at 100 mM final concentration. (C) Mean fluorescence intensity of the four linker variants of the 335P biosensor measured via flow cytometry. Biosensors were induced with 30 μM IPTG and maltose was added at 100 mM final concentration. Samples were run in biological triplicate, and error bars represent one standard deviation. (*$p \leq 0.001$ from a two-tailed students t-test). All data are representative of multiple independent experiments.

[0025] Figure 20. Transposon key features and scar options. (A) Cartoon representation of the transposon. The transposon is digested out of its storage vector pAY438 using the BglII restriction enzymes. This leaves the minimal 3' "A" that is essential for MuA mediated strand transfer during the transposition reaction. All bases outside of this "A" get cleaved from the transposon following transposition. Highlighted bases will remain in the gene of interest after the transposon has been excised out. The NotI site is embedded in the MuA recognition site. The MuA recognition sites are show in boxes. The CamR and SacB ORFs with their own constitutive promoters are contained within the MuA recognition sequences. (B) The transposon will insert irrespective of frame: therefore, for each of the three frames, we describe the potential bases that need to be added to the ZFP to ensure that both regions of MBP are in frame with the ZFP insertion. By adding a single base to the front

of the ZFP for frame 2, all the linkers will be alanines, except for the codon interrupted by transposon, and this cannot become a STOP codon. Frames 1 and 3 yielded poor linker options after the frame of the ZFP and MBP was preserved. Codons containing a "*" are controllable by varying the "X" base identity.

[0026] Figure 21. Representative graphic of the primers used to prepare library for deep sequencing. The zinc finger could potentially be anywhere inside MBP and is only drawn in the middle here for ease of visualization. All primers contained a variant of the common sequences necessary for the downstream amplification conducted by the next generation sequencing core. Primers were spaced apart in order to capture insertions in windows of ~300 bp, as the NGS required amplicons < 500 bp in length. The top cartoon describes the primers used for the forward facing ZFP, whereas the bottom cartoon described the reactions that were done to account for possible reverse ZFP insertions due to the palindromic nature of the transposon recognition sequence. Please see Table ??? (S3) for description of the 8 PCRs.

[0027] Figure 22. Flow Chart of NGS Analysis Pipeline. This diagram summarizes the logic of the custom NGS data analysis program written for this study. Functions were created to conduct each task are outlined in the boxes. The boxes indicate steps where a Needleman-Wunsch (N-W) alignment is performed or indicate post-processing steps that only require the Needleman-Wunsch output alignment file of the insert aligned against the template. Phred quality scores are a measure of the quality of identification of a particular nucleotide during the NGS process; a score of 20 corresponds to 99% confidence in the identity of that base.

[0028] Figure 23. 270A double ZFP versus 270A single ZFP. The effects the number of zinc finger inserts into MBP at position 270A on biosensor performance were evaluated using flow cytometry for this construct. IPTG was used to induce biosensor repression, and the maltose addition relieved repression. The plots shown use varying concentrations of IPTG

and are grouped by the number of zinc fingers present with (A) two zinc finger insertions, (B) one zinc finger insertion.

DETAILED DESCRIPTION

[0029] The present invention is described herein using several definitions, as set forth below and throughout the application.

[0030] Definitions

[0031] Unless otherwise specified or indicated by context, the terms "a", "an", and "the" mean "one or more." For example, "a component" should be interpreted to mean "one or more components."

[0032] As used herein, "about," "approximately," "substantially," and "significantly" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of these terms which are not clear to persons of ordinary skill in the art given the context in which they are used, "about" and "approximately" will mean plus or minus $\leq$10% of the particular term and "substantially" and "significantly" will mean plus or minus >10% of the particular term.

[0033] As used herein, the terms "include" and "including" have the same meaning as the terms "comprise" and "comprising" in that these latter terms are "open" transitional terms that do not limit claims only to the recited elements succeeding these transitional terms. The term "consisting of," while encompassed by the term "comprising," should be interpreted as a "closed" transitional term that limits claims only to the recited elements succeeding this transitional term. The term "consisting essentially of," while encompassed by the term "comprising," should be interpreted as a "partially closed" transitional term which permits additional elements succeeding this transitional term, but only if those additional elements do not materially affect the basic and novel characteristics of the claim.

[0034] The disclosed technology relates to "biosensors." As disclosed herein, a "biosensor" is a molecule or a system of molecules that can be used to bind to a ligand and provide a detectable response based on binding the ligand. In some cases, "biosensors" may be referred to as "molecular switches." Biosensors and molecular switches are disclosed in the art. (*See, e.g.*, Ostermeier, Protein Eng. Des. Sel. 2005 Aug; 18(8):359-64; Wright *et al.*, Curr. Opin. Chem. Biol. 2007 Jun; 11(3):342-6; Roberts, Chem. Biol. 2004 Nov; 11(11): 1475-6; and U.S. Patent Nos. 8,771,679; 8,679,753; and 8,338,138; the contents of which are incorporated herein by reference in their entireties). Biosensors and molecular switches have been utilized in recombinant microorganisms. (*See, e.g.*, Rogers *et al.*, Curr. Opin. Biotechnol. 2016 Mar 18;42:84-91; and U.S. Published Application Nos. 2010/0242345 and 2013/0059295; the contents of which are incorporated herein by reference in their entireties). As indicated, many results have been published describing the utility of using naturally occurring biosensors for novel purposes, and applications include both high throughput screening and feedback-mediated enhanced production of various products via biosynthetic pathways. However, to the present inventors' knowledge, no one has published a bottom-up and generalizable strategy for converting metabolite-binding proteins into metabolite-responsive biosensors.

[0035] As used herein, the term "metabolite-binding protein" may be used interchangeably with the term "ligand-binding protein." As contemplated herein, a "ligand-binding protein" may include any protein that binds to a ligand. For example, a ligand-binding protein may include a receptor for a ligand. A ligand-binding protein may include an enzyme, and where the ligand-binding protein is an enzyme, the substrate for the enzyme may corresponds to the ligand as contemplated herein. As such, the term "ligand" may be used interchangeably herein with the term "substrate." A ligand-binding protein may include a periplasmic binding protein that binds a ligand or substrate. A ligand-binding protein may include a transporter that binds a ligand or substrate.

[0036] The systems, components, and methods disclosed herein may be utilized for sensing a ligand or a substrate or a metabolite in a cell or a reaction mixture. The disclosed

systems, components, and methods typically include and/or utilize a fusion protein comprising a ligand-binding protein and a DNA-binding protein. The fusion protein of the disclosed systems and methods may otherwise be referred to as a "biosensor" as contemplated herein. The fusion proteins or biosensors disclosed herein bind the ligand of the ligand-binding protein and modulate expression of a reporter gene operably linked to a promoter that is engineered to include specific binding sites for the DNA-binding protein. The difference in expression of the reporter gene in the presence of the ligand versus expression of the reporter gene in the absence of the ligand can be correlated to the concentration of the ligand in a reaction mixture. As such, in some embodiments, the disclosed fusion proteins or biosensors may be referred to as metabolite-responsive transcription factors.

[0037] In some embodiments, the fusion protein or biosensor binds to the promoter that is engineered to include specific binding sites for the DNA-binding protein with an affinity (K_{d1}) in the absence of the ligand. When the ligand is present, the fusion protein or biosensor binds the ligand, and then the fusion protein has a second binding affinity (K_{d2}) for the promoter in the presence of the ligand. For example, the fusion protein or biosensor may bind the ligand and undergo a conformation change that alters the binding affinity of the fusion protein or biosensor for the promoter. In some embodiments of the disclosed systems $K_{d1} < K_{d2}$, and in other embodiments of the disclosed systems $K_{d1} > K_{d2}$. The difference in affinities in the presence and absence of the ligand may be based on a conformational change that the fusion protein exhibits in the presence of the ligand versus the absence of the ligand. The fusion protein or biosensor may modulate expression of the report gene based on whether the fusion protein or biosensor is bound to the promoter or the fusion protein or biosensor is not bound to the promoter, and the modulation may be correlated with the concentration of the ligand in the system.

[0038] As used herein, "modulating expression" may include "repressing expression" and/or "inhibiting expression," and "modulating expression may include "de-repressing expression" and/or "activating expression." As such, in some embodiments, when the fusion protein or biosensor is not bound to a ligand, the fusion protein or biosensor may repress

expression and/or inhibit expression from a promoter that is engineered to include specific binding sites for the DNA-binding protein, and when the fusion protein or biosensor is bound to the ligand the fusion protein may de-repress and/or activate expression from the promoter. De-repression and/or activation of the expression of the reporter gene then can be correlated with the presence of the ligand. In other embodiments, when the fusion protein or biosensor is bound to a ligand, the fusion protein or biosensor may repress expression and/or inhibit expression from the promoter that is engineered to include specific binding sites for the DNA-binding protein, and when the fusion protein or biosensor is not bound to the ligand the fusion protein or biosensor may de-repress expression and/or activate expression from the promoter. A decrease in expression of the reporter gene then can be correlated with the presence of the ligand.

[0039] In some embodiments, when the fusion protein or biosensor is bound to the promoter engineered to include specific binding sites for the DNA-binding protein, the fusion protein may repress and/or inhibit expression of the report gene. Then, in the presence of the ligand for the ligand binding protein, the fusion protein or biosensor may bind the ligand and de-repress and/or activate expression of the reporter gene that is operably linked to the promoter. For example, in the presence of the ligand the fusion protein or biosensor may no longer bind to the promoter or may bind to the promoter with a lower affinity such that expression of the reporter gene is de-repressed and/or activated (*i.e.*, $K_{d1} < K_{d2}$). In the absence of the ligand, the fusion protein or biosensor may undergo a conformational shift whereby the fusion protein or biosensor binds to the promoter or binds to the promoter with a higher affinity than in the presence of the ligand. De-repression and/or activation of the expression of the reporter gene then can be correlated with the presence of the ligand.

[0040] In other embodiments, when the fusion protein or biosensor is bound to the promoter engineered to include specific binding sites for the DNA-binding protein, the fusion protein or biosensor may activate expression of the reporter gene. Then, in the presence of the ligand for the ligand binding protein, the fusion protein or biosensor may bind the ligand and no longer activate expression of the reporter gene or may repress or inhibit expression of

the reporter gene, effectively decreasing expression of the reporter gene. For example, in the presence of the ligand the fusion protein or biosensor may bind to the promoter with a higher affinity than in the absence of the ligand (*i.e.*, $K_{d1} > K_{d2}$) and activate expression. In the absence of the ligand, the fusion protein or biosensor may undergo a conformational shift and may no longer bind the promoter or may bind the promoter with a lower affinity than in the presence of the ligand and no longer activate expression of the reporter gene. A decrease in expression of the reporter gene then can be correlated with the presence of the ligand.

[0041] The disclosed biosensors, systems, and methods may be utilized and/or performed using any suitable cell. Suitable cells may include prokaryotic cells and eukaryotic cells.

[0042] Reference is made herein to nucleic acid and nucleic acid sequences. The terms "nucleic acid" and "nucleic acid sequence" refer to a nucleotide, oligonucleotide, polynucleotide (which terms may be used interchangeably), or any fragment thereof. These phrases also refer to DNA or RNA of genomic or synthetic origin (which may be single-stranded or double-stranded and may represent the sense or the antisense strand).

[0043] Reference also is made herein to peptides, polypeptides, proteins and compositions comprising peptides, polypeptides, and proteins. As used herein, a polypeptide and/or protein is defined as a polymer of amino acids, typically of length $\geq$ 100 amino acids (Garrett & Grisham, Biochemistry, 2nd edition, 1999, Brooks/Cole, 110). A peptide is defined as a short polymer of amino acids, of a length typically of 20 or less amino acids, and more typically of a length of 12 or less amino acids (Garrett & Grisham, Biochemistry, 2nd edition, 1999, Brooks/Cole, 110).

[0044] As disclosed herein, exemplary peptides, polypeptides, proteins may comprise, consist essentially of, or consist of any reference amino acid sequence disclosed herein, or variants of the peptides, polypeptides, and proteins may comprise, consist essentially of, or consist of an amino acid sequence having at least about 80%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to any amino acid sequence disclosed herein. Variant peptides,

polypeptides, and proteins may include peptides, polypeptides, and proteins having one or more amino acid substitutions, deletions, additions and/or amino acid insertions relative to a reference peptide, polypeptide, or protein. Also disclosed are nucleic acid molecules that encode the disclosed peptides, polypeptides, and proteins (*e.g.*, polynucleotides that encode any of the peptides, polypeptides, and proteins disclosed herein and variants thereof).

[0045] The term "amino acid," includes but is not limited to amino acids contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues. The term "amino acid residue" also may include amino acid residues contained in the group consisting of homocysteine, 2-Aminoadipic acid, N-Ethylasparagine, 3-Aminoadipic acid, Hydroxylysine, β-alanine, β-Amino-propionic acid, allo-Hydroxylysine acid, 2-Aminobutyric acid, 3-Hydroxyproline, 4-Aminobutyric acid, 4-Hydroxyproline, piperidinic acid, 6-Aminocaproic acid, Isodesmosine, 2-Aminoheptanoic acid, allo-Isoleucine, 2-Aminoisobutyric acid, N-Methylglycine, sarcosine, 3-Aminoisobutyric acid, N-Methylisoleucine, 2-Aminopimelic acid, 6-N-Methyllysine, 2,4-Diaminobutyric acid, N-Methylvaline, Desmosine, Norvaline, 2,2'-Diaminopimelic acid, Norleucine, 2,3-Diaminopropionic acid, Ornithine, and N-Ethylglycine. Typically, the amide linkages of the peptides are formed from an amino group of the backbone of one amino acid and a carboxyl group of the backbone of another amino acid.

[0046] The amino acid sequences contemplated herein may include conservative amino acid substitutions relative to a reference amino acid sequence. For example, a variant peptides, polypeptides, and proteins as contemplated herein may include conservative amino acid substitutions relative to an amino acid sequence of a reference peptide, polypeptide, or protein. "Conservative amino acid substitutions" are those substitutions that are predicted to interfere least with the properties of the reference peptide, polypeptide, or protein. In other

words, conservative amino acid substitutions substantially conserve the structure and the function of the reference peptide, polypeptide, or protein. The following table provides a list of exemplary conservative amino acid substitutions.

[0047] Table of Conservative Amino Acid Substitutions

Original Residue	Conservative Substitution
Ala	Gly, Ser
Arg	His, Lys
Asn	Asp, Glu, His
Asp	Asn, Glu
Cys	Ala, Ser
Gln	Asn, Glu, His
Glu	Asp, Gln, His
Gly	Ala
His	Asn, Arg, Gln, Glu
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	His, Met, Leu, Trp, Tyr
Ser	Cys, Thr
Thr	Ser, Val
Trp	Phe, Tyr
Tyr	His, Phe, Trp
Val	Ile, Leu, Thr

[0048]

[0049] "Non-conservative amino acid substitutions" are those substitutions that are predicted to interfere most with the properties of the reference peptide, polypeptide, or protein. For example, a non-conservative amino acid substitution might replace a basic amino acid at physiological pH such as Arg, His, or Lys, with a non-basic or acidic amino acid at physiological pH such as Asp or Glu. A non-conservative amino acid substitution might replace a non-polar amino acid at physiological pH such as Ala, Gly, Ile, Leu, Phe, or Val, with a polar amino acid at physiological pH such as Arg, Asp, Glu, His, or Lys.

[0050] The peptides, polypeptides, and proteins disclosed herein may be modified to include non-amino acid moieties. Modifications may include but are not limited to

carboxylation (*e.g.*, N-terminal carboxylation via addition of a di-carboxylic acid having 4-7 straight-chain or branched carbon atoms, such as glutaric acid, succinic acid, adipic acid, and 4,4-dimethylglutaric acid), amidation (*e.g.*, C-terminal amidation via addition of an amide or substituted amide such as alkylamide or dialkylamide), PEGylation (*e.g.*, N-terminal or C-terminal PEGylation via additional of polyethylene glycol), acylation (*e.g.*, O-acylation (esters), N-acylation (amides), S-acylation (thioesters)), acetylation (*e.g.*, the addition of an acetyl group, either at the N-terminus of the protein or at lysine residues), formylation lipoylation (*e.g.*, attachment of a lipoate, a C8 functional group), myristoylation (*e.g.*, attachment of myristate, a C14 saturated acid), palmitoylation (*e.g.*, attachment of palmitate, a C16 saturated acid), alkylation (*e.g.*, the addition of an alkyl group, such as an methyl at a lysine or arginine residue), isoprenylation or prenylation (*e.g.*, the addition of an isoprenoid group such as farnesol or geranylgeraniol), amidation at C-terminus, glycosylation (*e.g.*, the addition of a glycosyl group to either asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein). Distinct from glycation, which is regarded as a nonenzymatic attachment of sugars, polysialylation (*e.g.*, the addition of polysialic acid), glypiation (*e.g.*, glycosylphosphatidylinositol (GPI) anchor formation, hydroxylation, iodination (e.g., of thyroid hormones), and phosphorylation (*e.g.*, the addition of a phosphate group, usually to serine, tyrosine, threonine or histidine).

[0051] Variants comprising deletions relative to a reference amino acid sequence or nucleotide sequence are contemplated herein. A "deletion" refers to a change in the amino acid or nucleotide sequence that results in the absence of one or more amino acid residues or nucleotides relative to a reference sequence. A deletion removes at least 1, 2, 3, 4, 5, 10, 20, 50, 100, or 200 amino acids residues or nucleotides. A deletion may include an internal deletion or a terminal deletion (*e.g.*, an N-terminal truncation or a C-terminal truncation or both of a reference polypeptide or a 5'-terminal or 3'-terminal truncation or both of a reference polynucleotide).

[0052] Variants comprising a fragment of a reference amino acid sequence or nucleotide sequence are contemplated herein. A "fragment" is a portion of an amino acid

sequence or a nucleotide sequence which is identical in sequence to but shorter in length than the reference sequence. A fragment may comprise up to the entire length of the reference sequence, minus at least one nucleotide/amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous nucleotides or contiguous amino acid residues of a reference polynucleotide or reference polypeptide, respectively. In some embodiments, a fragment may comprise at least 5, 10, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 50, 60, 70, 80, 90, 100, 150, 250, or 500 contiguous nucleotides or contiguous amino acid residues of a reference polynucleotide or reference polypeptide, respectively. Fragments may be preferentially selected from certain regions of a molecule, for example the N-terminal region and/or the C-terminal region of a polypeptide or the 5'-terminal region and/or the 3' terminal region of a polynucleotide. The term "at least a fragment" encompasses the full length polynucleotide or full length polypeptide.

[0053] Variants comprising insertions or additions relative to a reference sequence are contemplated herein. The words "insertion" and "addition" refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides. An insertion or addition may refer to 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, or 200 amino acid residues or nucleotides.

[0054] Fusion proteins and fusion polynucleotides also are contemplated herein. A "fusion protein" refers to a protein formed by the fusion of at least one peptide, polypeptide, protein or variant thereof as disclosed herein to at least one molecule of a heterologous peptide, polypeptide, protein or variant thereof. The heterologous protein(s) may be fused at the N-terminus, the C-terminus, or both termini. A fusion protein comprises at least a fragment or variant of the heterologous protein(s) that are fused with one another, preferably by genetic fusion (*i.e.*, the fusion protein is generated by translation of a nucleic acid in which a polynucleotide encoding all or a portion of a first heterologous protein is joined in-frame with a polynucleotide encoding all or a portion of a second heterologous protein). The heterologous protein(s), once part of the fusion protein, may each be referred to herein as a "portion", "region" or "moiety" of the fusion protein. For example, where the fusion protein

comprises at least a portion of a ligand binding protein and at least a portion of a DNA-binding portion, the portions of the fusion may be referred to as "a ligand binding portion" and "a DNA-binding portion," respectively.

[0055] A fusion polynucleotide refers to the fusion of the nucleotide sequence of a first polynucleotide to the nucleotide sequence of a second heterologous polynucleotide (e.g., the 3' end of a first polynucleotide to a 5' end of the second polynucleotide). Where the first and second polynucleotides encode proteins, the fusion may be such that the encoded proteins are in-frame and results in a fusion protein. The first and second polynucleotide may be fused such that the first and second polynucleotide are operably linked (e.g., as a promoter and a gene expressed by the promoter as discussed below).

[0056] "Homology" refers to sequence similarity or, interchangeably, sequence identity, between two or more polypeptide sequences or polynucleotide sequences. Homology, sequence similarity, and percentage sequence identity may be determined using methods in the art and described herein.

[0057] The phrases "percent identity" and "% identity," as applied to polypeptide sequences, refer to the percentage of residue matches between at least two polypeptide sequences aligned using a standardized algorithm. Methods of polypeptide sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail above, generally preserve the charge and hydrophobicity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide. Percent identity for amino acid sequences may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S. F. et al. (1990) J. Mol. Biol. 215:403 410), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes

various sequence analysis programs including "blastp," that is used to align a known amino acid sequence with other amino acids sequences from a variety of databases.

[0058] Percent identity may be measured over the length of an entire defined polypeptide sequence or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length may be used to describe a length over which percentage identity may be measured.

[0059] A "variant" of a particular polypeptide sequence may be defined as a polypeptide sequence having at least 50% sequence identity to the particular polypeptide sequence over a certain length of one of the polypeptide sequences using blastp with the "BLAST 2 Sequences" tool available at the National Center for Biotechnology Information's website. (*See* Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250). In some embodiments a variant polypeptide may show, for example, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length relative to a reference polypeptide.

[0060] A variant polypeptide may have substantially the same functional activity as a reference polypeptide. For example, a variant polypeptide may exhibit or more biological activities associated with binding a ligand and/or binding DNA at a specific binding site.

[0061] The terms "percent identity" and "% identity," as applied to polynucleotide sequences, refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences. Percent identity for a nucleic acid sequence may be

determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul, S. F. et al. (1990) J. Mol. Biol. 215:403 410), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including "blastn," that is used to align a known polynucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called "BLAST 2 Sequences" that is used for direct pairwise comparison of two nucleotide sequences. "BLAST 2 Sequences" can be accessed and used interactively at the NCBI website. The "BLAST 2 Sequences" tool can be used for both blastn and blastp (discussed above).

[0062] Percent identity may be measured over the length of an entire defined polynucleotide sequence or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined sequence, for instance, a fragment of at least 20, at least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such lengths are exemplary only, and it is understood that any fragment length may be used to describe a length over which percentage identity may be measured.

[0063] A "full length" polynucleotide sequence is one containing at least a translation initiation codon (*e.g.*, methionine) followed by an open reading frame and a translation termination codon. A "full length" polynucleotide sequence encodes a "full length" polypeptide sequence.

[0064] A "variant," "mutant," or "derivative" of a particular nucleic acid sequence may be defined as a nucleic acid sequence having at least 50% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the "BLAST 2 Sequences" tool available at the National Center for Biotechnology Information's website. (*See* Tatiana A. Tatusova, Thomas L. Madden (1999),

"Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250). In some embodiments a variant polynucleotide may show, for example, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length relative to a reference polynucleotide.

[0065] Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences due to the degeneracy of the genetic code. It is understood that changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein.

[0066] "Operably linked" refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with a second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame.

[0067] A "recombinant nucleic acid" is a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two or more otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques such as those described in Sambrook, J. *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1 3, Cold Spring Harbor Press, Plainview N.Y. The term recombinant includes nucleic acids that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid. Frequently, a recombinant nucleic acid may include a nucleic acid sequence operably linked to a promoter sequence. Such a recombinant nucleic acid may be part of a vector that is used, for example, to transform a cell.

[0068] "Transformation" describes a process by which exogenous DNA is introduced into a recipient cell. Transformation may occur under natural or artificial conditions according to various methods well known in the art, and may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method for transformation is selected based on the type of host cell being transformed and may include, but is not limited to, bacteriophage or viral infection, electroporation, heat shock, lipofection, and particle bombardment. The term "transformed cells" includes stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome, as well as transiently transformed cells which express the inserted DNA or RNA for limited periods of time.

[0069] A "composition comprising a given polypeptide" and a "composition comprising a given polynucleotide" refer broadly to any composition containing the given polynucleotide or amino acid sequence. The composition may comprise a dry formulation or an aqueous solution. The compositions may be stored in any suitable form including, but not limited to, freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. The compositions may be aqueous solution containing salts (*e.g.*, NaCl), detergents (*e.g.*, sodium dodecyl sulfate; SDS), and other components.

[0070] "Substantially isolated or purified" nucleic acid or amino acid sequences are contemplated herein. The term "substantially isolated or purified" refers to nucleic acid or amino acid sequences that are removed from their natural environment, and are at least 60% free, preferably at least 75% free, and more preferably at least 90% free, even more preferably at least 95% free from other components with which they are naturally associated.

ILLUSTRATIVE EMBODIMENTS

[0071] The disclosed subject matter relates to metabolite-responsive transcription regulator biosensors. The following embodiments are illustrative and do not limit the scope of the claimed subject matter.

[0072] The disclosed subject matter may include systems and methods that comprise or utilize a biosensor. In some embodiments, the disclosed systems and methods comprises or utilize: (a) a fusion protein comprising a ligand-binding protein (LBP) and a DNA-binding protein (DBP) or portions or fragments thereof as described herein, the fusion protein comprising an amino acid sequence represented as: (N-terminal portion of the LBP) – (DBP) – (C-terminal portion of the LBP); and (b) a promoter which can be operably linked to a reporter gene. In the disclosed systems and methods, the promoter typically includes at least one heterologous binding site that is specific for the DBP and the fusion protein binds to the binding site and represses and/or inhibits transcription of the reporter gene in a cell or a reaction mixture when the ligand for the LBP is not present in the cell or the reaction mixture.

[0073] In the disclosed systems and methods, the fusion protein may bind to the promoter with an affinity (K_{d1}) in the absence of the ligand. When the ligand is present, the fusion protein preferably binds the ligand and the fusion protein then has a second binding affinity (K_{d2}) for the promoter in the presence of the ligand (or the fusion protein no longer binds the promoter). In some embodiments, the difference in affinities in the presence and absence of the ligand may be based on a conformational change that the fusion protein exhibits in the presence of the ligand versus the absence of the ligand.

[0074] Where $K_{d1} < K_{d2}$, transcription of the reporter gene may be de-repressed or activated in the presence of the ligand. In some embodiments, de-repression or activation in the presence of the ligand is proportional to the concentration of the ligand in the cell or the reaction mixture. In this embodiment, preferably the fusion protein binds to the promoter with a relatively high K_{d1} in the absence of the ligand (*e.g.*, with a K_{d1} < about 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, 0.01 nM or lower). In this embodiment, preferably the fusion protein binds to the promoter with a relatively low K_{d2} in the presence of ligand (*e.g.*, with a K_{d2} > about 0.02 nM, 0.05 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM or higher). In this embodiment, preferably the ratio K_{d2}:K_{d1} is at least about 5, 10, 20, 50, 100, 500, 1000 or more.

[0075] Where $K_{d1} > K_{d2}$, transcription of the reporter gene may be activated by the fusion protein when the fusion protein is bound to the ligand and expression may be no longer activated, repressed, or inhibited in the absence of the ligand, effectively decreasing expression in the absence of the ligand. In this embodiment, preferably the fusion protein binds to the promoter with a relatively high K_{d1} in the presence of the ligand (*e.g.*, with a K_{d1} < about 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, 0.01 nM or lower). In this embodiment, preferably the fusion protein binds to the promoter with a relatively low K_{d2} in the absence of ligand (*e.g.*, with a K_{d2} > about 0.02 nM, 0.05 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM or higher). In this embodiment, preferably the ratio K_{d1}:K_{d2} is at least about 5, 10, 20, 50, 100, 500, 1000 or more.

[0076] In the disclosed systems and methods, the fusion protein includes at least an N-terminal portion of the ligand-binding protein (LBP) fused at the N-terminus to at least a portion of the DNA-binding protein (DBP). In some embodiments, the fusion protein comprises an amino acid sequence of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids from the N-terminus of the LBP fused at the N-terminus to at least a portion of the DBP.

[0077] In the disclosed systems and methods, the fusion protein includes at least a C-terminal portion of the ligand-binding protein (LBP)-fused at the C-terminus to at least a portion of the DNA-binding protein (DBP). In some embodiments, the fusion protein comprises an amino acid sequence of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids from the C-terminus of the LBP fused at the C-terminus to at least a portion of the DBP.

[0078] In the disclosed systems and methods, the ligand-binding protein (LBP) binds to a ligand. In some embodiments of the systems and methods disclosed herein, the ligand is a cellular metabolite and the fusion protein and systems and methods disclosed herein may be utilized to detect and measure cellular metabolism. In some embodiments, the LBP is maltose binding protein and the ligand is maltose.

[0079] In the disclosed systems and methods, the fusion protein includes at least a portion of a DNA-binding protein (DBP). In some embodiments, the DBP comprises one or more DNA-binding domains selected from the group consisting of a zinc-finger protein (ZFP) DNA-binding domain, a transcription activator-like effector (TALE) DNA-binding domain, and a clustered regularly interspaced short palindromic repeat (CRISPR) DNA-binding domain. Suitable zinc-finger proteins may include, but are not limited to BCR-ABL1.

[0080] The disclosed systems and methods typically include or utilize a polynucleotide comprising a promoter, which may be operably linked to a reporter gene. The polynucleotide comprising a promoter, which may be operably linked to a reporter gene may be referred to as a reporter cassette. Suitable promoters include, but are not limited to, prokaryotic promoters. The promoter of the disclosed systems and methods typically is modified by inserting into the promoter a heterologous sequence that comprises one or more binding sites for the DNA-binding protein (DBP). In some embodiments, the promoter includes two, three, or more binding sites for the DBP. The binding sites are inserted in the promoter at positions such that when the DBP binds to the binding sites, the expression of a reporter gene that is operably linked to the promoter is repressed. In some embodiments, the binding sites are located at one or more positions in the promoter selected from: (i) between the -10 box (TATA box) and the -35 box (GC-rich region); (ii) adjacent to the -10 box or within 5 nucleotides of the -10 box; and/or (iii) adjacent to the -35 box or within 5 nucleotides of the -35 box.

[0081] The disclosed systems and methods may include or utilize a reporter gene that is operably linked to the promoter of the systems and methods, for example as part of a reporter cassette. Suitable reporter genes may provide a detectable signal when expressed (*e.g.*, fluorescence of GFP) and/or may provide a selectable marker when expressed (*e.g.*, a marker for anti-biotic resistance such as β-lactamase).

[0082] Also disclosed herein are methods for making and selecting components for use in the disclosed systems including methods for making and selecting biosensors or fusion

proteins as discussed herein In some embodiments, the disclosed methods may be performed to prepare and select a fusion proteins comprising a ligand-binding protein (LBP) and a DNA-binding protein (DBP) or portions or fragments thereof as described herein, the fusion protein comprising an amino acid sequence represented as: (N-terminal portion of the LBP) – (DBP) – (C-terminal portion of the LBP). A library of fusion proteins may be prepared by inserting the DBP randomly into the LBP, for example, by performing a recombinant DNA method such as transposon-mediated recombination. One or more fusion proteins of the library then may be tested and selected for use as biosensors in the systems and methods disclosed herein. For example, the fusion proteins of the library may be tested for repressing transcription from a promoter that includes at least one heterologous binding site that is specific for the DBP, where the fusion protein binds to the binding site of the promoter and represses and/or inhibits transcription from the promoter when the ligand for the LBP is not present.

[0083] Applications for the disclosed technology include, but are not limited to: (i) novel sensing in which the novel metabolite-responsive biosensors enable real time monitoring of small molecules in living cells; (ii) high-throughput screening in which the novel metabolite-responsive biosensors can be used to rapidly screening very large $<10^8$ genetic libraries for high-producing strains; and (iii) dynamic feedback control in which the novel metabolite-responsive biosensors that regulate transcription can enable the engineering of feedback control to optimize production of product molecules via natural and/or engineered biosynthetic pathways.

[0084] Advantages of the disclosed technology include, but are not limited to: (i) the disclosed technology enables the use of biosensors to monitor the many metabolites not recognized by natural biosensors; (ii) the disclosed technology is generalizable in that the disclosed technology enables leveraging the wealth of naturally occurring metabolite-binding proteins into metabolite responsive transcriptional regulator proteins; (iii) the disclosed technology is broadly applicable because it utilizes modular DNA binding proteins, such as zinc finger proteins, such that novel biosensors can be easily programmed to regulate specific target genes; and (iv) the disclosed technology provides a library of zinc finger-responsive

promoters, which we have built and characterized, that exhibit a range of response profiles, and as such, a user can predictably implement a desired biosensor-regulated function by pairing an engineered biosensor with a desired promoter design based upon the provided library.

[0085] In one embodiment, the present inventors developed a generalizable strategy for engineering novel metabolite-responsive transcriptional regulators. The inventors explored several strategies for converting a ligand-binding protein into a functioning biosensor, applied quantitative analysis to identify rules for designing biosensor-regulated promoters, and quantitatively characterized these novel biological parts. The inventor's systematic investigation guides the engineering of customized metabolite-responsive biosensors.

ILLUSTRATIVE EMBODIMENTS

[0086] The following embodiments are illustrative and should not be interpreted to limit the scope of the claimed subject matter.

[0087] Embodiment 1. A system comprising: (a) a fusion protein that functions as a biosensor, the fusion protein comprising a ligand-binding protein (LBP) and a DNA-binding protein (DBP), the fusion protein comprising an amino acid sequence represented as: (N-terminal portion of the LBP) – (DBP) – (C-terminal portion of the LBP); and (b) a promoter which can be operably linked to a reporter gene, wherein the promoter comprises at least one heterologous binding site that is specific for the DBP and the fusion protein binds to the ligand and modulates expression from the promoter.

[0088] Embodiment 2. The system of embodiment 1, wherein the fusion protein has a first binding affinity (K_{d1}) for the promoter in the absence of the ligand, and the fusion protein has a second binding affinity (K_{d2}) for the promoter in the presence of the ligand, such that $K_{d1} < K_{d2}$ and transcription of the reporter gene is de-repressed or activated in the presence of the ligand.

[0089] Embodiment 3. The system of embodiment 2, wherein de-repression or activation is proportional to concentration of the ligand in the system.

[0090] Embodiment 4. The system of embodiment 2 or 3, wherein K_{d1} is < about 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, 0.01 nM or lower.

[0091] Embodiment 5. The system of any of embodiments 2-4, wherein K_{d2} is > about 0.02 nM, 0.05 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM or higher.

[0092] Embodiment 6. The system of any of embodiments 2-5, wherein the ratio K_{d2}:K_{d1} is at least about 5, 10, 20, 50, 100, 500, 1000 or more.

[0093] Embodiment 7. The system of embodiment 1, wherein the fusion protein has a first binding affinity (K_{d1}) for the promoter in the absence of the ligand, and the fusion protein has a second binding affinity (K_{d2}) for the promoter in the presence of the ligand, such that $K_{d1} > K_{d2}$ and transcription of the reporter gene is repressed or de-activated in the presence of the ligand.

[0094] Embodiment 8. The system of embodiment 7, wherein repression or de-activation is proportional to concentration of the ligand in the system.

[0095] Embodiment 9. The system of embodiment 7 or 8, wherein K_{d2} is < about 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, 0.01 nM or lower.

[0096] Embodiment 10. The system of any of embodiments 7-9, wherein K_{d1} is > about 0.02 nM, 0.05 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM or higher.

[0097] Embodiment 11. The system of any of embodiments 7-10, wherein the ratio K_{d1}:K_{d2} is at least about 5, 10, 20, 50, 100, 500, 1000 or more.

[0098] Embodiment 12. The system of any of the foregoing embodiments, wherein the N-terminal portion of the LBP within the fusion protein comprises an amino acid

sequence of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids from the N-terminus of the LBP.

[0099] Embodiment 13. The system of any of the foregoing embodiments, wherein the C-terminal portion of the LBP within the fusion protein comprises an amino acid sequence of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids from the C-terminus of the LBP.

[00100] Embodiment 14. The system of any of the foregoing embodiments, wherein the ligand is a cellular metabolite.

[00101] Embodiment 15. The system of any of the foregoing embodiments, wherein the LBP is maltose binding protein.

[00102] Embodiment 16. The system of any of the foregoing embodiments, wherein the DBP comprises one or more DNA-binding domains selected from the group consisting of a zinc-finger protein (ZFP) DNA-binding domain, a transcription activator-like effector (TALE) DNA-binding domain, and a clustered regularly interspaced short palindromic repeat (CRISPR) DNA-binding domain.

[00103] Embodiment 17. The system of embodiment 16, wherein the ZFP is BCR-ABL1.

[00104] Embodiment 18. The system of any of the foregoing embodiments, wherein the promoter is a prokaryotic promoter.

[00105] Embodiment 19. The system of embodiment 18, wherein the promoter comprises two or more binding sites for the DBP.

[00106] Embodiment 20. The system of embodiment 19, wherein the binding sites are located at one or more positions selected from: (i) between the -10 box (TATA box) and the -35 box (GC-rich region); (ii) adjacent to the -10 box (TATA box) or within 5 nucleotides of

the -10 box (TATA box); and (iii) adjacent to the -35 box (GC-rich region) or within 5 nucleotide of the -35 box (GC-rich region).

[00107] Embodiment 21. A method for preparing a fusion protein for use as a biosensor, the fusion protein comprising a ligand-binding protein (LBP) and a DNA-binding protein (DBP) or portions or fragments thereof, where the fusion protein comprises an amino acid sequence represented as (N-terminal portion of the LBP) – (DBP) – (C-terminal portion of the LBP), the method comprising preparing a library of fusion proteins by inserting the DBP randomly into the amino acid sequence of the LBP via performing a recombinant DNA method, and selecting a fusion protein from the library of fusion proteins as a biosensor.

[00108] Embodiment 22. The method of embodiment 21, wherein the recombinant DNA method is transposon-mediated DNA recombination.

[00109] Embodiment 23. The method of embodiment 21, wherein the fusion protein is selected as a biosensor via testing whether the fusion protein modulates transcription from a promoter comprising a binding site for the DBP of the fusion protein in the presence or absence of the ligand for the LBP of the fusion protein.

EXAMPLES

[00110] The following Examples are illustrative and are not intended to limit the scope of the claimed subject matter.

[00111] Example 1

[00112] Reference is made to Younger *et al.*, "Engineering novel modular biosensors to confer metabolite-responsive regulation of transcription," ACS Synth. Biol. 2017 Feb 17;6(2):311-325, the content of which is incorporated herein by reference in its entirety.

[00113] Title: Engineering modular biosensors to confer metabolite-responsive regulation of transcription

[00114] Abstract

[00115] Efforts to engineer microbial factories have benefitted from mining biological diversity and high throughput synthesis of novel enzymatic pathways, yet screening and optimizing metabolic pathways remain rate-limiting steps. Metabolite-responsive biosensors may help to address these persistent challenges by enabling the monitoring of metabolite levels in individual cells and metabolite-responsive feedback control. We are currently limited to naturally-evolved biosensors, which are insufficient for monitoring many metabolites of interest. Thus, a method for engineering novel biosensors would be powerful, yet we lack a generalizable approach that enables the construction of a wide range of biosensors. As a step towards this goal, we here explore several strategies for converting a metabolite-binding protein into a metabolite-responsive transcriptional regulator. By pairing a modular protein design approach with a library of synthetic promoters and applying robust statistical analyses, we identified strategies for engineering biosensor-regulated bacterial promoters and for achieving design-driven improvements of biosensor performance. We demonstrated the feasibility of this strategy by fusing a programmable DNA binding motif (zinc finger module) with a model ligand binding protein (maltose binding protein), to generate a novel biosensor conferring maltose-regulated gene expression. This systematic investigation provides insights that may guide the development of additional novel biosensors for diverse synthetic biology applications.

[00116] Introduction

[00117] Cells evaluate and respond to their internal states through a range of mechanisms, including the wide use of molecular biosensors. In a general sense, a biosensor may be understood to comprise a species that senses one or more analytes, typically through a molecular recognition event involving binding to the analyte, such that recognition of the analyte is transduced into a change in the biosensor that enables it to effect a change in cell state. Biosensors may be composed of a range of biomolecules, most commonly including RNA [1-3] or protein [4-15]. Early applications included the generation of whole-cell biosensors, in

which an environmental analyte enters a cell through active or passive transport. Upon recognition of the analyte by an intracellular biosensor, an output signal such as fluorescence, luminescence, or color-change is generated, most commonly by biosensor-induced expression of a reporter gene or by analyte binding-induced changes in the activity of a fluorescent or enzymatic biosensor protein. A particularly exciting frontier is the use of biosensors to sense not external factors, but rather a cell's internal metabolic state.

[00118] Metabolite-responsive biosensors may help to address several pervasive and persistent challenges in the fields of synthetic biology and metabolic engineering [3-7, 9, 11, 13-16]. First, biosensors may help overcome the costliest and rate-limiting step in the development of new biosynthetic pathways – screening and evaluating pathway or strain variants to both identify well-performing constructs and glean insights into pathway function that may be utilized in subsequent iterative rounds of the design-build-test engineering cycle. By coupling metabolite-binding to outputs such as fluorescence or antibiotic resistance, biosensors can enable the screening of large libraries (e.g., $>10^8$ members), which remain beyond the capacity of even contemporary automated platforms for performing clonal evaluations. For example, the naturally occurring transcription factor BmoR was harnessed to confer growth in the presence of butanol, which enabled the screening of a plasmid library to identify strains exhibiting robust production of 1-butanol [6]. Similar approaches have been harnessed to screen plasmid libraries to achieve enhanced production of mevalonate [17], triacetic acid lactone [18], and L-lysine [19]. Such an approach may be extended to screen for high-performing variants generated through genomic mutation, including both random mutagenesis, which has been utilized to optimize L-lysine production via mutation of endogenous enzymes [20], and targeted genome-wide mutagenesis, which has been used to optimize naringenin and glucaric acid production via combinatorial perturbation of endogenous gene regulation [4]. While most investigations to date have applied these methods to bacterial chasses, such approaches may also be extended to yeast and other organisms [9]. In general, "digital" biosensor outputs, such as expression of antibiotic resistance, are most useful for screening, while "analog" biosensor outputs, such as fluorescence, enable both screening and characterization of internal

metabolite concentrations, potentially at the single-cell level, to guide construct analysis and iterative refinement.

[00119] A powerful, yet less explored extension of this approach is the use of metabolite-responsive transcriptional regulators to implement feedback control in order to optimize system performance. An early demonstration of this opportunity was the use of an acetyl phosphate biosensor to sense excess glycolytic flux, and in response, regulate the expression of limiting genes in the lycopene biosynthesis pathway. This activity resulted in both enhanced lycopene production and diminished growth defects [16]. More recently, feedback control was used to achieve balanced flux through several pathways that led to enhanced yields and improved cell survival during the production of a biofuel (fatty acid ethyl ester) [7]. This investigation made use of the natural FadR biosensor, which is antagonized by Acyl-CoA, paired with synthetic promoters engineered to achieve robust regulation by FadR. Similarly, lysine-responsive riboswitches were utilized to control the expression of citrate synthase and thereby increase lysine production by controlling flux in the TCA cycle [3]. The potential utility of biosensor-mediated feedback control is now widely recognized [11, 21, 22], and further implementation is currently limited largely by the pool of suitable biosensors.

[00120] A general challenge in the use of biosensors is that the pool of metabolites one would like to measure and potentially utilize for feedback control is much larger than the pool of metabolite-responsive transcriptional regulators that have been identified. Bioinformatic approaches and surveys of published literature may identify a number of useful biosensors that have simply not yet been utilized as such. For example, a recent study elegantly applied a systematic characterization of known metabolite-responsive transcriptional regulators to generate quantitative fingerprints enabling these biological "parts" to be harnessed for engineering applications [5]. However, since the entire pool of naturally-evolved biosensors is likely much smaller than the pool of metabolite targets, it would be attractive to develop approaches for engineering novel biosensors. Ideally, such a biosensor could be constructed to recognize an analyte of interest (with some practical degree of specificity), would exhibit a dynamic range suitable (or tunable) to the application of interest, and could be directed to

regulate a gene (or genes) of interest in a ligand-dependent fashion. Although this comprises a daunting protein engineering challenge, a number of smaller-scale successes suggest strategies that may help to achieve this goal.

[00121] The most widely used approach for engineering novel biosensors is to genetically fuse a ligand-binding protein with a distinct functional domain, such that the fusion causes the activity of the functional domain to be conditional upon the presence or absence of the ligand of interest [10, 23-29]. Most commonly, the functional domain comprises a fluorescent protein or enzyme conferring antibiotic resistance, each of which comprises an output amenable to screening the large libraries required to identify functional fusion proteins. For example, maltose binding protein (MBP) and β-lactamase (BLA) were circularly permutated to generate a library of fusion proteins, such that successful fusions exhibited high BLA activity only in the presence of maltose [24]. Calmodulin, which experiences a conformational change upon binding to Ca^{2+}, is similarly amenable to such a fusion strategy to create fusion proteins based upon BLA [26] or GFP and its derivatives [30]. Indeed, many similar approaches have harnessed proteins in which ligand binding induces a conformational change in order to generate biosensors in which fluorescence, often via FRET, provides a metric of intracellular metabolite concentration (reviewed in [10, 31-33]). Furthermore, zinc finger proteins (ZFP), transcription activator-like effectors (TALE), and CRISPR-based DNA binding domains have been fused to putative repressor and activator domains to create novel transcription factors to regulate both prokaryotic and eukaryotic transcription, although such functions are not generally regulated by ligand binding to the transcription factor ([34-39]). However, recently an allosterically regulated version of Cas9 has been developed by fusing the estrogen receptor-α to create a protein that represses transcription in the presence of the ligand, 4-hydroxytamoxifen[40]. Given the broad homology within the LacI/GalR family of ligand-responsive transcription factors, novel biosensors have also been constructed by fusing the ligand-binding domains from LacI paralogs to the LacI DNA binding domain, conferring regulation of the *lac* promoter by fructose, ribose, or other species [17, 18, 41, 42]. Ultimately, computational protein design could guide the development of novel biosensors. To date, such

methods have been used primarily to shift ligand specificity of existing biosensor proteins [17, 43-47], although *de novo* design of novel ligand-binding proteins and biosensors is another promising frontier [48, 49]. Overall, these approaches bespeak the promise of engineering novel biosensor proteins, but to date no generalizable approach for engineering novel metabolite-responsive transcriptional regulators has been described.

[00122] In this study, we investigated, validated, and developed a strategy for engineering novel metabolite-responsive transcriptional regulators. Our central goals were to quantitatively evaluate several strategies for converting a ligand-binding protein into a functioning biosensor that regulates transcription, and to elucidate design principles governing the performance of biosensors constructed in such a fashion. To this end, we leveraged the facts that MBP is a well-characterized ligand-binding protein, and that zinc finger proteins (ZFP) are well-characterized and programmable DNA binding domains. Furthermore, we applied quantitative analyses to identify rules for designing biosensor-regulated promoters and quantitatively characterize these new biological parts. This systematic investigation establishes a foundation for applying a potentially generalizable strategy towards the ultimate goal of engineering customized metabolite-responsive biosensors.

[00123] Results

[00124] Developing novel zinc finger protein-regulated constitutive promoters. In this investigation, we sought to develop a readily generalizable strategy for engineering novel biosensor proteins from the ground up. We hypothesized that such a goal might be achieved by first using an orthogonal DNA binding protein to regulate transcription of an engineered promoter, and then fusing this DNA binding domain to a distinct protein capable of binding the target ligand, such that when the fusion protein binds ligand, DNA binding (and thus transcriptional regulation) is either disrupted or enhanced.

[00125] To begin investigating this overall strategy, we first sought to engineer a novel transcriptional regulator by leveraging the modular, programmable DNA binding properties

conferred by the zinc finger protein (ZFP) architecture [50-52]. ZFPs are small (compared to alternative architectures such as TALEs [38, 39]), easy to manipulate, and can be designed to bind to nearly any sequence. The ZFP architecture has previously been utilized to create novel transcription factors in *E. coli* [36], as well as in eukaryotes [34, 35, 53]. The Cys_2-His_2 class of ZFPs is an attractive DNA binding domain, since each "finger" of the ZFP binds to a distinct 3 bp DNA sequence (Figure 1A) [54], and thus sequence specific binding is achieved by engineering ZFPs comprising multiple "fingers" fused in tandem. Therefore, to initially investigate our strategy for biosensor engineering, we utilized the BCR-ABL1 ZFP as our DNA binding domain. BCR-ABL1 is well-characterized, exhibits a low equilibrium dissociation constant when binding its cognate 9 bp DNA binding site with three tandem fingers (K_d ~78 pM), and has been shown to exhibit conditional DNA binding when genetically split and reconstituted [50, 55]. Notably, although the BCR-ABL1 consensus binding sequence is known, no *E. coli* promoters have been previously repressed by BCR-ABL1.

[00126] In order to begin elucidating the rules for building novel ZFP-regulatable promoters, a library of 68 different constitutive promoters was designed (Figure 7). The library was built by inserting BCR-ABL1 binding site(s) at various locations around the consensus -10 box (TATAAT) and -35 region (TTGACA). The -10 box and -35 region are critical for recruitment of the σ^{70} factor of RNA polymerase, and these sequences are necessary and sufficient to create a constitutive promoter[56, 57]. Design features that varied across the library included BCR-ABL1 binding site location(s), relative to the consensus elements, and spacing between BCR-ABL1 binding sites. Although this design did not presuppose that ZFP-binding would repress transcription from these constitutive promoters, this was the anticipated mechanism because BCR-ABL1 was not fused to a transactivation domain [36]. Each promoter was encoded on a low copy number plasmid and drove expression of *E. coli*-optimized GFP as a reporter.

[00127] To quantify the extent to which each promoter was repressed or activated by BCR-ABL1 during exponential growth, we defined a metric of "relative expression" that describes how induction of BCR-ABL1 expression impacts expression from the promoter as

compared to a "No Sites" control promoter lacking BCR-ABL1 binding sites (see Material and Methods). This relative expression normalization strategy was utilized in order to implicitly correct for any effects that arabinose many confer on GFP/ OD_{600} in a manner unrelated to expression of the ZFP. Thus, low relative expression indicates that a promoter is highly repressed by BCR-ABL1. The library of promoters exhibited wide ranges of basal expression (Figure 8) and repressibility by BCR-ABL1 (Figure 1B). Nearly all of the promoters exhibited a decrease in GFP expression upon the induction of the BCR-ABL1 ZFP, and no promoters showed any level of activation upon BCR-ABL1 induction. When relative expression was quantified at 10 h post-induction, at which point cultures had reached stationary phase, the observed repressibility was much more pronounced (Figure 1C). To investigate how relative expression patterns differed between cells within each population, we also examined several representative promoter cases at the single cell level using flow cytometry (Figure 1D). Overall, responses were unimodal, such that population-averaged fluorescence measured by flow cytometry corresponded well to comparable metrics obtained by microplate-based assays (Figure 9), and thus the latter method was used for subsequent analyses. Given this wide range of phenotypes, we next investigated the relationship between promoter design and repressibility.

[00128] We first examined the impact of promoter design on repressibility by inspection. As depicted in Figure 2, promoters representing variations on a particular design feature were first grouped together in order to identify simple trends, with the caveat that such trends are potentially restricted to the scope of promoters evaluated in our library. Generally, placing two ZFP binding sites in between the -10 box and the -35 region (the promoter "core") led to greater repressibility than did insertion of a single ZFP binding site, regardless of its position (Figure 2A). Go15 is an exception to this trend, as it was not dramatically less repressible than was Go14. Furthermore, promoters with two ZFP binding sites in the core were more repressible than were comparable promoters that lacked these core sites but shared other ZFP binding sites (Figure 2B). Compared to promoters including all three ZFP binding sites required for recognition by BCR-ABL1, promoters in which only two sites were present

exhibited reduced repressibility by BCR-ABL1 (Figure 2C). Adding additional ZFP binding sites downstream of the -10 box did not increase repressibility (Figure 2D). However, adding additional binding sites upstream of the -35 box did effectively increase repressibility (Figure 2E). Decreasing the spacer distance between the -10 box and the first downstream binding site increased repressibility (Figure 2F). Similarly, removing any spacer between the ZFP binding sites and the -10 box and -35 region resulted in greater repressibility than did removing spacers adjacent to either the -10 box or -35 region alone (Figure 2G). To explain the increase in repressibility observed for Go85 compared to Go66, we hypothesize that BCR-ABL1 cannot simultaneously occupy adjacent binding sites, and furthermore, that binding to the sites directly upstream of the -10 box and downstream of the -35 region results in greater repressibility than is conferred by BCR-ABL1 binding to the more distal binding sites present in Go66. Altogether, promoter Go92 exhibited the greatest repressibility of any library member, and this promoter appears to follow many of the design rules suggested by the above inspection-based analysis. However, since only a subset of our promoter library was amenable to this direct inspection-based analysis, we next pursued a systematic analysis of the entire library to further refine our understanding of the applicable design rules.

[00129] Computational identification of promoter design features conferring ZFP-mediated repression. Statistical methods can provide insights into large or diverse data sets that are difficult to compare qualitatively or by inspection alone. Therefore, we performed a series of statistical analyses termed computational "feature selection" in order to determine which promoter features are important for predicting the relative repressibility of a given promoter in the presence of the ZFP. Given a set of feature "inputs," feature selection seeks to eliminate those features that are redundant or irrelevant to the prediction of a particular output. In our analysis, the output was defined as the repressibility (the negative of relative expression) exhibited by each promoter in the library. To generate the input list, we defined a set of 17 quantitative features that described each promoter in the library. Because we sought to elucidate general design rules and avoid over-fitting our particular promoter library, we

defined the 17 features strictly on the basis of describing the locations of each ZFP binding site relative to the -10 box, the -35 region, and to other ZFP binding sites (Figure 3A).

[00130] Three different feature selection methods were applied to analyze BCR-ABL1-mediated repression of our promoter library. We first used partial least squares regression (PLSR), in which the regression coefficient associated with each feature indicates the degree to which that feature explains variations in repressibility within our dataset. Each coefficient was scaled using a permutation test to correct for the coefficient one would calculate for a randomized (meaningless) output vector [58], and these corrected coefficients were used to generate a ranked lists of features (for PLSR coefficients, see Figure 10). By repeating the PLSR using only fixed numbers of most important features, we found that the first few features explained much of the variance in the overall dataset (Figure 3B). A similar trend was observed when repeating the PLSR with a limited number of principal components, indicating that principal components do not provide deeper understanding of this system than do single features. The second method used was Random Forest, which creates a predictive model of the system using decision trees. By iteratively generating decision trees using a subset of features, this method calculates the mean loss of accuracy when a given feature is removed, and this quantity is used to generate a ranked list of features by importance (Figure 10). The final method used was Lasso regression, which performs least squares regression with an additional penalty placed on the magnitudes of regression coefficients [59]. This penalty is weighted by a parameter, λ, such that as λ is increased, the coefficients of unimportant features shrink to zero; features are thus ranked by the number of iterations that they retain a non-zero coefficient as λ is increased. For each value of λ, the regression fit was tested with 10-fold cross validation, to obtain a mean squared error (MSE) and number of retained coefficients for each value of λ (Figure 3C). A reasonable fit of the system was obtained using 3-5 features, with lower MSE for larger feature numbers likely representing over-fitting of noise in the dataset.

[00131] Overall, the three feature selection methods (PLSR, Random Forest, and Lasso) generated similar but not identical ranked lists of features (Figure 3D). The most

important features included *spacer inside -10* and *spacer -10*, which were undesirable for repressibility, and *BS in core, core middle space,* and *pairs in core,* which were desirable for repressibility. Together, these findings indicate that placing binding sites as close as possible to the -10 box and -35 region are predicted to confer strong repressibility. Interpreting other features is less straightforward, such as *core middle space*, which describes whether there exist base pairs in the core other than ZFP binding sites; the three methods appear to disagree on the importance of *core middle space*. One possible explanation for this discrepancy is the influence of Go22 and Go92 on this analysis, since both of these promoters contain space in the core but no space on either side of the -10 box (Go22 and Go92) and no space on either side of the -35 region (Go92). As such, for these promoters, containing space in the core may be merely associated with the presence of other promoter features conferring repressibility. These cases may well emphasize the importance of *spacer inside -10* and *spacer -10*. Moreover, Lasso, which is considered the most robust method for feature selection, ranked *core middle space* as unimportant, potentially by resolving this contradiction better than did the other methods. Altogether, this analysis enabled us to leverage our diverse promoter library to glean general, quantitative design principles for engineering ZFP-repressible promoters. However, it was not yet clear whether such rules would extend to the design of promoters regulated by ZFP-based biosensors.

[00132] Conversion of transcriptional repressors into ligand-responsive biosensors. Having established that BCR-ABL1 functions as a transcriptional repressor, we next investigated two strategies for converting this repressor into a biosensor. Here, the primary goal was to investigate general strategies for converting a ligand-binding protein into a ligand-responsive transcription factor. As described above, we hypothesized that such conditional regulation of gene expression may be achieved by fusing BCR-ABL1 to a ligand-binding domain. To investigate the feasibility of this approach, we chose the uniquely well-studied maltose binding protein (MBP), in part because this protein experiences a substantial and well-characterized conformational change (~9 Å decrease in separation between N and C termini) upon ligand binding [60-65]. The first strategy we explored was termed the Split Zinc

Finger (SZF) approach, in which BCR-ABL1 was split genetically such that the N and C termini of MBP were fused to BCR-ABL1-derived ZFPs. This strategy leverages prior observations in which the N and C termini of MBP were fused to FRET-paired fluorophores [66-69], split GFP fragments [28], or context-dependent fluorophores [45], each enabling the monitoring of ligand binding-induced conformational changes in MBP. Further rationale for this strategy is that ZFPs exhibited conditional DNA binding when this domain was genetically split and then reconstituted using either self-splicing inteins or protein-protein interactions [55, 70]. The second strategy we explored was termed the Split Protein (SP) approach, in which MBP was genetically split, with the halves fused to the N and C termini of intact BCR-ABL1. We hypothesized that such a construct may permit ZFP-DNA interactions in a manner that depends upon whether MBP is bound to maltose. The three most repressible reporters (Go66, Go85, and Go92) were used to evaluate the feasibility of each of these proposed biosensor mechanisms.

[00133] To investigate the SZF strategy, BCR-ABL1 was split between the first and second zinc fingers as previously described [55] (see Figure 4A for the proposed mechanism). None of the reporters evaluated exhibited repression upon the induction of SZF biosensor expression (Figure 4B). One potential explanation is that the three zinc fingers could not localize with the spacing or geometric orientation required to simultaneously bind a single BCR-ABL1 DNA binding site. While it may be possible to improve SZF biosensor-mediated repression by identifying promoters that place BCR-ABL1 binding sites in geometries that enable a split ZFP to bind, there is no guarantee that such a configuration would exist. Moreover, even if such a promoter were identified, such a design is likely to be a "one-off" solution specific to the MBP SZF biosensor. Therefore, we next evaluated the alternative SP biosensor design strategy, which has the potential to be more generalizable.

[00134] To initially investigate the SP strategy, MBP was genetically split at the point previously reported to generate a functional chimera with beta-lactamase (BLA), termed "RG13" [24], and BCR-ABL1 was inserted between these N and C terminal fragments (see Figure 4C for the proposed mechanism). We hypothesized that such a split site might support

the SP mechanism because (a) in RG13, MBP retains the capacity to bind maltose and (b) in RG13, binding of maltose to MBP likely induces a conformational rearrangement of the overall fusion protein (or at least the stabilization of a conformation that alleviates disruption of the BLA active site [71]). This SP biosensor was again evaluated against a select set of reporters. Notably, induction of biosensor expression suppressed reporter output in a manner that was significantly alleviated in the presence of maltose (Figure 4D). These data thus support the fundamental feasibility of the SP strategy. This observation is also consistent with our hypothesis that DNA binding was impaired in the SZF architecture, at least compared to the SP architecture. Notably, repressibility conferred by the SP biosensor was only slightly less than that observed with BCR-ABL1 ZFP alone (Figure 4E). This correspondence suggests that the rules for predicting robust ZFP-mediated repression of reporter output (Figures 2, 3) seem to hold true for the SP biosensor, which also supports the generalizability of the SP strategy with respect to reporter construct design. As was observed for BCR-ABL1-mediated repression (Figure 1D), SP biosensor-mediated repression and alleviation was also unimodal when analyzed by flow cytometry (Figure 11).

[00135] Given these promising initial results, we next investigated how the method of SP biosensor implementation impacts the performance of the system. We first investigated how biosensor expression levels impact performance (Figure 4F). With increasing biosensor expression levels, repression of the promoter increased (bottom curve), although maltose-mediated alleviation of reporter output also decreased (upper curve). Such a trend is the expected behavior of this system if, at higher levels of biosensor expression, intracellular concentrations of maltose are insufficient to drive all of the biosensors into the ligand-bound (alleviated) state. We would propose two hypotheses that could explain this phenomenon. First, at higher concentrations of maltose, transport limitations may confer an upper bound on the intracellular concentration of maltose, irrespective of the extracellular maltose concentration. Second, it remains possible that even at saturating intracellular concentrations of maltose, the maltose-bound form of our biosensor may still bind DNA (and repress transcription) to a lesser but finite extent, compared to the apo form of the biosensor. Thus,

we determined that for the case of a relatively high extracellular concentration of maltose (100 mM in medium, which is expected to be substantially higher than the concentration in the cytoplasm), induction of biosensor expression with 30 μM IPTG conferred a robust balance between repression and maltose-mediated alleviation of reporter output. To exclude the possibility that some other aspect of cell biology or ZFP function could explain the maltose-mediated alleviation of reporter output, a similar analysis was performed using cells expressing the ZFP alone (i.e., in place of the SP biosensor). As expected, the addition of maltose had no significant impact on ZFP-mediated repression of the reporter (Figure 12). Moreover, the addition of maltose did not substantially impact cell growth (Figure 13). We next investigated how extracellular maltose concentration impacts biosensor performance (Figure 4G). With the caveat that intracellular concentrations of maltose are expected to be substantially lower than are extracellular concentrations, we observed that biosensor responsiveness varied with extracellular maltose concentration, and for the most sensitive reporters (Go92 and Go85), modest but significant alleviation was observed at extracellular maltose concentrations as low as 0.1 mM. To further investigate how maltose binding to MBP domains impacts SP biosensor performance, we repeated this dose-response analysis after making a mutation in the MBP domain (W340A), which has been reported to substantially weaken, but not ablate, MBP binding to maltose [72] (Figure 4H). As expected, a substantially higher (~400x) extracellular concentration of maltose was required to alleviate reporter output in the W340A SP mutant case. Altogether, these observations demonstrate the feasibility of engineering novel biosensors using the SP strategy. Although the MBP split site used in this initial construct was not selected to optimally implement the SP strategy, our overall goal was to evaluate the SP strategy in general. Therefore, we carried this functional SP biosensor forward for further development.

[00136] Contributions of biosensor biophysical properties to biosensor performance. In order to investigate how general biophysical properties of a biosensor impact its performance, we next performed a series of rational modifications of the SP biosensor protein. First, we hypothesized that if DNA binding affinity limits the degree to which our

biosensors repress transcription, then replacing the BCR-ABL1 domain with a ZFP that binds to DNA with higher affinity would improve transcriptional repressibility in the absence of maltose. However, since BCR-ABL1 interacts with its binding site with a K_d ~78 pM [50], a simple model of binding equilibrium would suggest that promoter occupancy should not vary much with changes in this high affinity binding constant. As a point of reference, we note that the dimeric tetracycline repressor (TetR) binds to its operator sequence (tetO) with a similar K_d ~20 pM [73], although tetR is understood to achieve exquisite transcriptional repression through contorting the target DNA rather than through high affinity binding alone[74]. In order to directly investigate the relationship between affinity and repression in our system, and to investigate the modularity of our biosensor vis-à-vis ZFP domain choice, we replaced BCR-ABL1 with the Zif268 ZFP domain from the human EGR1 protein. Zif268 binds its 9 bp binding site with a K_d ~8 pM (~10 times tighter than that of BCR-ABL1) [50]. Go92 was converted to Zif268-responsive promoter by replacing the BCR-ABL1 binding sites with Zif268 binding sites (GCAGAAGCC versus GCGTGGGCG, respectively). The SP biosensor was also modified to replace the BCR-ABL1 ZFP with Zif268 (SP-Zif268). SP-Zif268 did not exhibit an enhanced capacity to suppress reporter output, although it instead exhibited reduced fold-alleviation in the presence of maltose compared to the original SP biosensor (Figure 5A). Altogether, these observations are consistent with a simple model wherein increasing the affinity of the biosensor for its target DNA did not increase repression, presumably because such a change would not impact promoter occupancy. Moreover, the fact that the SP-Zif268 biosensor exhibited significant (if somewhat diminished) functionality indicates that the ZFP domains within SP biosensors may be exchanged in a modular fashion.

[00137] We next investigated how biosensors size may impact reporter repression, for example by sterically occluding RNA polymerase binding to the -10 box and -35 region. To this end, the fluorescent protein mCherry was fused to either the N- or C- terminus of the SP biosensor to generate mC-SP or SP-mC, respectively. In this experiment, mCherry was selected as a functionally "neutral" fusion partner in order to investigate the impact of increasing the bulk of the biosensor alone. Although the SP-mC modification did not improve

biosensor performance, the mC-SP construct notably exhibited both improved repression and increased fold-induction of reporter output upon the addition of maltose (Figure 5). Altogether, these data suggest a useful strategy for building novel biosensors in which the tradeoff between desired performance characteristics may be optimized for a particular application. In general, adding steric "bulk" may outperform enhancing DNA binding for increasing repression in the "off" state without sacrificing the degree to which the regulated gene is expressed when in the "on" state.

[00138] Discussion

[00139] In this study, we investigated a potentially generalizable strategy for converting metabolite-binding proteins into metabolite-responsive transcription factors. By systematically and quantitatively evaluating the design principles governing the performance of such biosensors, which was the focus of this investigation, this work establishes a foundation for pursuing the long-term goal of engineering repertoires of customized metabolite-responsive biosensors. By leveraging modular design of both promoter libraries and biosensor proteins, these investigations elucidated a number of design principles that are useful for both explaining the variations observed in our libraries and for guiding the design of novel biosensors in subsequent work.

[00140] Using a library of engineered promoters, we identified several important rules by which binding of a ZFP to DNA confers a repression of transcription. Interestingly, nearly all promoters evaluated were repressed, at least to some degree, and none exhibited increased expression in the presence of the ZFP. The BCR-AB1 ZFP alone was sufficient to achieve significant transcriptional repression, even though this protein is smaller than canonical natural transcription factors, such as TetR and LacI (106 aa compared to 221 aa (TetR) and 374 aa (LacI)). This minimal ZFP also regulated gene expression in manner somewhat different from that conferred by a previously described fusion between a ZFP and a transactivation domain from CRP. Lee *at al.* observed that this ZFP-CRP fusion conferred transcriptional activation when bound upstream of the +1 site and repression when bound

downstream of the +1 site [36], while our minimal ZFP (which lacks a transactivation domain) conferred repression even when bound upstream of the +1 site (Figure 2). We determined that placing ZFP binding sites as close as possible to the consensus -10 box and -35 region of the promoter yielded the highest level of transcriptional repression. The -10 box and -35 region are the sites at which the transcription initiation factor σ70 binds in order to mediate recruitment and assembly of the RNA polymerase (RNAP) complex. Therefore, we hypothesize that placing the ZFP binding sites very close to the -10 box and -35 region effectively prevents the σ70 from binding to this region of DNA and/or σ70-mediated recruitment of RNAP. The greatest repression was observed when both the -10 box and -35 region were abutted with ZFP binding sites, and blocking the -10 box may confer greater repression than does blocking the -35 region (Figures 2G, 3D). It is possible that these observations may be leveraged to achieve greater repression by overlapping the ZFP binding sites with the conserved -10 box and -35 region, since binding of biosensors to these sites may more efficiently block σ70-mediated recruitment of RNAP. One potential challenge associated with this strategy is the potential to repress endogenous genes that share the consensus -10 box and -35 regions, although minimizing overlap with these consensus sequences could mitigate this problem.

[00141] Our comparison of two potential biosensor engineering strategies – the SP (split protein) and SZF (split zinc finger) architectures – revealed several insights into the feasibility and generalizability of each approach. The SP biosensors repressed the most-repressible reporters to nearly the same extent as did the ZFPs alone, suggesting that the rules governing promoter design may be generalizable across SP biosensors (Figure 4D). In contract, the SZF biosensor evaluated conferred no repression of reporter output (Figure 4B). We hypothesize that insertion of MBP between the fingers of BCR-ABL1 precluded simultaneous binding of DNA by all three zinc fingers. Even if this geometric constraint were alleviated by modulating the protein or DNA sequences, it is likely that such a solution would be unique to each biosensor. Therefore, the SZF approach may be generalizable, but not

readily so. In contrast, the SP approach was both more effective and may also be more readily generalizable.

[00142] Our investigation also provided several insights into the mechanism by which this initial SP biosensor functions and the prospects for extending this approach to generate novel biosensors. In many ways, these insights leverage the wealth of information available to describe our model ligand-binding domain, MBP. The SP biosensors utilized the MBP split sites that were identified by using a random domain insertion approach to generate the "RG13" MBP/BLA fusion protein [24]; the N terminal half of SP comprises the first 316 aa of MBP, and the C terminal half comprises residues 319-370 of MBP. In the crystal structures of both MBP and RG13, residues 316R and 319A are ~10 Å apart [62, 71]. However, it should be noted that the RG13 crystal structure was obtained in the presence of saturating Zn^{2+}, a condition which ablated the activity of the BLA subdomain of the protein, and that no maltose-bound (or zinc-free) structure of RG13 has been obtained. Thus, these distances should be treated as estimates as to how RG13 residues 316R and 319A are positioned when the protein is expressed under physiological conditions. When MBP binds maltose, the separation of these residues increases by no more than ~3 Å [60]. In contrast, when a Cys_2-His_2 class ZFP binds to its cognate 9 bp of DNA, the distance separating the N- and C-termini of the ZFP is ~40 Å [75]. Therefore, we hypothesize that in order for the ZFP domain of the SP biosensor to adopt a conformation capable of binding its 9 bp DNA target, residues 316R and 319A may be separated by as much as 40 Å. Furthermore, since the addition of maltose alleviates biosensor-mediated repression of transcription (and therefore impairs or ablates DNA binding), we hypothesize that maltose binding to the SP biosensor stabilizes interactions between the split MBP fragments, such that residues 316R and 319A are retained in a close (~13 Å) spacing, which prevents the ZFP domain from adopting a conformation capable of DNA binding (Figure 4C). Importantly, if the SP biosensor operates via this ligand binding-induced stabilization mechanism, then biosensor function need not rely upon a ligand-binding induced conformational change in MBP. Thus, this mechanism could be extended to ligand-binding proteins that do not experience a ligand binding-induced

conformation change as dramatic as that exhibited by MBP. Moreover, the proposed ligand binding-induced stabilization mechanism is consistent with the "induced fit" model of substrate binding, in which ligand binding causes a shift in protein structure that results in an increase in the stability of the ligand-bound complex. Indeed, ligand binding-induced stabilization may confer allosteric regulation of many proteins, and this property may even be engineerable [76]. Thus, we speculate that the mechanism of the MBP-based SP biosensor may be extended to biosensors based upon distinct ligand-binding domains. Moreover, there exist many methods by which proteins can be split, fused, and screened, including *in vitro* methods such as circular permutation and domain insertions, as well as computational methods for predicting effective split sites, such that evaluating whether a given ligand-binding protein is amenable to conversion into a biosensor using the SP approach is relatively straightforward [25, 77-80]. In fact, periplasmic binding proteins such as MBP may be generally amenable to conversion into molecular switches via domain insertion, as was demonstrated by the insertion of TEM-1 beta-lactamase into ribose binding protein, glucose binding protein, and xylose binding protein[81]. Additionally, an allosterically regulated version of Cas9 has been developed by using domain insertion to fuse Cas9 to estrogen receptor-α to create a repressor that is inducible by the addition of 4-hydroxytamoxifen[40]. In this study, a site in Cas9 that is permissive to protein insertion was first identified using random domain insertion of a PDZ domain. Thereafter, the estrogen receptor-α was inserted into this site following the rationale that this receptor undergoes a substantial conformation change upon ligand binding, bringing its termini within 21 Å of one another in the presence of ligand, such that only the ligand bound conformation of the receptor may exhibit a structure that avoids disruption of the Cas9 structure (and, presumably, function). Thus, while this technology is of substantial utility for regulating Cas9, it is not yet clear whether or how this approach may be extended to generate Cas9-based regulators that are responsive to a range of metabolites or ligands. Altogether, the SP strategy appears to be a promising and potentially generalizable method for generating novel biosensors, although further investigation is required to determine which types of ligand-binding proteins may be most readily converted into biosensors via this approach.

[00143] Our investigation also provided several insights into how biophysical properties of the biosensor itself could impact its overall performance. First, comparing SP biosensors based upon BCR-ABL1 to those based upon Zif268, the latter of which binds its cognate DNA with approximately 10-fold greater affinity, we observed that the SP-Zif268 biosensor repressed transcription to a similar extent but exhibited a reduced response to the addition of maltose. As discussed above, the observed comparable degree of repression is consistent with a simple model of high affinity binding, in which both SP and SP-Zif268 biosensors achieve a similar level of promoter occupancy. To interpret the reduced response to maltose, we hypothesize that due to the tighter binding of Zif268 to DNA, even the maltose-bound state may interact with DNA to some extent that represses reporter output (indeed, the same may be true to a lesser extent for the original SP biosensor). For example, if each maltose-bound biosensor exists in an equilibrium between states that are competent (disfavored) versus incompetent (favored) for DNA binding, then the higher affinity with which Zif268 binds DNA may cause biosensors based upon this protein to become "trapped" in a DNA-bound state, even when bound to maltose. Finally, the fact that the SP-Zif268 biosensor nonetheless exhibited significant (if somewhat diminished) functionality indicates that, within the SP framework, the ZFP domains may be exchanged to tune biosensor performance or to regulate novel reporter constructs.

[00144] We also investigated the role of biosensor size on performance, which provided some insights into how biosensor performance may be tuned. We observed that fusing mCherry to the N terminus of the SP biosensor (mC-SP) improved both reporter repression and fold induction upon the addition of maltose, although no such effect was observed when mCherry was fused to the C terminus of the SP biosensor (SP-mC). While it is not possible to provide a specific structural explanation for these effects, a reasonable speculation is that the mC-SP biosensor sterically occludes recruitment of the RNAP to a greater extent than does the original SP biosensor. If this were true, it could be possible to achieve even greater repression of reporter expression by exploring the addition of "bulky" domains of various sizes, shapes, and linker geometries to a candidate SP biosensor.

[00145] We also attempted to compare the performance of our initial SP biosensors to that of some naturally-evolved biosensors, using the systematic characterization of the latter that was recently reported by Rogers *et al.* [5]. Rogers et al. evaluated fold-induction after cells had reached stationary phase, while we evaluated both repression and alleviation during exponential growth, so we re-analyzed our data from the experiments reported in Figure 5 using a later time point at which cell growth had slowed, to facilitate this comparison (Figure 14). While this analysis did indeed lead to higher calculated values for degree of repression and fold-alleviation upon the addition of ligand (6 ± 0.4 fold-repression and 3.8 ± 0.3 fold-alleviation for the mC-SP biosensor), the natural biosensors generally achieved a greater fold-induction, due in large part to the more efficient suppression of output gene expression when in the ligand-free "off" state. While such nuances reflect the manner in which biosensor performance is evaluated to some extent, this investigation more importantly identifies specific performance attributes of SP biosensors that might be targeted to better approach the performance of naturally-evolved biosensors.

[00146] Although we evaluated and identified several promising strategies for improving biosensor performance, it is possible that when extending the SP approach to target applications, biosensor performance may be further improved by either design-driven or screening-based methods. Some strategies could entail refining reporter design. Depending on the application requirements, fold-induction may be improved by locating the reporters on single-copy plasmids or on chromosomal DNA (instead of on low copy number plasmids as described here) to increase promoter occupancy for a given quantity of biosensors. Alternatively, the promoter sequence could be altered to partially diminish interactions with σ70, potentially using either targeted mutations or random promoter mutagenesis followed by selection to "tune" a promoter to match the properties of a given biosensor. Other strategies could improve the biosensor proteins. In particular, although utilizing the RG13 split site for MBP proved to be feasible for generating our initial SP biosensors, it is likely that evaluating all possible ZFP insertion sites into a ligand binding protein may identify fusion proteins that are specifically suited to the SP mechanism. Based upon our observations of the factors

limiting SP biosensor performance, candidate biosensors may also be improved by random mutation and directed evolution (e.g., optimizing allosteric regulation to enhance ligand binding-induced alleviation of DNA binding). A final strategy could be to process the output of our existing biosensor/reporter(s) system to achieve preferable overall performance characteristics. For example, reporter output could be coupled to additional genetic circuitry, such as RNA-based toe hold switches or positive feedback circuits to amplify reporter output, and with some tuning, increase fold-induction[82, 83]. By leveraging the modularity conferred by programmable ZFP binding [50, 75], it may also be possible to implement multiple SP biosensors in a single cell. Moreover, high throughput genome engineering approaches such as MAGE [84] could make it possible to place even endogenous genes under partial or total control of such engineered biosensors. In sum, a modular approach to biosensor engineering is likely to accelerate the generation of novel biosensors, iterative improvement of biosensor performance, and adaptation of biosensors for novel applications in metabolic engineering and synthetic biology.

[00147] Materials and Methods

[00148] Bacterial strains and culturing. All experiments were conducted in TOP10 *Escherichia coli* cells (F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-) (Life Technologies). Cells were maintained in Lysogeny Broth (LB) Lennox formulation (10 g/L of tryptone, 5 g/L of yeast extract, 5 g/L of NaCl) supplemented with appropriate antibiotics (Ampicillin 100 μg/mL or Kanamycin 50 μg/mL). All experimental analysis was conducted in M9 minimal media (1X M9 salts, 0.2% Casamino Acids, 2 mM $MgSO^4$, 0.1 mM $CaCl^2$, 1 mM Thiamine HCl) containing glycerol (0.4%) as the primary carbon source. 1% arabinose and variable amounts of maltose monohydrate and isopropyl β-D-1-thiogalactopyranoside (IPTG) were added as indicated. M9 medium containing both Ampicillin and Kanamycin was used to maintain the strains that contained both a reporter plasmid and a biosensor plasmid.

[00149] Plasmid construction. All plasmids were assembled using standard molecular biology techniques. Plasmid backbones containing "plug-and-play" multiple cloning sites and compatible plasmids containing synthetic parts (mCherry, GFPmut3b, pBAD, AraC, pTrc2) were generously provided by Jim Collins (MIT) [85]. Custom RBS sequences were designed using the RBS Calculator [86]. The pA15 low copy number origin was obtained from the Registry of Standard Biological Parts, plasmid pSB3K3. Template sequences derived from published descriptions were used for terminators [87], BCR-ABL1 [55], and the Zif268 portion of human EGR1 [88] (AddGene #52724). MBP was PCR amplified directly from TOP10 genomic DNA. The library of constitutive reporters was cloned in a low copy number pA15 backbone (~10 copies per cell) with the ampicillin resistance cassette. All of the pBAD-based inducible ZFP and pTrc-based inducible biosensor expression constructs included a ColE1 backbone (~300 copies per cell) and kanamycin resistance cassette. The mCherry gene was cloned behind each ZFP or biosensor gene to act as a co-cistronic reporter to confirm arabinose and IPTG mediated induction of gene expression. Representative plasmid maps are included in Figure 6.

[00150] Microplate-based fluorescence assays and analysis. Cultures were inoculated from single colonies into 2 mL of M9 media and grown overnight to stationary phase. Overnight cultures were diluted 1:20 and grown into exponential phase (OD_{600} ~0.5). Cultures were again diluted to an OD_{600} ~0.05, plated in black-walled clear bottom 96-well plates in biological triplicate, and induced with 1% arabinose (to drive expression of the ZFP) or IPTG as indicated (to drive expression of the biosensor), +/- maltose as specified. In each experiment, IPTG-induced expression of biosensor constructs was confirmed via the co-cistronic expression of mCherry (data not shown). Plates with lids were incubated and shaken in a continuous double orbital pattern at 548 cpm (2 mm) inside a BioTek Synergy H1 plate reader for 10 h with GFP, mCherry, and OD_{600} measurements taken every 15 min. Monochrometer settings were 481/511 nm for GFP and 585/620 nm for mCherry.

[00151] To quantify reporter output, GFP fluorescence per OD_{600} was quantified and averaged over 7 time points that span ~1.5 h of exponential growth (unless otherwise

indicated). The specific fluorescence of each sample was defined as the mean (GFP/ OD_{600}) averaged across these 7 time points, and this specific fluorescence was averaged across 3 biological replicate samples. To quantify fluorescence attributable to GFP, each sample was background-subtracted using a control sample comprising cells expressing no fluorescent proteins. To enable comparisons between promoters, each specific fluorescence value from the arabinose or IPTG-induced condition was normalized to the specific fluorescence of the uninduced condition, yielding "relative specific fluorescence". To normalize this metric of promoter performance to the base case, the relative specific fluorescence calculated for each promoter-ZFP (or promoter-biosensor) combination was then normalized to the same value calculated for that repressor using the "No sites" promoter, yielding a quantity we termed, "relative expression". This normalization strategy was utilized in order to implicitly correct for any minor effects that arabinose, IPTG, or maltose many confer on GFP/ OD_{600} in a manner that is unrelated to expression of the ZFP or biosensor. Thus, when quantifying biosensor performance, relevant control samples for the "+maltose" case (e.g., the uninduced case and No sites control case) were also quantified in the presence of maltose. For each metric, error was propagated according to the division rule to generate reported standard deviations.

[00152] Flow cytometry. Flow cytometry was used to quantify fluorescent reporter output on a single cell basis. Cells were grown and induced as described for the microplate-based fluorescence assays. Samples were collected after 5 hours of growth. Cells were then placed on ice, diluted 1:2 in chilled phosphate buffer saline (PBS) supplemented with 5 mM EDTA, and analyzed on an LSR II flow cytometer (BD). A minimum of 100,000 events were collected per sample. Mean fluorescent intensity was calculated using a minimum of 20,000 cells per sample using FlowJo software (Treestar), and relative expression calculations and error propagation were conducted as described for the microplate assays.

[00153] Statistical analysis of promoter design features. In order to use computational analysis to compare promoter designs, it was necessary to define quantitative descriptors, or features, that capture distinguishing architectural aspects of each promoter. Because our goal

was to elucidate general design principles, we chose to limit our features to those describing the quantity and location of the various 9 bp ZFP binding sites. Following this approach, we defined 17 features that describe the locations of ZFP binding sites relative to both the -10 box (TATA box) and -35 region and relative positioning amongst the ZFP binding sites. In order to determine which promoter features were important for explaining variation in performance between promoters, several feature selection methods were applied to each set of input and output data (both of which were mean-centered and variance-scaled) to generate rankings of feature importance, noting that feature independence was not assumed. For these analyses, features always served as the regression inputs. The output was the "repressibility", which we defined as the negative of relative expression, such that a promoter-ZFP combination with a high repressibility exhibits low relative expression.

[00154] Three feature selection techniques were utilized: partial least squares regression (PLSR), Random Forest, and Lasso. PLSR was executed using the built-in MATLAB function, *plsregress*. To determine feature importance, a permutation test was used [58]. Briefly, the output vector was randomly permuted, and PLSR was executed for this meaningless output vector, such that when this process was repeated multiple times, we calculated the standard deviation associated with each coefficient (one coefficient per feature); thereby, the ratio of true coefficient magnitude to the standard deviation associated with this coefficient provided a metric by which features can be ranked in order of importance. To implement the Random Forest method, we modified a MATLAB script developed by Jaiantilal (https://code.google.com/p/randomforest-matlab/), which was based upon a method originally described by Breiman and Cutler (http://www.stat.berkeley.edu/~breiman/RandomForests/). The last feature selection method used was Lasso regression, also known as sparse or regularized regression [59]. Lasso feature selection is generally considered more robust than a permutation test or Random Forest, because the selection is built into model generation and does not require removing features from a predictive model. Each of these methods is described in full detail below.

[00155] Statistical analysis of promoter design features. In order to apply computational methods to describe the library of promoters, it was necessary to choose quantitative descriptors, or features, that describe architectural properties of each promoter. We chose 17 features to describe the location of binding sites, relative to the -10 and -35 boxes, and to other binding sites. Two assumptions are associated with our choice of features. First, features were not assumed to be independent. Second, the expression of the reporter gene was assumed to depend solely on the repression of a bound biosensor or zinc finger protein.

[00156] In order to determine which promoter features are important, three feature selection methods were used. All three methods used the same input and output data to generate a ranking of feature importance. All data were mean-centered and variance-scaled before these methods were applied. Input data consisted of a matrix of promoter indices and feature variables (62 promoter indices in rows, 17 feature variables in columns). Output data are described above. The "repressibility" value for each promoter was defined as the negative relative expression of the reporter gene (GFP).

[00157] The following three feature selection techniques generated rankings of the features in order of importance.

[00158] *Partial Least Squares Regression.* The first feature selection method used was a permutation test using partial least squares regression (PLSR). First, PLSR was executed using the built-in MATLAB function, *plsregress*. This function returns a predictive model for the output values through regression coefficients for each feature. To determine feature importance, a permutation test was used (Janes *et al.*, 2004). The output vector was randomly permuted, and PLSR was executed for the meaningless output vector. Regression coefficients were recorded for 1000 permutations, and a mean and standard deviation was calculated for the coefficient for each feature. After many permutations, coefficient means approached zero, as is expected for random permutations, but the standard deviations associated with each coefficient approached a different finite value for each feature, which indicates the degree to

which that coefficient fluctuates randomly. Features for which the coefficients were greater in magnitude than the random variance are likely to be more significant. Therefore, the ratio of coefficient magnitude to the standard deviation associated with each coefficient provided a metric by which features were ranked in order of importance. In addition to rankings described in the main text (Figure 3), coefficients and standard deviations for each feature are reported Figure 10.

[00159] The MATLAB function, *plsregress,* also provides a vector of output variance explained by each feature. Summing these gives the overall variance explained in the output data by the regression. PLSR was executed with one feature removed, and the loss of output variance explained was recorded for each feature. It is important to note that ranking through this loss of output variance explained, or loss of predictive power, yields the same ranking as the permutation test. Using this ranking, PLSR was executed with an increasing number of features, in order of importance, with the output variance explained recorded each time. The output variance explained was also recorded for an increasing number of principal components used in the regression. These both were plotted to show the contribution of each feature or principal component to the regression model (Figure 3B).

[00160] *Random Forest.* The second feature selection method utilized was Random Forest. To implement the Random Forest method, we modified a MATLAB script developed by Jaiantilal (https://code.google.com/p/randomforest-matlab/), which was based upon a method originally described by Breiman and Cutler (http://www.stat.berkeley.edu/~breiman/RandomForests/). First, the promoter library was divided into 54 promoters selected randomly to comprise a training set, leaving 8 promoters as a test set. Next, 6 features were "bagged" into a subset by random selection without replacement. The size of this subset is traditionally one third of the total set of features, which in this case rounds to 6 features (http://statweb.stanford.edu/~tibs/ElemStatLearn/). Next, a subset of promoters from the training set was randomly selected with replacement. The size of this subset is similarly one third of the total number of promoters in the training set, yielding an 18 promoter subset. A decision tree was then generated, its predictions were tested against

the data from the test set of promoters, and the mean square error was recorded. This process was repeated for a large number of bagged promoter training sets, while retaining the same subset of 6 features. This overall sequence was then repeated for a large number of feature subsets, generating a total of 100 decision trees, each of which used the same test set. Finally, this entire process was repeated for 100 different random choices of test set, generating a total of 10,000 decision trees. To assess the importance of a feature, the input data within the test set were perturbed such that the feature values associated with each promoter (e.g., number of ZFP binding sites) were randomly permuted by shuffling. Any decision tree that included the feature of interest was then retested using the perturbed input data. The increase in mean squared error (i.e., reduction in predictive power) was averaged over all trees containing this feature. This metric (average increase in mean square error) was thus used to generate a ranking of features by importance, such that features with a greater average increase in mean square error were ranked as more important. (Figure 10).

[00161] *Lasso regression.* The last feature selection method used was Lasso regression, also known as sparse or regularized regression. This type of feature selection is generally considered more robust than a permutation test or random forest, because the selection is built into the model generation, and does not require removing features from a predictive model (Tibshirani, R. (1996)). Lasso regression uses the least squares method, and is regularized by placing a constraint on the sum of the absolute value of the regression coefficients. Mathematically, the method places a penalty on large coefficient magnitudes by minimizing the following expression:

[00162]
$$\sum_{i=1}^{N}(y_i - \sum_j \beta_j x_{ij})^2 + \lambda \sum_j |\beta_j|$$

[00163] In this expression, *yi* represents output data for the *i*th promoter, β is the regression coefficient for the *j*th feature, and *xij* is input data (feature variable *j* for promoter *i*). The value of λ is a tunable parameter that determines the extent of regularization. With this

method, coefficients of unimportant features shrink to zero as λ is increased. Using the MATLAB function, *lasso*, Lasso regression was executed for 100 increasing values of lambda, with the number of features with non-zero coefficients shrinking from 17 to 0. Each regression iteration (corresponding to each value of λ) was tested using 10-fold cross validation, and a mean squared error was recorded for each iteration. For each feature, the number of regression iterations for which it had a non-zero coefficient was recorded. This metric was used to generate a ranking of features in order of importance, with the most important features having non-zero coefficients for larger values of λ. In addition to the feature ranking, the mean squared error and number of features with non-zero coefficients were plotted together versus the value of λ (Figure 3C).

[00130] Abbreviations

[00131] MBP – Maltose binding protein

[00132] ZFP – Zinc finger protein

[00133] TALE – Transcription activator-like effector

[00134] PLSR – Partial Least squares regression

[00135] MSE – Mean squared error

[00136] BLA – Beta-lactamase

[00137] SZF – Split zinc finger

[00138] SP – Split protein

[00139] IPTG - Isopropyl β-D-1-thiogalactopyranoside

[00140] LB – Lysogeny broth

[00141] OD – Optical density at 600 nm

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[00231] Example 2

[00232] Title: Development of novel metabolite-responsive transcription factors via transposon-mediated protein fusion

[00233] Abstract

[00234] Naturally evolved metabolite-responsive biosensors enable applications in metabolic engineering, ranging from screening large genetic libraries to dynamically regulating biosynthetic pathways. However, there are many metabolites for which a natural

biosensor does not exist. To address this need, we developed a general method for converting metabolite-binding proteins into metabolite-responsive transcription factors – Biosensor Engineering by Random Domain Insertion (BERDI). This approach takes advantage of an unbiased *in vitro* transposon insertion reaction to generate all possible insertions of a DNA-binding domain into a metabolite-binding protein, followed by fluorescence activated cell sorting (FACS) to isolate functional biosensors. To develop and evaluate the BERDI method, we generated a library of candidate biosensors in which a zinc finger DNA-binding domain was inserted into maltose binding protein, which served as a model well-studied metabolite binding protein. Library diversity was characterized by several methods, a selection scheme was deployed, and ultimately several distinct and functional maltose-responsive transcriptional biosensors were identified. The BERDI method comprises a generalizable strategy that may ultimately be applied to convert a wide range of metabolite-binding proteins into novel biosensors for applications in metabolic engineering and synthetic biology.

[00235] Introduction

[00236] Metabolite-responsive biosensors have a wide variety of uses, from basic research and discovery, to diagnostics, to engineered biosynthesis (Khalil and Collins, 2010). Such biosensors include diverse sensing and output modalities, including fluorescent and FRET-based biosensors (Golynskiy *et al.*, 2011, Strianese *et al.*, 2012), RNA-based biosensors (Kang *et al.*, 2014, Michener *et al.*, 2012), and transcription factor biosensors (Brockman and Prather, 2015, Dietrich *et al.*, 2010, Venayak *et al.*, 2015). Transcription factor biosensors have proven to be especially powerful for bioengineering, facilitating dynamic profiling of intracellular glucaric acid (Rogers *et al.*, 2015) and malonyl-CoA (Li *et al.*, 2015), and enabling high-throughput screening of large genetic libraries constructed to achieve biosynthesis of 1-butantol, succinate, and adipate (Dietrich *et al.*, 2013), benzoic acids (van Sint Fiet *et al.*, 2006), and $_{L}$-Lysine (Binder *et al.*, 2012). Moreover, transcription factor biosensors have been harnessed to implement dynamic intracellular feedback control, balancing metabolic fluxes to increase production titers and yields of lycopene (Farmer and Liao, 2000), fatty acid ethyl ester (Zhang *et al.*, 2012), amorphadiene (Dahl *et al.*, 2013), 1-

butanol (Dietrich *et al.*, 2013), and malonyl-CoA (Liu *et al.*, 2015). Notably, these examples relied upon naturally-evolved transcription factor biosensors, and broader utilization of such approaches is currently restricted by the limited pool of naturally evolved (and known) metabolite-responsive biosensors. Thus, approaches for generating novel metabolite biosensors are required.

[00237] Several strategies for generating new biosensors have been explored. One approach for generating new transcription factor biosensors is fusion of the ligand-binding domain from one transcription factor to the DNA-binding domain from a different transcription factor. However, these chimeric biosensors are generally limited to fusions within families of structurally-related transcription factors, such as the LacI/GalR family, in order to preserve mechanisms of ligand-responsiveness that arise from allosteric regulation (Meinhardt *et al.*, 2012, Shis *et al.*, 2014). An alternative approach is to fuse a metabolite-binding protein (which multimerizes upon ligand-binding) to a natural transcription factor, such as AraC, to generate chimeras in which metabolite binding modulates transcription factor activity (Chou and Keasling, 2013). Additionally, the binding pockets of transcription factors such as LuxR (Collins *et al.*, 2005, Collins *et al.*, 2006), AraC (Tang and Cirino, 2011, Tang *et al.*, 2008, Tang *et al.*, 2013), and XylR (Mohn *et al.*, 2006) have been mutagenized and evolved to bind new, albeit structurally similar, ligands. In a recently reported strategy for engineering novel biosensors in eukaryotes, fusion proteins were engineered to be unstable in the absence of ligand, such that the addition of the ligand stabilized the protein and enabled it to carry out its functional role (Feng *et al.*, 2015). While these findings have generated both useful biosensors and novel insights into biosensor design, most methods include some inherent limitation on the extent to which they may be generalized to build biosensors for any metabolite of interest. Therefore, there remains an outstanding need for new methods for generating novel transcription factor biosensors.

[00238] We recently reported a new strategy for converting a ligand-binding protein into a transcription factor biosensor (Younger *et al.*, 2016). In this proof-of-principle investigation, the *Escherichia coli* maltose binding protein (MBP) was genetically fused with

a modular zinc finger DNA binding domain (ZFP) to generate a novel maltose-responsive transcription factor, in which the addition of maltose alleviated transcriptional repression of an engineered promoter. This demonstration leveraged a wealth of prior knowledge pertaining to MBP; specifically the ZFP was inserted into MBP at a position that was previously identified via random fusion between MBP and TEM1 β-lactamase (bla) to generate a maltose-regulated bla (Guntas *et al.*, 2004). Whether other fusions between MBP and a ZFP could generate a functional biosensor, and whether a functional biosensor could be generated in the absence of such prior knowledge remained open questions.

[00239] To address these questions, here we developed an efficient method for generating combinatorial fusions between a ligand-binding protein and a ZFP, followed by isolation of functional biosensors from this diverse library, which we term Biosensor Engineering by Random Domain Insertion (BERDI). To develop and validate this method, a library of fusions between a ZFP and MBP was generated, analyzed, and screened. Notably, we successfully identified three novel maltose-responsive biosensors, which validates this overall approach. Thus, BERDI comprises a generalizable strategy that may ultimately be applied to convert a wide range of metabolite-binding proteins into novel biosensors.

[00240] Materials and Methods

[00241] *Bacterial strains and culturing.* All experiments were conducted in DS941 Z1 *Escherichia coli* cells (AB1157, recF143, $lacI^q$ lacZ ΔM15, P_{laciq}-LacI, P_{N25}-TetR). Cells were maintained in Lysogeny Broth (LB) Lennox formulation (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with appropriate antibiotics (Ampicillin 100 μg/mL, Kanamycin 50 μg/mL, and/or Chloramphenicol 34 μg/mL). All experimental analyses were conducted in M9 minimal media (1X M9 salts, 0.2% Cas amino acids, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM Thiamine HCl) containing glycerol (0.4%) as the primary carbon source. Variable amounts of isopropyl β-D-1-thiogalactopyranoside (IPTG) were added, as indicated, to induce biosensor expression. Maltose monohydrate was added to the media at a final concentration of 100 mM, where indicated.

[00242] The biosensor expression vector was built using standard molecular biology techniques using parts (GFPmut3b and pTrc2) gifted by Jim Collins (MIT) (Litcofsky *et al.*, 2012). The green fluorescent protein (GFP) reporter plasmid driven by the pGo92 zinc finger-responsive promoter was previously described (Younger, Dalvie, Rottinghaus and Leonard, 2016). Custom RBS sequences for the biosensor and reporter plasmids were designed using the RBS calculator (Salis *et al.*, 2009). The camR and sacB ORFs were transferred from pKM154, which was gifted by Kenan Murphy (University of Massachusetts) (Murphy *et al.*, 2000) (Addgene plasmid #13036), into a storage vector containing MuA transposon recognition sequences, flanked by BglII restriction sites (pAY438). The BCR-ABL1 ZFP was subcloned into a storage vector flanked by NotI restriction sites (pAY437). Description of all plasmids used in this study can be found in in Table 1 below.

[00243] Table 1. Plasmids used in this study

Plasmid name	Description	Resistance	Origin	Reference
pAY430	pGo92-GFP. Zinc finger repressible promoter driving GFP.	Amp^R	pA15	Younger *et al.*, 2016
pAY447	MBP (no promoter)	Amp^R	ColE1	This work
pAY438	BglII and transposon recognition sequences flanking CmR and SacB	Kan^R Cm^R	ColE1	This work
pAY437	BCR-ABL1 zinc finger, without a stop codon or promoter, flanked by NotI sites	Amp^R	ColE1	This work
pAY431	pTrc2 promoter and RBS followed by KpnI and SphI MCS, followed by a terminator	Kan^R	ColE1	This work
pAY419	316R reference biosensor (SP from Younger *et al.* 2016)	Kan^R	ColE1	Younger *et al.*, 2016
pAY470	277A biosensor (in pAY431)	Kan^R	ColE1	This work
pAY451	270A double ZFP biosensor (in pAY431)	Kan^R	ColE1	This work
pAY453	270A single biosensor (in pAY431)	Kan^R	ColE1	This work
pAY452	335P (3AA) biosensor (in pAY431)	Kan^R	ColE1	This work
pAY469	335P (2AA) biosensor (in pAY431)	Kan^R	ColE1	This work
pAY468	335P (1AA) biosensor (in pAY431)	Kan^R	ColE1	This work
pAY460	335P (0AA) biosensor (in pAY431)	Kan^R	ColE1	This work

[00244] *Candidate biosensor library construction.* The MuA transposase inserts its transposon randomly, and in either a forward or reverse direction, into any DNA sequence (Haapa *et al.*, 1999). Furthermore, the transposon can be inserted in any of the 3 possible codon frames in MBP. A detailed description of the transposon sequence and potential scar options can be found in Figure 20.

[00245] First, a library representing all possible random insertions within MBP was generated. Double-stranded DNA comprising a transposon conferring chloramphenicol resistance as well as containing the *sacB* gene for negative selection with sucrose was digested out of a storage plasmid (pAY438) using BglII, gel extracted, and cleaned by ethanol precipitation/resuspension in 40 µL of TE buffer. *In vitro* transposition reactions were carried out using the Mutation Generation System kit (Thermo Scientific # F701), as per the manufacturer's protocol. Briefly, 100 ng of purified transposon was mixed with 200 ng of target plasmid encoding MBP (pAY447), and the mixture was incubated with 1 µL of 0.22 ng/µL MuA transposase for 4 h at 30^0 C. MuA was heat-inactivated (10 min at 75 ^{0}C), and a PCR cleanup (IBI Scientific) was conducted to recover the library. The entire library was electroporated into two tubes of electrically-competent *E. coli* cells (~250 µL final volume each). Transformed cells were selected on plates containing chloramphenicol (transposon) as well as ampicillin (plasmid backbone). Serial dilutions were made at each cloning step and extrapolated to estimate library size. The MBP gene was digested out with restriction enzymes KpnI and SphI and purified by agarose gel electrophoresis to separate the band representing MBP *with* transposon insertion (3923 bp) from the band representing WT MBP (1122 bp). The MBP *with* transposon band was purified and cloned into an expression plasmid under the control of a lac-inducible promoter pTrc2 (pAY431). Finally, restriction digestion (using the NotI site present in the transposon scar) was used to replace the transposon with the sequence encoding the ZFP (BCR-ABL1), and this ligation was transformed into competent *E. coli* cells that already contained the ZFP-responsive GFP reporter plasmid (pAY430). Cells were selected with ampicillin and kanamycin for both

plasmids as well as 10% sucrose to maximize loss of the transposon, yielding the naïve (unselected) candidate biosensor library.

[00246] *Microplate-based fluorescent assays and analysis.* Cultures were inoculated from single colonies into 2 mL of M9 media and grown overnight to stationary phase. Overnight cultures were diluted 1:10 and grown for 1-2 h (OD600 ~ 0.5). Cultures were again diluted 1:10 (OD600 ~0.05), plated in black-walled clear bottom 96-well plates in biological triplicate, and induced with 30 μM IPTG and/or 100 mM maltose. Plates with lids were incubated and shaken in a continuous double orbital pattern at 548 cpm (2 mm) inside a BioTek Synergy H1 plate reader for 10 h with GFP fluorescence and OD600 absorption measurements taken every 15 min. Monochrometer settings were 485/515 nm for GFP.

[00247] *Flow cytometry and fluorescence activated cell sorting (FACS).* Overnight cultures (2 mL) were diluted 1:10 into a fresh 2 mL aliquot of M9 media and grown for 1-2 hours (OD600 ~ 0.5). Cultures were again diluted 1:10 (OD600 ~0.05) in a fresh 2 mL of either M9 media, or M9 media containing 100 μM IPTG. Cultures were grown for 4 h post-induction prior to FACS sorting. Cells were then diluted down to a concentration of 10^7 cells/mL in 4° PBS. Sorting was performed on a BD FACS Aria II instrument (BD Biosciences, San Jose, USA) using an 85 μm tip with a 488 nm excitation laser and a FITC emission filter (530/30 nm). This FITC channel was used for analysis of GFP expression. Cells were first gated based upon forward and side scatter, then the population of single cells were plotted on a GFP histogram. To set a gate for recovering cells exhibiting biosensor-mediated repression of reporter (GFP) output, a distribution of GFP fluorescence in cells was obtained from the population (100,000 events), and gating was set such that no more than 1% of this "ON" (uninduced) population would be recovered. This gate was used to recover biosensor candidates capable of reporter repression. To recover "reversible" repressors (minimize false positive repressors), the same gating definition described above was used, but this time (in the absence of IPTG), "ON" cells were recovered. For each round of sorting, 100,000 cells were recovered into 3 mL of M9 minimal media containing ampicillin and kanamycin, and this culture was subsequently inoculated into 50 mL of M9 containing

ampicillin and kanamycin and grown overnight at 37 °C. Subsequent sorts were performed (as indicated) the next day using the sorted and expanded population, as above. Traditional flow cytometry was performed on a LSRII flow cytometer (BD Biosciences, San Jose, USA). For all flow cytometry analyses, mean fluorescent intensity was calculated based on the GFP histograms of single cells (gated by forward and side scatter) using FlowJo Software (Tree Star).

[00248] *Digest-based evaluation of library diversity.* All gel electrophoresis experiments were conducted with a 1% agarose gel and run in 1x TAE (tris acetate EDTA) at 120 volts. DNA was stained using SYBR Safe (Thermo Scientific) and imaged under blue light. Band sizes were estimated using a 1 kb ladder (New England BioLabs). Exposure time was adjusted to maximize the differences between the lows and highs in the gel. Plot profiles of resulting gel images were analyzed using ImageJ's "plot profile" function. Intensity profiles for each lane were generated by subtracting the "gray value" from an empty gel lane from the grey value evaluated along the length of the lane of interest.

[00249] *Next-generation sequencing (NGS) and analysis.* The naïve library of candidate biosensors was digested using KpnI and SphI, and this DNA fragment was gel extracted and subjected to probe sonication on ice (QSonica Q700 probe sonicator; 45 minutes of 30 seconds on, 15 seconds off at 10% maximum intensity) to shear the library into fragments less than 500 bp in length. Library preparation was done by PCR amplifying four equally sized regions of MBP, using primers incorporating common sequences derived from Fluidigm's AccessArray system (Moonsamy *et al.*, 2013) to be used for NGS. The PCR primers were designed to bind either the MBP or ZFP sequence, and an MBP primer was always paired with a ZFP primer for library preparation, such that each amplicon would contain one MBP-ZFP boundary, and thus the ZFP insertion site could be determined. In total, eight unique PCR reactions were run on the naïve library: four evenly spaced MBP primers, each paired with either the forward or reverse ZFP primer. See Figure 21 for a visual layout of the primers and Table 2 for a list of PCR reactions and primer sequences. Two biological replicates of the library were prepared for a total of sixteen reactions. Samples were

submitted to the University of Illinois at Chicago (UIC) Sequencing Core, where Illumina adapters and custom barcodes were further appended to the amplicons via another PCR reaction. All samples were run together on an Illumina MiSeq lane using paired end DNA sequencing. In total, ~8M reads were generated from the sixteen samples.

[00250] Table 2. PCR primer pairs for NGS. CS – Common sequence

PCR Number	Forward primer (common sequence #) Primer 5'-3'	Reverse primer (common sequence #) Primer 5'-3'	ZFP direction
1	AYP828 (CS1) acactgacgacatggttctacacaacgatt catacatagctaaaaggtacc	AYP829 (CS2) tacggtagcagagacttggtctctagggct agctctagccat	Forward
2	AYP830 (CS1) acactgacgacatggttctacaggcaagc tgattgcttaccc	AYP829 (CS2) tacggtagcagagacttggtctctagggct agctctagccat	Forward
3	AYP831 (CS1) acactgacgacatggttctacaacaggcg agaagggatcc	AYP833 (CS2) tacggtagcagagacttggtctgccagctct ttgttcggac	Forward
4	AYP831 (CS1) acactgacgacatggttctacaacaggcg agaagggatcc	AYP832 (CS2) tacggtagcagagacttggtctgggtgttca ataattgggcatgc	Forward
5	AYP828 (CS1) acactgacgacatggttctacacaacgatt catacatagctaaaaggtacc	AYP835 (CS2) tacggtagcagagacttggtctacaggcga gaagggatcc	Reverse
6	AYP830 (CS1) acactgacgacatggttctacaggcaagc tgattg cttaccc	AYP835 (CS2) tacggtagcagagacttggtctacaggcga gaagg gatcc	Reverse
7	AYP834 (CS1) acactgacgacatggttctacatctagggc tagctctagccat	AYP833 (CS2) tacggtagcagagacttggtctgccagctct ttgttcggac	Reverse
8	AYP834 (CS1) acactgacgacatggttctacatctagggc tagctctagccat	AYP832 (CS2) tacggtagcagagacttggtctgggtgttca ataattgggcatgc	Reverse

[00251] All data analysis was performed using customized software (written in Python). Briefly, reads were first filtered to retain only those reads containing the transposon

scar sequence as well as the sequences of the primers used to generate the amplicon corresponding to that sample. Next, we identified viable paired end reads as those in which at least 12 contiguous bases at the end of one read perfectly matched the reverse complement of its paired read. Only such viable paired end reads were carried forward. Next, to discard low-quality reads, we filtered out reads with Phred quality scores below 20 (a score of 20 corresponds to 99% confidence in the identity of that base). Next, reads were partitioned into bins by read length, in roughly 50 bp increments, to enable subsequent normalization of scores by read length. Reads were then aligned to the MBP template sequence using the Needleman-Wunsch algorithm (using a gap opening penalty of 10.0, gap extension penalty of 0.5, and the EDNAFULL scoring matrix). The resulting alignment scores were normalized by read length (i.e., by bin), since scores generally increase with length of alignment. Alignments that generally had less than 2 gaps per 50 bases aligned were carried forward. The resulting alignments were then analyzed to identify ZFP insertion sites, which we defined as the first or last base to align to MBP, depending on the orientation of the amplicon being analyzed. Insertion sites were only classified as "identified" if the alignment comprised a block of perfect alignment with MBP between the identified insertion site and the end of the read (i.e., reads were discarded if the alignment generated gaps or mismatches *within* the block of sequence where the read aligned to the MBP template). A graphical representation of this pipeline is presented in Figure 22. In all cases, the insertion site refers to the last base of MBP upstream (5') of the transposon insertion. All of the NGS analysis code and data is available at https://github.com/PeterSu92/BERDI-NGS-insertion-analysis.

[00252] Results

[00253] *Generation of random domain insertion libraries via transposon mutagenesis.* Here we sought to develop an efficient method for generating novel transcription factor biosensors, which we term Biosensor Engineering by Random Domain Insertion (BERDI). The overall BERDI strategy is summarized in Figure 15. This method utilizes the MuA transposase to insert a transposon nonspecifically into a plasmid encoding the gene sequence for the metabolite binding protein. Transposon mutagenesis provides a simple and efficient

method for generating a library of insertions due to its nonspecific and single insertion into each target DNA molecule, minimal scar sequence, and ability to generate > 10^5 variants in a single pot *in vitro* reaction (Haapa *et al.*, 1999, Mehta *et al.*, 2012, Nadler *et al.* 2016). The transposon is later exchanged for a ZFP coding sequence to generate a library of candidate biosensors. Transposase insertion has been used to successfully circularly permute proteins as well as to profile proteins for permissible insertion points (Edwards *et al.*, 2008, Mehta *et al.*, 2012, Nadler *et al.*, 2016, Oakes *et al.*, 2016, Segall-Shapiro *et al.*, 2011). Here, we investigated whether this technique can be used to generate novel biosensors by randomly inserting a ZFP into a ligand-binding protein. We reasoned that choosing MBP as a model ligand-binding domain would be the most effective strategy for evaluating the BERDI method, since (a) we know that at least one such feasible biosensor should exist, based upon our prior work (Younger *et al.*, 2016), and (b) such an approach enabled us to utilize established reporter constructs and biosensor evaluation methods.

[00254] First, a library of candidate biosensors was generated. MuA inserts randomly into target DNA molecules (in either forward or reverse direction), such that for a plasmid of length n bases, the total number of possible insertions is $2n$. Additionally, multiplying the three frames in which the transposon can insert by the two directions in which the transposon can insert (i.e., forward or reverse) yields six possible insertions for a given codon of target DNA. However, only one of these six insertions (forward and in-frame) will generate a *productive* insertion. The transposase also leaves a partially controllable scar (i.e., one has some choice in the design of this scar sequence). Therefore, we designed the transposon such that when the ZFP is inserted in frame with the rest of MBP, the resulting scars encode for linkers comprising three alanine residues on either side of the ZFP domain (see Figure 20B for details).

[00255] Given that transposon insertions are random and independent of one another, we aimed to achieve a library size at least 10x greater than the maximum possible number of insertions – 6,288 (the number of directions in which the transposon can insert, 2, multiplied by the size of the target plasmid, 3144 bp); this yields a target library size of 62,880 members.

Our initial transposition library generated over 8 x 10^5 colonies, or ~125x the maximum library diversity. Next, the library was subcloned to eliminate MBP variants lacking a transposon (i.e. when the transposon inserted elsewhere in the plasmid backbone), and then the transposon was replaced with a sequence encoding the ZFP to yield a naïve candidate biosensor library. At each step, we confirmed that library diversity exceeded the target of 10x oversampling (Table 3).

[00256] Table 3. Targeted and experimental library sizes

Experimental Step	Theoretical library size	Targeted library size (10x oversampling)	Experimental Results	Experimental Oversampling
Generate library	3144 (bp MBP plasmid) x 2 (forward or reverse) = 6,288	62,880	~800,000	~125x
Clone transposed gene into expression plasmid	1116 (bp of MBP) x 2 (forward or reverse) = 2,232	22,320	~30,000	~14x
Exchange transposon for ZFP	1116 (bp of MBP) x 2 (forward or reverse) = 2,232	22,320	~30,000	~14x

[00257] *Analyzing diversity of the naïve library.* In order to evaluate library diversity prior to sorting for functional biosensors, we analyzed the naïve library using three distinct methods. First, sequences from the naïve library were digested out of the expression vector library, and these biosensor-encoding fragments were then subsequently digested with a restriction enzyme recognizing a unique site in the sequence encoding the ZFP. These digests were evaluated by gel electrophoresis (Figure 16A,B), which yielded a smear consistent with the presence of many diverse insertions. We next performed both Sanger sequencing on 46

individual colonies and next-generation sequencing (NGS) on the library, by PCR amplifying regions containing both the MBP and ZFP and then subjecting these amplicons to NGS (Figure 21). Confirmed insertions were identified by sequence analysis (Figure 16C). Colony sequencing was performed to complement NGS, since the latter is expected to be impacted by biases introduced during PCR-based library preparation. A full list of insertion counts identified by sequencing individual colonies and NGS can be found in Table 4.

[00258] Table 4. List of all insertions found by NGS and Sanger (colony) sequencing

Insertion Position	Count by NGS	Count by Sanger	Insertion Position	Count by NGS	Count by Sanger	Insertion Position	Count by NGS	Count by Sanger
1	7	0	384	2	0	771	1	0
2	35358	0	391	2	0	779	1	0
9	2	1	394	3	0	785	5	0
10	1	0	404	1	0	786	662975	0
12	1	0	405	1	0	787	666	0
13	1	0	410	2	0	788	1	0
14	13	0	412	2	0	789	1	0
15	868175	1	417	1	0	791	4	0
16	4	0	422	1	0	794	1	0
18	1	0	423	1	0	807	66	0
32	2	0	442	1	0	808	2	0
40	1	0	443	66	1	809	262424	0
59	4	0	444	318427	0	810	192	0
60	24625	0	445	1	0	812	6	0
74	2	0	448	1	0	814	2	0
152	15	0	494	1	0	867	2	4
201	42	0	550	1	0	870	21	0
285	1	0	561	1	0	896	0	1
286	2708	4	563	1	0	897	0	1
287	2	0	564	7093	4	898	0	1
300	3366	0	565	130462	0	899	2840	0
306	0	1	566	4	0	900	7824	2
325	12	0	570	1	0	901	41428	15
326	4	0	580	2299	0	902	1	0
329	1	0	581	8766	0	903	44428	5
330	10	0	594	27159	2	904	3	0
331	4	0	595	68187	0	907	1	0
332	3	0	614	2642	0	938	2	0
333	3	0	615	2269	0	953	1	0
334	1	0	616	1	0	976	4	0
336	3	0	623	8349	0	978	32589	0
337	2	0	637	39957	1	979	14	0
340	8	0	638	5345	0	987	3932	0
341	1	0	639	1	0	993	1	0

346	1	0	680	8378	0	1004	3219	0
348	1	0	681	118	0	1068	595	0
349	5	0	670	0	1	1080	1	0
350	7	0	706	1	0	1086	72	0
351	2	0	711	201312	0	1087	183665	1
354	4	0	712	1659	0	1088	3	0
360	1	0	713	8	0	1090	2	0
361	2	0	715	1	0	1099	1	0
363	3	0	716	1	0	1102	1	0
364	1	0	717	1	0	1103	1	0
365	2	0	719	1	0	1104	1	0
366	14	0	723	1	0	1105	1	0
367	3	0	748	1	0	1110	1	0
371	1	0	757	1	0	1111	4	0
373	16	0	758	181930	0	1112	236842	0
382	8	0	759	884	0	1113	113	0
383	3	0	762	5	0	1115	1	0

[00259] When using conservative NGS analysis parameters (e.g., utilizing only alignments wherein the read perfectly matched the template, without gaps) to identify insertions, we observed at least 148 insertions across MBP, representing 13.2% of all possible insertions. Out of these, 47 (31%) were in frame, and the percentage of insertions containing a forward-facing ZFP was 48%, which is consistent with the expectation that MuA-mediated insertion is random (Table 5). We identified 37 *productive* insertions (forward-facing and in-frame) from these analyses.

[00260] Table 5. Frequency of insertion types observed in the naïve candidate biosensor library

Type of insertion	Expected if unbiased	Observed by NGS (n= 148 insertions)	Observed by colony (Sanger) sequencing (n = 17 insertions)
In frame	33%	30%	12%
Out of frame	66%	70%	88%
Forward ZFP	50%	49%	48%

Reverse ZFP	50%	51%	52%

[00261] To evaluate the bias that occurred during the PCR amplification performed in preparation for NGS, we analyzed several parameters. In our NGS data analysis, we observed insertion counts ranging from very abundant (10^6 counts) to very rare (1 count), and the top five most abundant insertions encompassed 64.8% of all insertion counts. 12 insertions out of the 46 found by Sanger sequencing individual colonies (counting both productive and non-productive insertions) were also identified in the NGS analysis, yet only one insertion site identified by colony sequencing matched any of the top five most frequent insertions identified by NGS analysis. Also, five of the insertion sites identified by sequencing colonies represented insertions not found by NGS. We therefore concluded that amplification bias significantly affected our NGS results, which suggests that our naïve library diversity was broader than the limited set of insertions confirmed by our conservative NGS analysis. Moreover, since the transposon insertion ratios (forward/reverse, in-frame vs. out of frame) demonstrated that the transposase functioned as expected (Table 5), and since our library size exceeded 10x oversampling, we concluded that the library likely contained sufficient diversity and we proceeded to investigate whether the library contained any functional biosensors.

[00262] *Identification of functional biosensors.* Our overall strategy was to first enrich for candidate biosensors capable of repressing the reporter in the absence of maltose, then to reselect to eliminate false positives, and then to identify maltose-responsive biosensors from within that pool (Figure 17A). The naïve library was first screened by FACS to enrich for candidate biosensor clones which, when expressed at a high level (100 μM IPTG induction), repressed the GFP reporter output. The selected pool was recovered and regrown, and this process was iterated on three consecutive days to enrich for candidate biosensors that were at least capable of repressing reporter output. Next, this population of candidate biosensors was re-screened by FACS in the absence of IPTG, to recover "reversible" repressors (clones

which express GFP in the absence of IPTG) in order to minimize false positives. Following these four rounds of screening, the recovered cells were plated and clonally assayed for maltose-responsiveness. Candidate biosensors were initially evaluated under conditions of 30 μM IPTG to induce the expression of the biosensor and 100 mM maltose, which are conditions under which we previously observed maximal maltose-responsiveness of the reference biosensor (Younger *et al.,* 2016). At this point, library members exhibiting both (a) at least a 2-fold repression of reporter output in the absence of maltose and (b) any significant maltose responsive were defined as "functional biosensors" and were sequenced. Of the 672 colonies analyzed, 340 demonstrated the ability to repress the reporter, but only 159 of those met both criteria for potential biosensors. These 159 colonies represented three unique biosensors (270A, 277A, and 335P). The other 181 repressors represented out-of-frame insertions at six positions (5E, 31G, 188G, 194T, 213I, and 262V). Moreover, none of the three novel biosensors identified were detected in the original naïve library, via either colony (Sanger) sequencing or NGS. Together, these observations indicate that our strategy substantially enriched for functional biosensor constructs, even if they were rather rare in the original naïve library.

[00263] The three functional biosensors, representing a ZFP insertion at 277A, an insertion of two ZFPs at 270A, and a single ZFP insertion at 335P, were next examined in greater detail. These insertions are depicted graphically in Figure 17B, along with the position at which the ZFP was previously inserted by design in the reference biosensor, at position 316R (Younger *et al.*, 2016). Interestingly, these four insertion points are distributed in three distinct regions of MBP. All four are on the outside of the protein and are either in a loop (270A) or at the end of an α-helix, near a loop (316R, 277A, and 335P). Given the sample size, and the lack of crystal structures of the new biosensors, it is not yet possible to predict whether they share other features that lend themselves to maltose-responsive transcriptional regulation. Performance of the new biosensors was next compared to that achieved by the reference biosensor (Figure 17C). Both the 277A and 270A biosensors exhibited similar repression to that conferred by the reference biosensor (~3 to 4-fold), however, neither 277A

nor 270A exhibited as much maltose-responsiveness as did the reference biosensor. Notably, the 335P biosensor exhibited much better repression (~10-fold) compared to the other three constructs, while also exhibiting substantial responsiveness to maltose. We next investigated why our BERDI method recovered a 270A double ZFP insertion but not a 270A single ZFP insertion. Such a double ZFP insertion is indeed a potential product that could be generated when cloning the ZFP cassette in place of the transposon cassette (both ends of the ZFP cassette use the same NotI restriction site), but given the ligation ratios used, we expected such a double insertion to be substantially less frequent than a single insertion. Therefore, we next generated the 270A single ZFP insertion biosensor to evaluate its performance. First, the 270A single ZFP construct exhibited milder repression compared to the 270A double ZFP variant (Figure 23), which would explain why the single insertion variant was not (or was less) enriched during the initial three rounds of FACS screening. Furthermore, the single ZFP insertion at 270A exhibited less responsiveness to maltose than did the double insertion biosensor variant. Taken together, these clonal observations support the conclusion that the BERDI method generated and selected for functional biosensors, based upon their performance, as intended.

[00264] *Biosensor performance characteristics: dose and linker analysis.* Having identified several novel functional biosensors, we next evaluated their performance characteristics. First, to investigate the impact of biosensor dose on reporter output repression and maltose sensitivity, the strongest repressor, 335P, was induced at a range of IPTG concentrations (Figure 18). At the highest IPTG concentration evaluated here (60 μM), the repression was not significantly greater than that observed at 25 or 30 μM IPTG, indicating that at these lower concentrations, saturating levels of the biosensor already achieve maximal repression. However, at 60 μM IPTG, the system was not sensitive to maltose, indicating that the biosensor was in excess relative to intracellular maltose levels (which would be lower than extracellular maltose levels). As the level of IPTG (and thus biosensor expression) decreased, the sensitivity of the 335P biosensor to maltose increased, but once the IPTG level dropped below 20 μM IPTG, the overall repression of the reporter decreases, as expected

given the decrease in biosensor protein. Thus, there exists an optimal window of biosensor expression that confers maximal maltose sensitivity while maintaining sufficient promoter repression (in the absence of maltose), corresponding to around 25 μM IPTG (different from the 100 μM IPTG used during selection). Each of these phenomena is consistent with observations previously characterized with the reference biosensor (Younger *et al.,* 2016), suggesting that at least at this functional level, the reference biosensor and this novel BERDI-generated biosensor exhibit qualitatively similar performance characteristics.

[00265] Intriguingly, however, a biosensor matching the original reference biosensor (e.g., a ZFP insertion 316R) was not recovered by the BERDI method, and we next investigated why this may be. One possible explanation is that the linkers introduced via the BERDI method differ from those included in the reference biosensor. The reference biosensor has two amino linkers on either side of the ZFP – lysine and leucine on the 5' end of the ZFP insertion and an asparagine and valine on the 3' end – whereas the BERDI method introduces three alanines on either side of the ZFP (Figure 20). To investigate the impact of this difference in linkers, a biosensor was generated in which the ZFP was inserted at 316R, with three alanines on each side of the ZFP, and this construct was compared to the original reference biosensor (Figure 19A). Surprisingly, the 316R biosensor with 3 Ala-linkers (mimicking the transposon scar) completely lost its ability to repress the GFP reporter. This observation could explain why the BERDI method did not recover a single ZFP insertion at 316R, and moreover, it demonstrates the importance of linker length (and potentially composition) on impacting overall biosensor performance. To further investigate how linker length may affect biosensor performance, three variants of the 335P biosensor (with 3AA linkers) were generated, with reduced linker lengths (0-2 AA on each side of the ZFP). As the linker length shortened, the repression of the biosensor decreased (Figures 17B-C). Interestingly, the maltose-responsiveness also varied with linker length, such that the 1AA 335P biosensor exhibited the best combination of promoter repression and maltose responsiveness. Altogether, these observations both validate the BERDI method for

generating novel functional biosensors and indicate that this modular biosensor design is amenable to further refinement of biosensor performance.

[00266] Discussion

[00267] In this study, we developed and implemented the BERDI method for the generation of maltose-responsive MBP-ZFP fusion proteins in a rapid and efficient manner to find three new biosensors. The fact that multiple insertions produced a bi-functional protein is not surprising given that a previous study found twelve functional insertions for a circular permuted GFP into MBP using a similar method (Nadler, Morgan, Flamholz, Kortright and Savage, 2016). Additionally, another transposon insertion study demonstrated multiple bi-functional insertions of a cytochrome into β-lactamase (Edwards, Busse, Allemann and Jones, 2008), demonstrating that if multiple possible insertions exist, this method is capable of identifying them. A possible explanation for the tolerance of the proteins studied both here and in previous research is that many circularly permuted proteins are able to retain their function, demonstrated in a study that found 15 unique functional circular permutations of an adenylate kinase using transposon mutagenesis (Mehta, Liu and Silberg, 2012). A second explanation may be that these are monomeric proteins, as homodimers would require the protein complex to tolerate two changes simultaneously. These findings emphasize the need for library based approaches, like the one described here, given the propensity for a given protein to have multiple positions where functional fusions can be created.

[00268] Despite some indication that our library did not sample the entire insertion space due to limitations such as amplification bias, we found three novel functional biosensors in our library, and six variants that were out-of-frame, but that still exhibited mild (less than 2-fold) inducible repression (5E, 31G, 188G, 194T, 213I, and 262V). The three biosensors that were enriched in the screening process were not detected by either colony (Sanger) sequencing or NGS, indicating that our screening method can isolate infrequent mutants from the initial library. As for the out-of-frame "repressors", we hypothesize that this is due to non-specific translation, since the start codon of the ZFP remained in the final

constructs. Therefore, it is possible that ribosomes still translated the full ZFP along with the downstream portion of MBP (out of frame), leading to a functional repressor. Thus, in future implementations of the BERDI method, it may be desirable to remove the start codon from the ZFP prior to library construction to minimize this issue and further enrich for *productive* biosensors over these false positives. In any event, this phenomenon did not preclude our identification of functional biosensors, but rather it necessitated clonal analysis of more candidate biosensors in the final step of the selection. Should the problem of false positive repressors prove intractable, an alternative solution would be to add a FACS-based screen to enrich for ligand-responsive biosensors prior to clonal analysis.

[00269] One of our newly discovered biosensors (270A) had a double ZFP insertion, which outperformed a similar biosensor comprising a single ZFP insertion at this position. It is possible that the presence of two ZFP domains, if correctly folded, increased repression due to the higher potential conformational shift. While this phenomenon is neither problematic or advantageous, it is inherently tied to our use of a unique restriction site within the transposon recognition sequences during library generation, such that it seems reasonable to accept this rare occurrence as a possible event that may occur during library creation. If this phenomenon were to prove problematic, library generation could be performed with a greater ratio of backbone plasmid to ZFP cassette during the applicable ligation step. Alternatively, mutagenesis of the transposon recognition sequences might reveal an alternative method that removes this possibility, although such a study is outside the scope of this investigation, and our results suggest that such a modification of the library generation method is not necessary.

[00270] Biosensor dose is critical when evaluating biosensor performance. Increasing biosensor expression increases the repression of the reporter, however it also limits the sensitivity to maltose. It is possible that the binding of maltose does not ever completely ablate the ability of the biosensor to bind DNA, therefore, regardless of intracellular maltose concentrations, the reporter may never be completely unbound by biosensors. This performance characteristic is likely to be unique for every biosensor created, which can be

characterized by a dose response analysis of both biosensor and ligand to tune desired biosensor properties.

[00271] Because protein structure and functional are so often intertwined, linker composition is vital to biosensor performance. The reference biosensor was not found in the transposon based screen due to the differences in the linkers. However, three novel biosensors were found. This implies that not only does linker composition matter, but that if we had chosen different linkers in the transposon design, we likely still would have found biosensors, albeit potentially at entirely different sites. We hypothesized that using too long of a linker would reduce the degree to which ligand binding induces conformational changes that are translated through the protein (e.g., via an allosteric mechanism) to impair DNA binding. Conversely, using no linker may prevent the ZFP from folding in a conformation conferring DNA binding in the absence of ligand. Therefore, we hypothesized that designing our library to include three alanine linkers on each side of the ZFP would potentially provide an inert and flexible linker that balances these effects. Indeed, the performance of the linker variants of the 335P biosensor largely supported this understanding of biosensor function; shortening linkers reduced biosensor-mediated repression and changed the maltose responsiveness, potentially indicating that the ZFP bound DNA to a lesser extent. Furthermore, the reference biosensor bound DNA when it included lysine and leucine residues flanking the N-terminus of the ZFP and asparagine and valine flanking the C-terminus of the ZFP, but when these linkers were each replaced with three alanines, promoter repression was ablated, implying that both length and composition of the linkers are important for performance. As is the case with biosensor expression levels, the specific linkers are likely to impact every biosensor differently. Importantly, this phenomenon may also provide an additional handle for tuning biosensor performance. Although we did not investigate this possibility in this study, systematically varying linker sequence and length, as a perturbation to a candidate biosensor, may indeed confer improved performance for some biosensors.

[00272] The 335P biosensor has the performance characteristics that make it capable of distinguishing between a high and low state of maltose that could be utilized for high-

throughput screening or feedback control mechanisms. However, the biosensors found by the BERDI method may not always have the performance characteristic desired for a particular application. Therefore, using the BERDI method as a starting point to generate functional biosensors, it may be possible to improve any biosensor by saturation mutagenesis on the three alanine linkers, or even the whole protein, followed by additional rounds of sorting to enrich for different performance characteristics.

[00273] The three novel biosensors described here were all found by sorting for inducible repressors, then clonal examination for maltose responsiveness. However, if ligand-responsive biosensors prove to be exceptionally rare, it could be useful to use FACS to enrich for ligand-responsive biosensors, prior to clonal analysis, as noted above. Additionally, instead of using GFP and FACS as the screening system for ligand responsive biosensor, GFP could be replaced with a gene conferring a survival selection. For example, if the reporter drove expression of the tetA gene, encoding the tetracycline/H+ antiporter, since the ZFP represses transcription, cells that could not alleviate this repression in the presence of the ligand would be selected against when challenged with tetracycline. Therefore, growth on tetracycline could be used as another way to enrich for rare, ligand-responsive, biosensor variants.

[00274] Here we demonstrate that the BERDI method is capable of generating novel metabolite-responsive biosensors from a metabolite-binding protein. Furthermore, we found that insertion of the ZFP into several positions of this model metabolite-binding protein resulted in functional biosensors. Importantly, the BERDI method can potentially be generalized to convert any metabolite binding protein, since care was taken to ensure that no part of the method was specific to maltose binding protein by design. Finally, the BERDI method enables the development of novel biosensors without relying upon prior knowledge about permissive sites within the ligand-binding protein, potentially enabling this method to be applied to less studied proteins. Ultimately, this approach could potentially be extended to generate novel biosensors from virtually any candidate metabolite binding protein for a range of applications in microbiology and synthetic biology.

[00275] Abbreviations

[00276] MBP, Maltose binding protein; ZFP, Zinc finger protein; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LB, Lysogeny broth; OD, Optical density at 600 nm; NGS, Next generation sequencing; GFP, Green fluorescent protein; TAE, tris acetate EDTA; EDTA, ethylenediaminetetraacetic acid; bla, TEM1 β-lactamase.

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[00318] In the foregoing description, it will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood

that although the present invention has been illustrated by specific embodiments and optional features, modification and/or variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00319] Citations to a number of patent and non-patent references are made herein. The cited references are incorporated by reference herein in their entireties. In the event that there is an inconsistency between a definition of a term in the specification as compared to a definition of the term in a cited reference, the term should be interpreted based on the definition in the specification.

CLAIMS

We claim:

1. A system comprising:

(a) a fusion protein that functions as a biosensor, the fusion protein comprising a ligand-binding protein (LBP) and a DNA-binding protein (DBP), the fusion protein comprising an amino acid sequence represented as:
(N-terminal portion of the LBP) – (DBP) – (C-terminal portion of the LBP); and

(b) a promoter which can be operably linked to a reporter gene,

wherein the promoter comprises at least one heterologous binding site that is specific for the DBP and the fusion protein binds to the ligand and modulates expression from the promoter.

2. The system of claim 1, wherein the fusion protein has a first binding affinity (K_{d1}) for the promoter in the absence of the ligand, and the fusion protein has a second binding affinity (K_{d2}) for the promoter in the presence of the ligand, such that $K_{d1} < K_{d2}$ and transcription of the reporter gene is de-repressed or activated in the presence of the ligand.

3. The system of claim 2, wherein de-repression or activation is proportional to concentration of the ligand in the system.

4. The system of claim 2, wherein K_{d1} is < about 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, 0.01 nM or lower.

5. The system of claim 2, wherein K_{d2} is > about 0.02 nM, 0.05 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM or higher.

6. The system of claim 2, wherein the ratio K_{d2}:K_{d1} is at least about 5, 10, 20, 50, 100, 500, 1000 or more.

7. The system of claim 1, wherein the fusion protein has a first binding affinity (K_{d1}) for the promoter in the absence of the ligand, and the fusion protein has a second binding affinity (K_{d2}) for the promoter in the presence of the ligand, such that $K_{d1} > K_{d2}$ and transcription of the reporter gene is repressed or de-activated in the presence of the ligand.

8. The system of claim 7, wherein repression or de-activation is proportional to concentration of the ligand in the system.

9. The system of claim 7, wherein K_{d2} is < about 1 nM, 0.5 nM, 0.2 nM, 0.1 nM, 0.05 nM, 0.02 nM, 0.01 nM or lower.

10. The system of claim 7, wherein K_{d1} is > about 0.02 nM, 0.05 nM, 0.1 nM, 0.2 nM, 0.5 nM, 1 nM, 2 nM or higher.

11. The system of claim 7, wherein the ratio K_{d1}:K_{d2} is at least about 5, 10, 20, 50, 100, 500, 1000 or more.

12. The system of claim 1, wherein the N-terminal portion of the LBP within the fusion protein comprises an amino acid sequence of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids from the N-terminus of the LBP.

13. The system of claim 1, wherein the C-terminal portion of the LBP within the fusion protein comprises an amino acid sequence of at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids from the C-terminus of the LBP.

14. The system of claim 1, wherein the ligand is a cellular metabolite.

15. The system of claim 1, wherein the LBP is maltose binding protein.

16. The system of claim 1, wherein the DBP comprises one or more DNA-binding domains selected from the group consisting of a zinc-finger protein (ZFP) DNA-

binding domain, a transcription activator-like effector (TALE) DNA-binding domain, and a clustered regularly interspaced short palindromic repeat (CRISPR) DNA-binding domain.

17. The system of claim 16, wherein the ZFP is BCR-ABL1.

18. The system of claim 1, wherein the promoter is a prokaryotic promoter.

19. The system of claim 18, wherein the promoter comprises two or more binding sites for the DBP.

20. The system of claim 19, wherein the binding sites are located at one or more positions selected from:

(i) between the -10 box (TATA box) and the -35 box (GC-rich region);

(ii) adjacent to the -10 box (TATA box) or within 5 nucleotides of the -10 box (TATA box); and

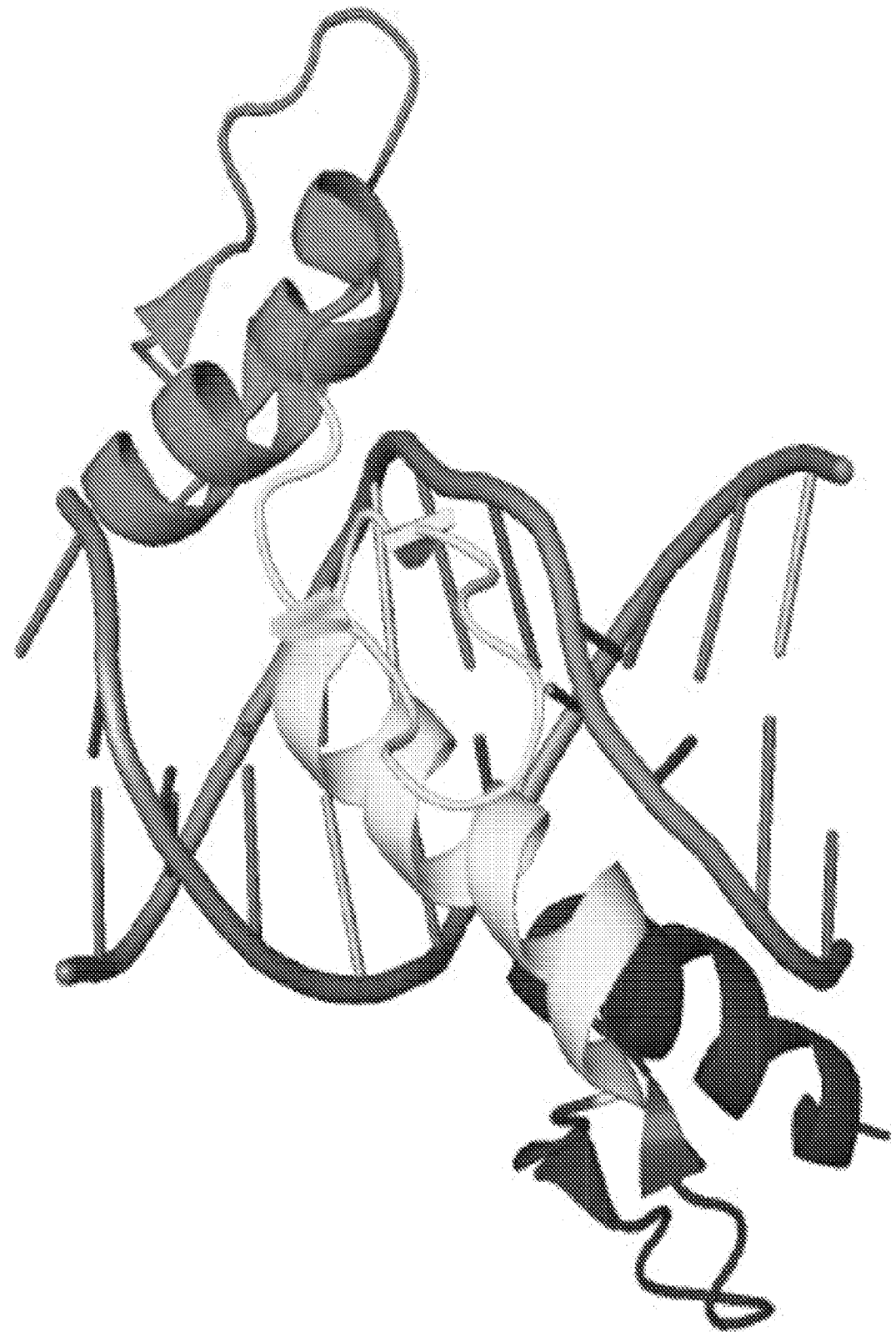
(iii) adjacent to the -35 box (GC-rich region) or within 5 nucleotide of the -35 box (GC-rich region).

21. A method for preparing a fusion protein for use as a biosensor, the fusion protein comprising a ligand-binding protein (LBP) and a DNA-binding protein (DBP) or portions or fragments thereof, where the fusion protein comprises an amino acid sequence represented as (N-terminal portion of the LBP) – (DBP) – (C-terminal portion of the LBP), the method comprising preparing a library of fusion proteins by inserting the DBP randomly into the amino acid sequence of the LBP via performing a recombinant DNA method, and selecting a fusion protein from the library of fusion proteins as a biosensor.

22. The method of claim 21, wherein the recombinant DNA method is transposon-mediated DNA recombination.

23. The method of claim 21, wherein the fusion protein is selected as a biosensor via testing whether the fusion protein modulates transcription from a promoter

comprising a binding site for the DBP of the fusion protein in the presence or absence of the ligand for the LBP of the fusion protein.



A

Figure 1

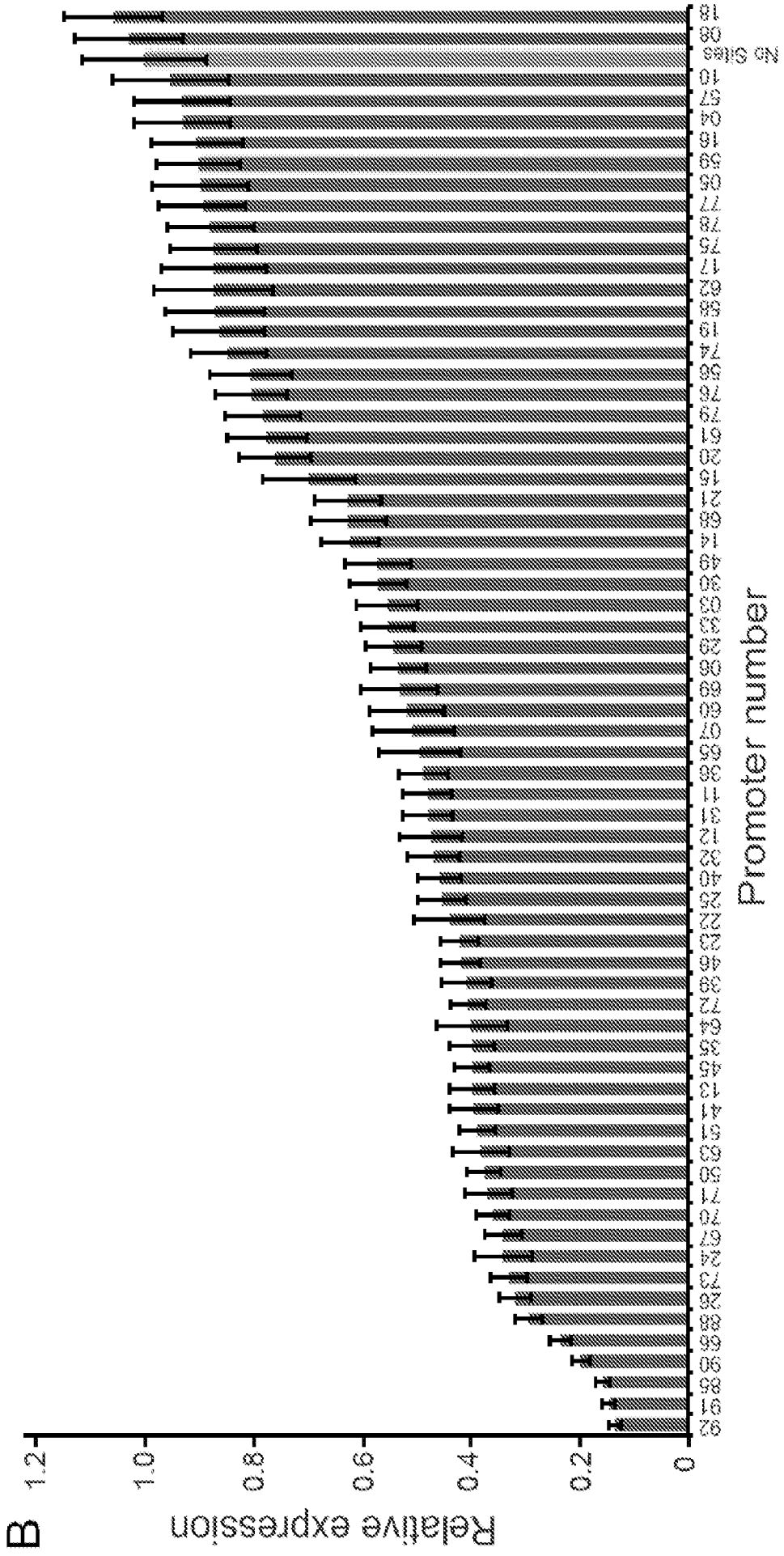
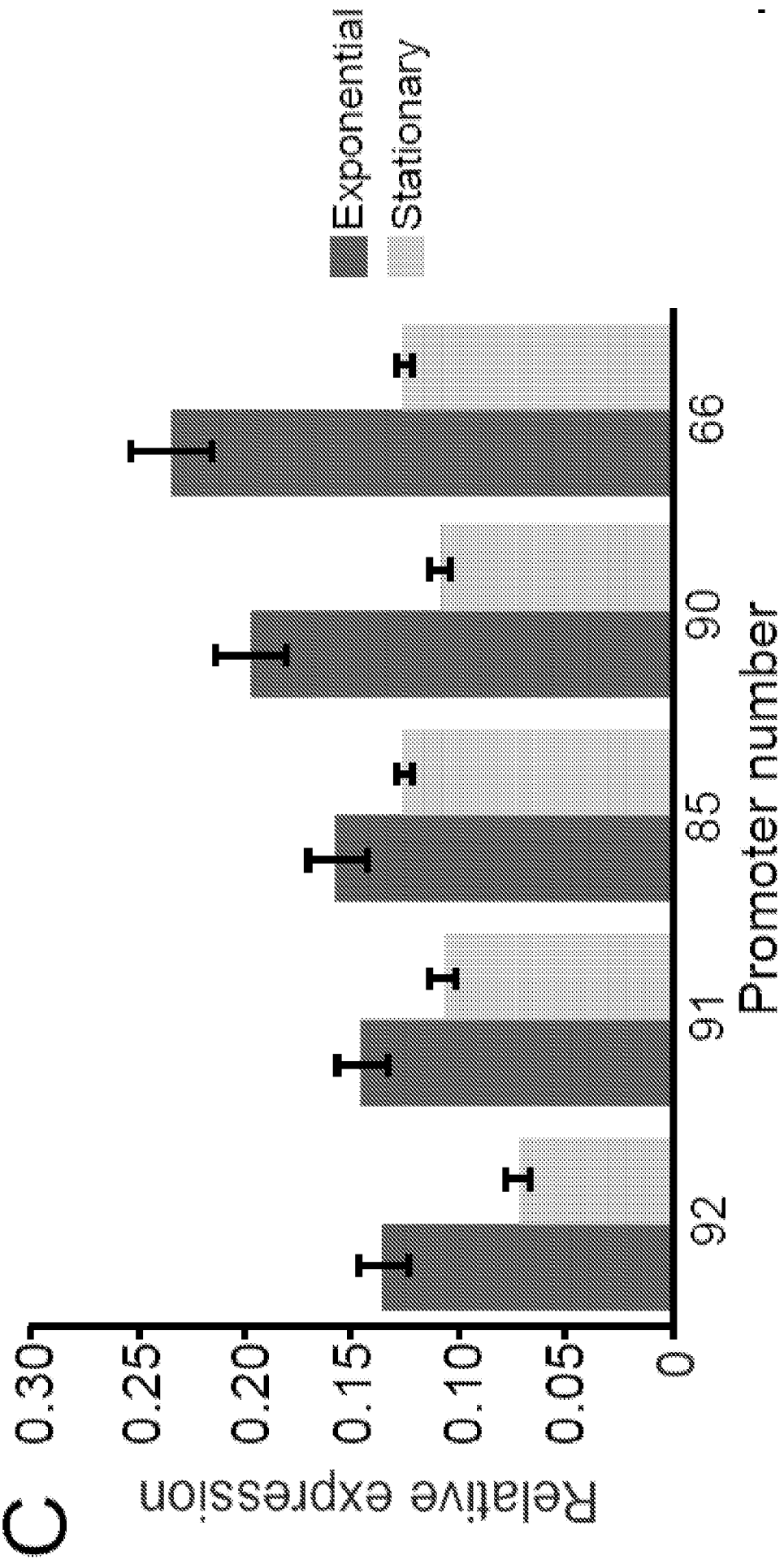


Figure 1 (continued)



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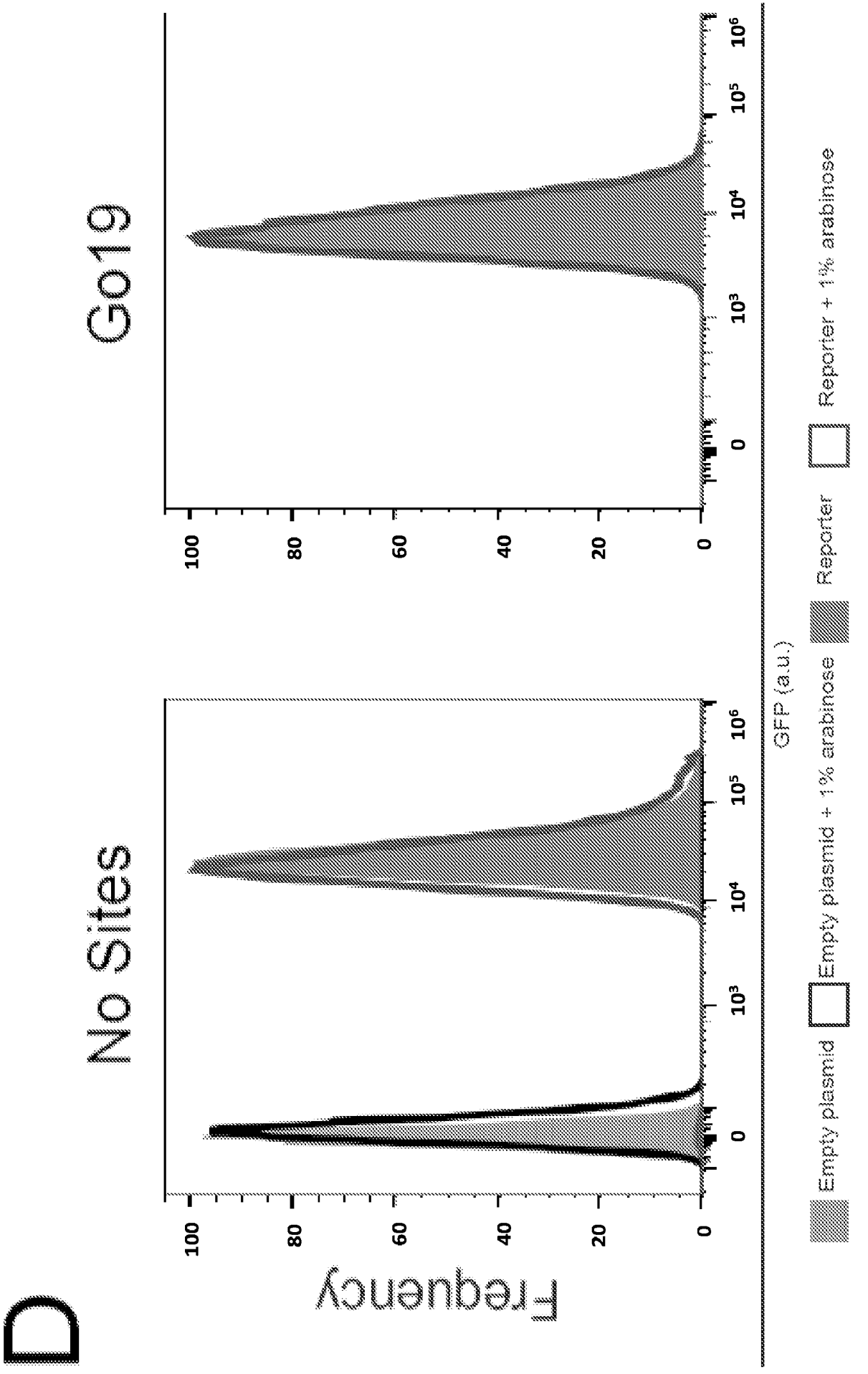


Figure 1 (continued)

E

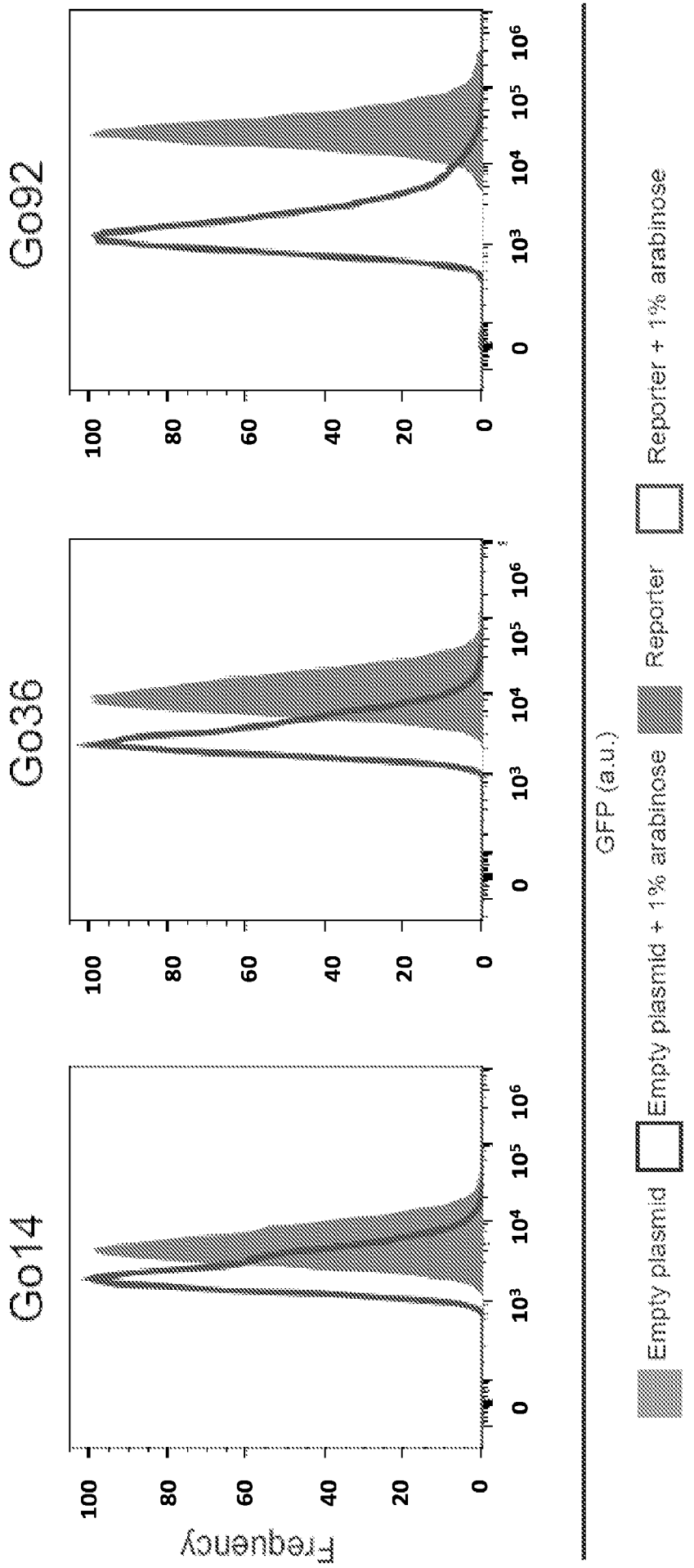
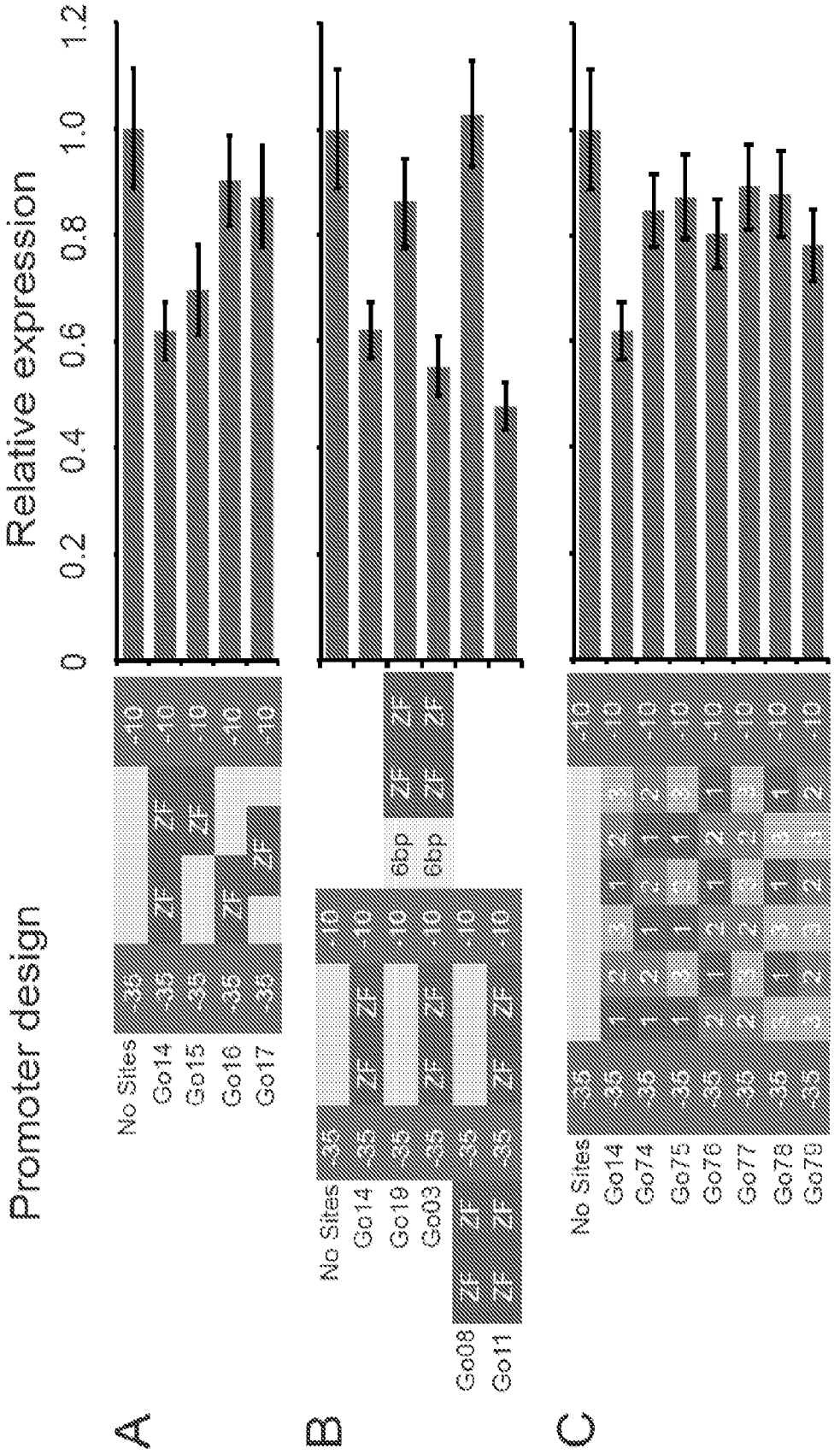


Figure 1 (continued)


Promoter design
Relative expression
0
0.2
0.4
0.6
0.8
1.0
1.2
A
B
C
No Sites
Go14
Go15
Go16
Go17
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-10
ZF
6bp

Figure 2

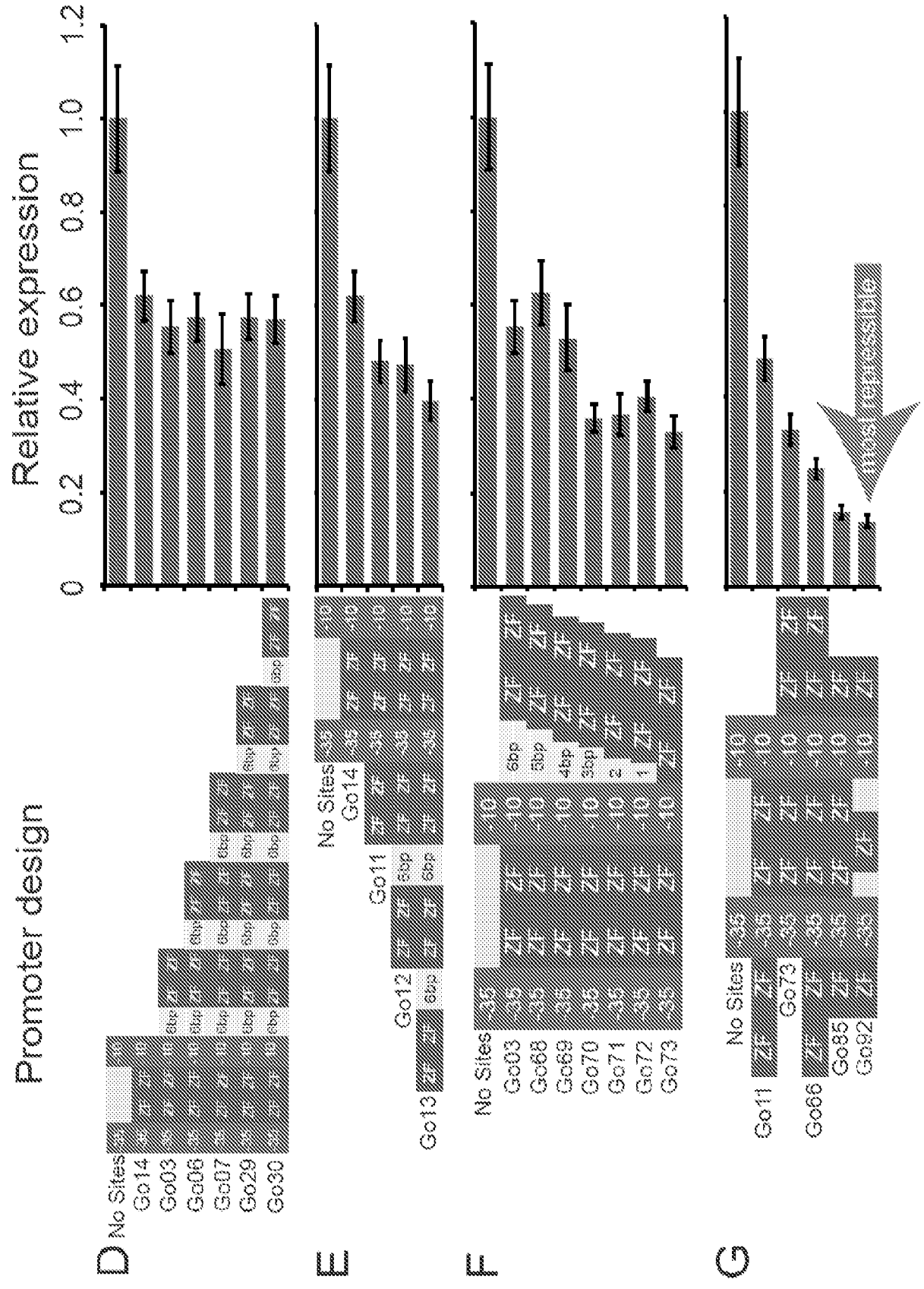


Figure 2 (continued)

A

Shorthand	Description of feature
BS in core	Number of binding sites in the core
pairs in core	Number of pairs in the core
BS upstream	Number of binding sites upstream of -35 region
pairs upstream	Number of pairs upstream of -35 region
BS downstream	Number of binding sites downstream of -10 box
pairs downstream	Number of pairs downstream of -10 box
length	Length of the promoter in base pairs
spacer -10	Base pairs downstream of the -10 box, before the first binding site
spacer -35	Base pairs upstream of the -35 region, before the first binding site
spacer inside -10	Base pairs upstream of the -10 box, before the first binding site
spacer inside -35	Base pairs downstream of the -35 region, before the first binding site
core middle space	Non-binding site base pairs in the core
ave down space	Average number of base pairs between binding sites downstream
ave up space	Average number of base pairs between binding sites up stream
down length	Base pairs downstream of the -10 box
up length	Base pairs upstream of the -35 region
lopsidedness	Absolute value of (binding sites upstream) - (binding sites downstream)

Figure 3

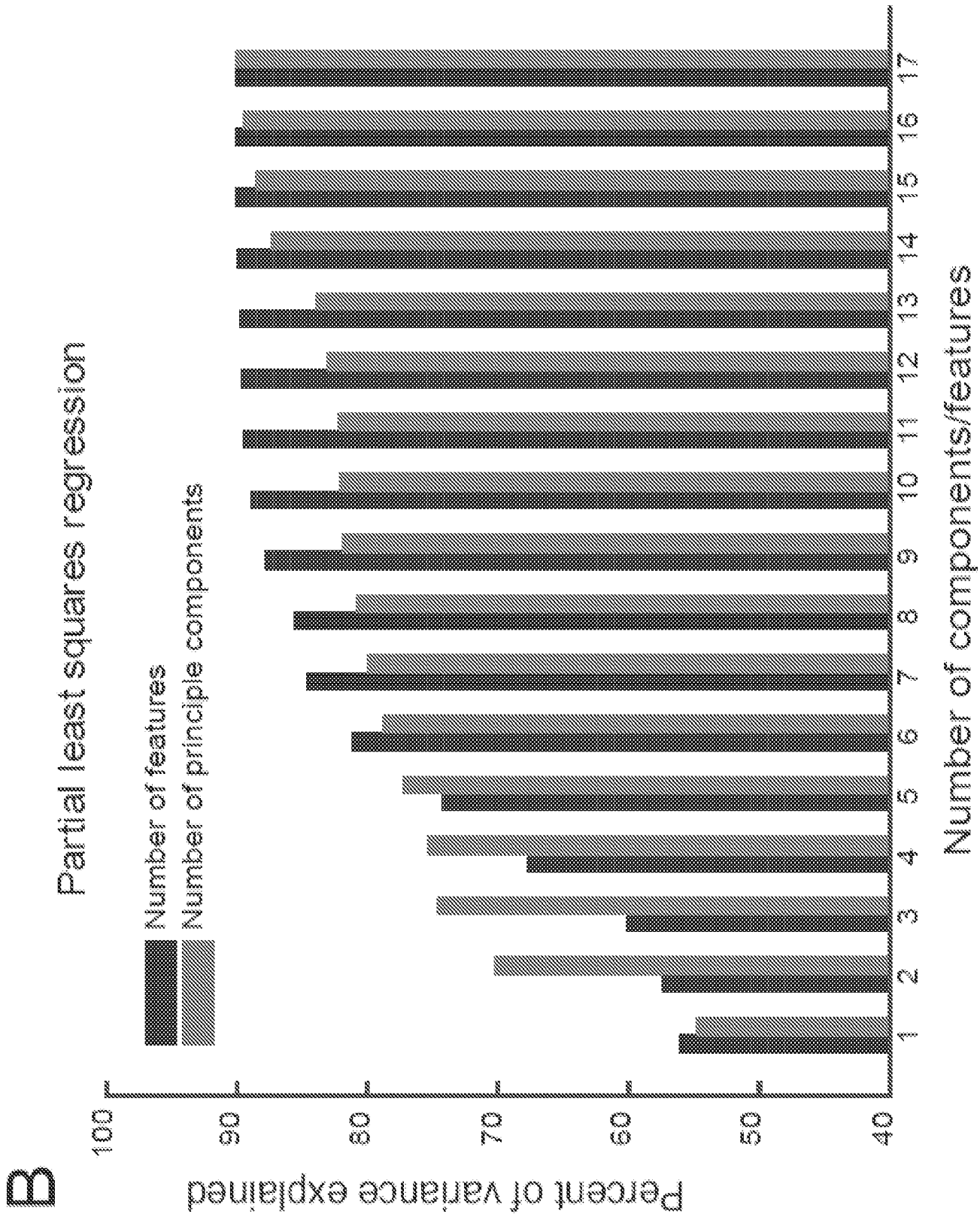


Figure 3 (continued)

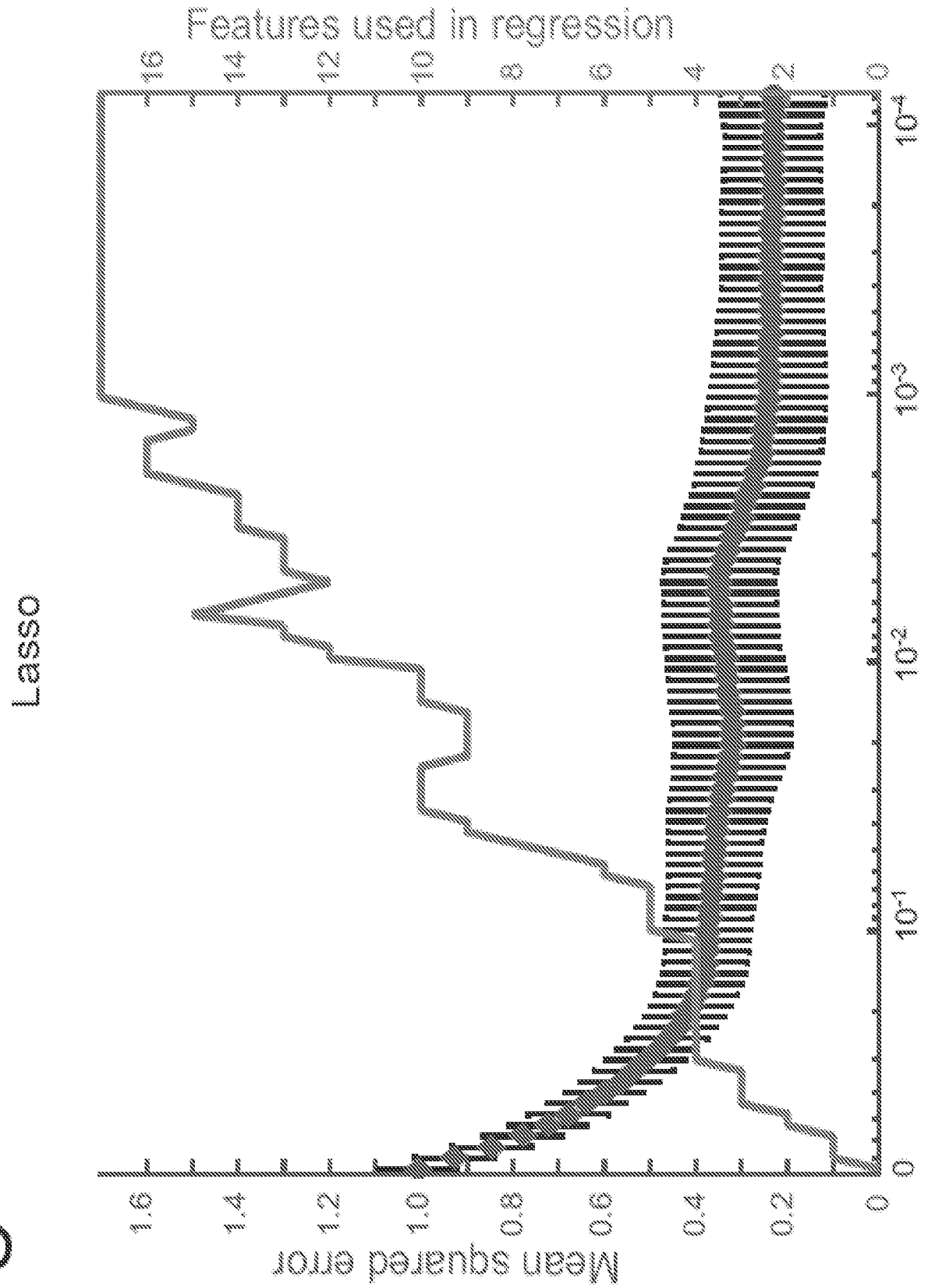


Figure 3 (continued)

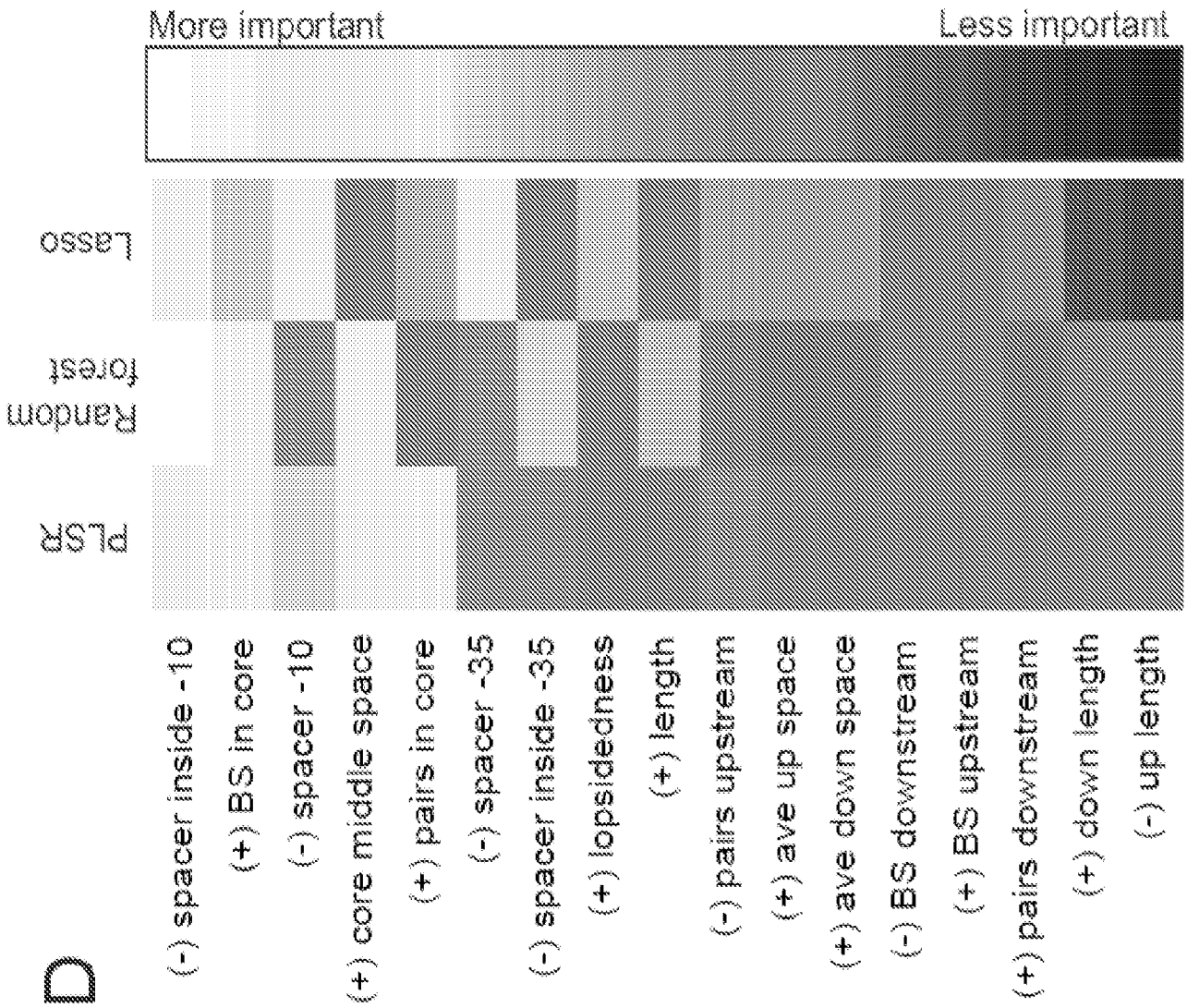


Figure 3 (continued)

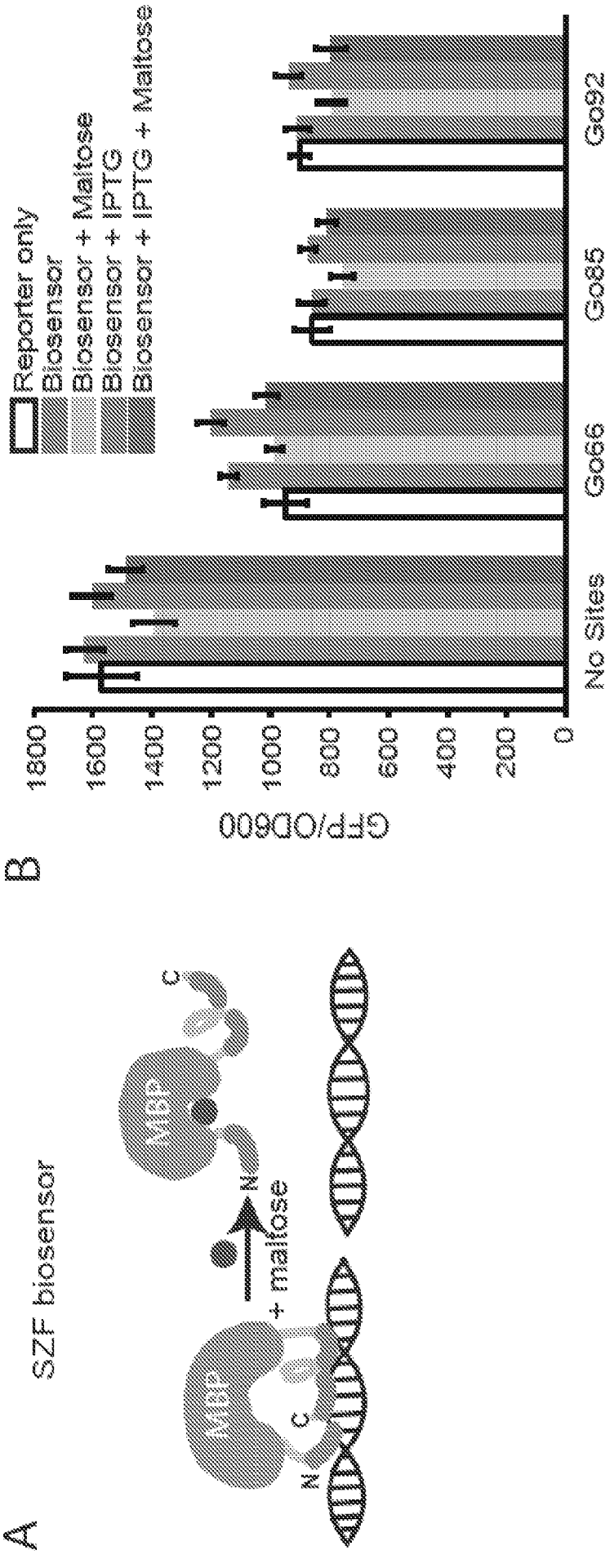

A
SZF biosensor
MBP
C
N
+ maltose
MBP
C
N
B
GFP/OD600
1800
1600
1400
1200
1000
800
600
400
200
0
No Sites
Go66
Go85
Go92
Reporter only
Biosensor
Biosensor + Maltose
Biosensor + IPTG
Biosensor + IPTG + Maltose

Figure 4

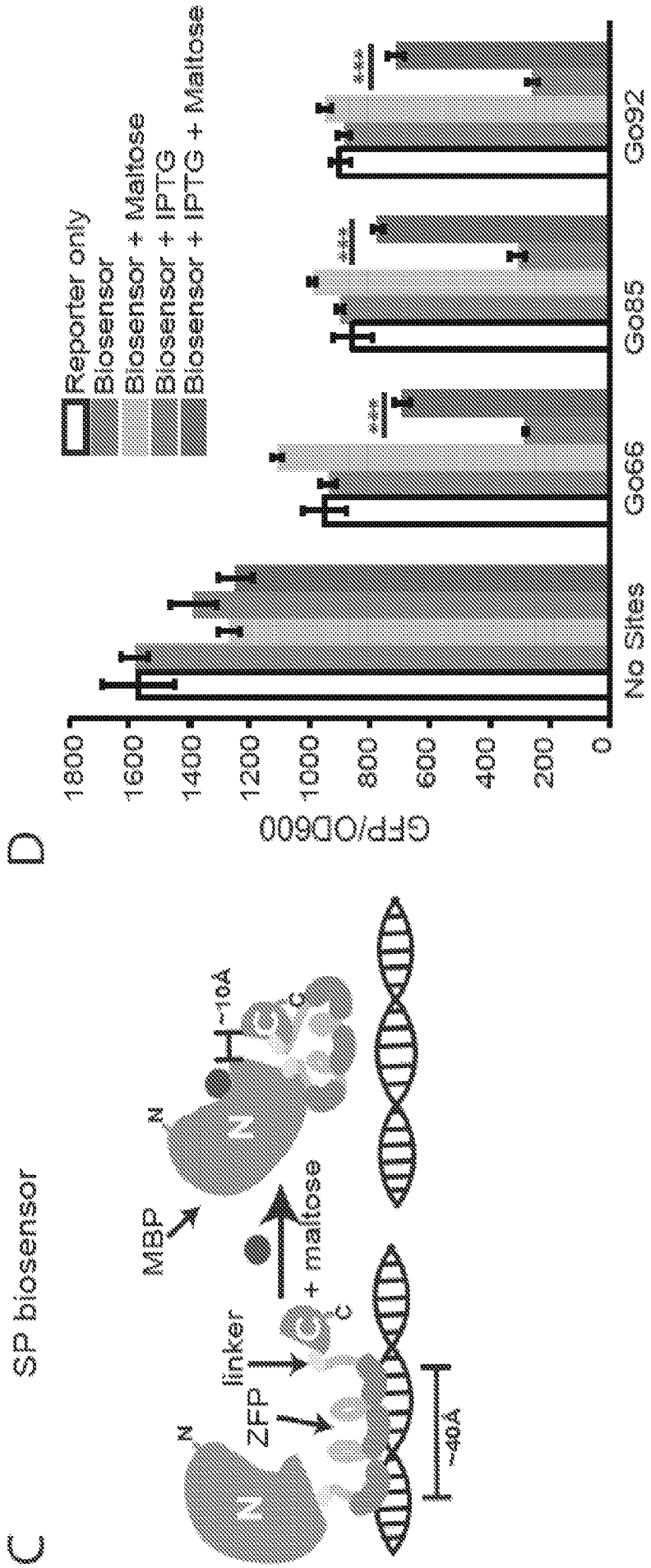
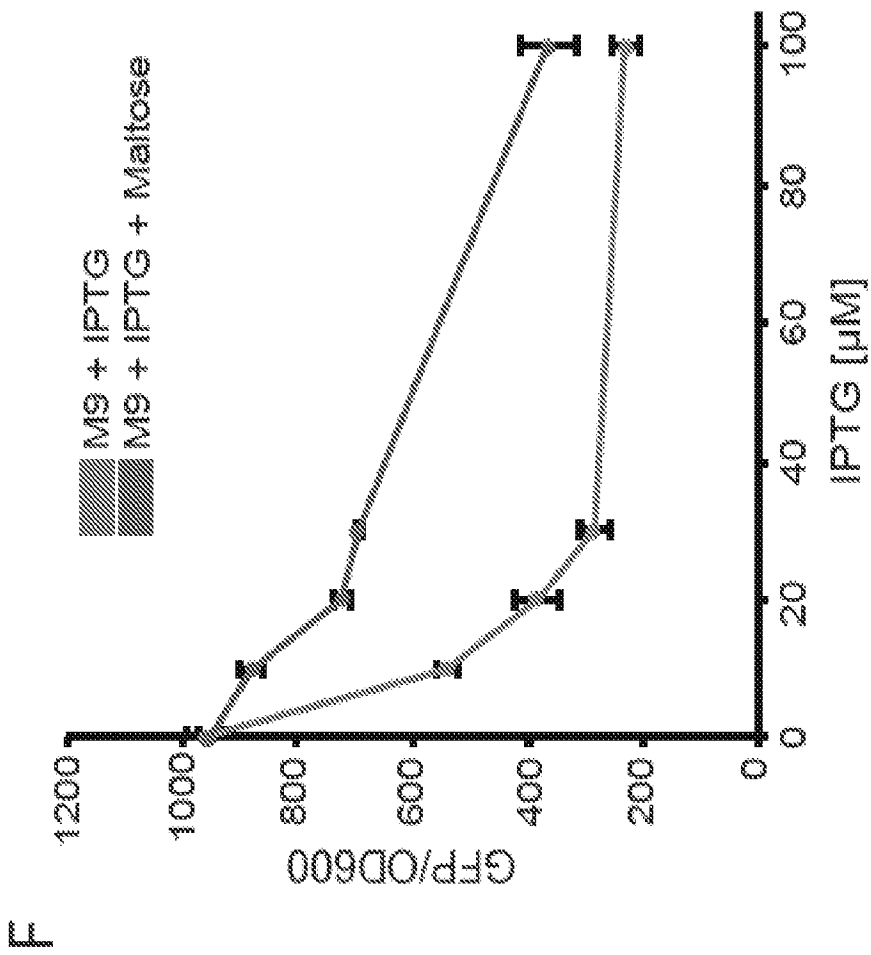


Figure 4 (continued)



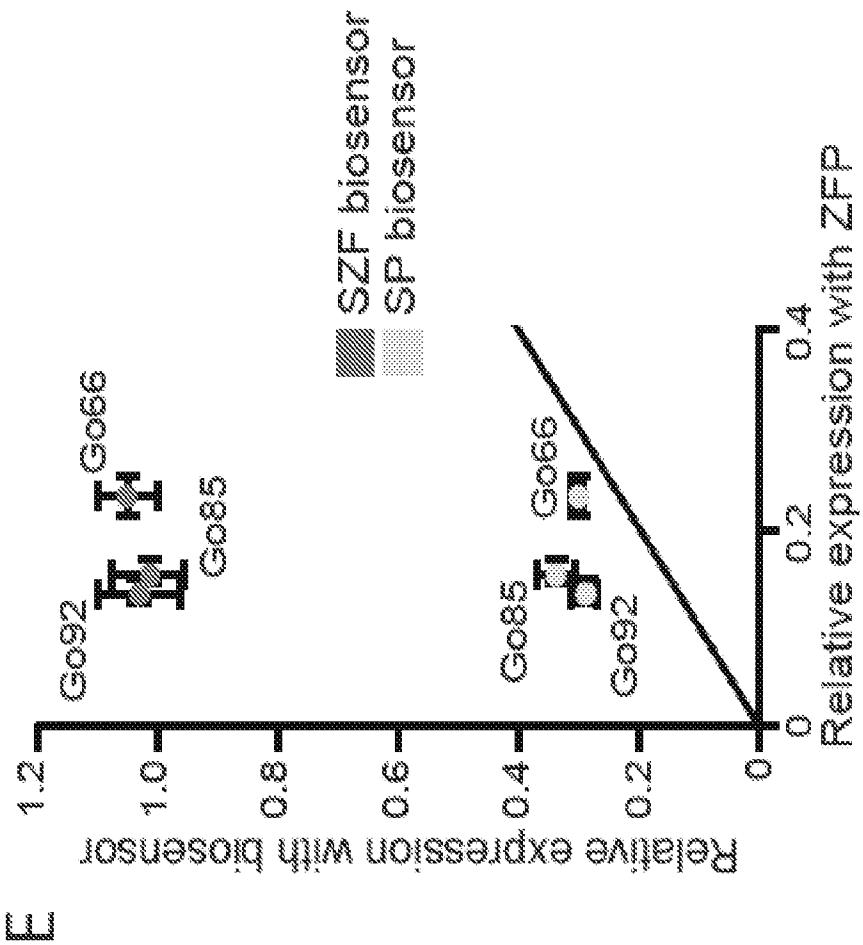


Figure 4 (continued)

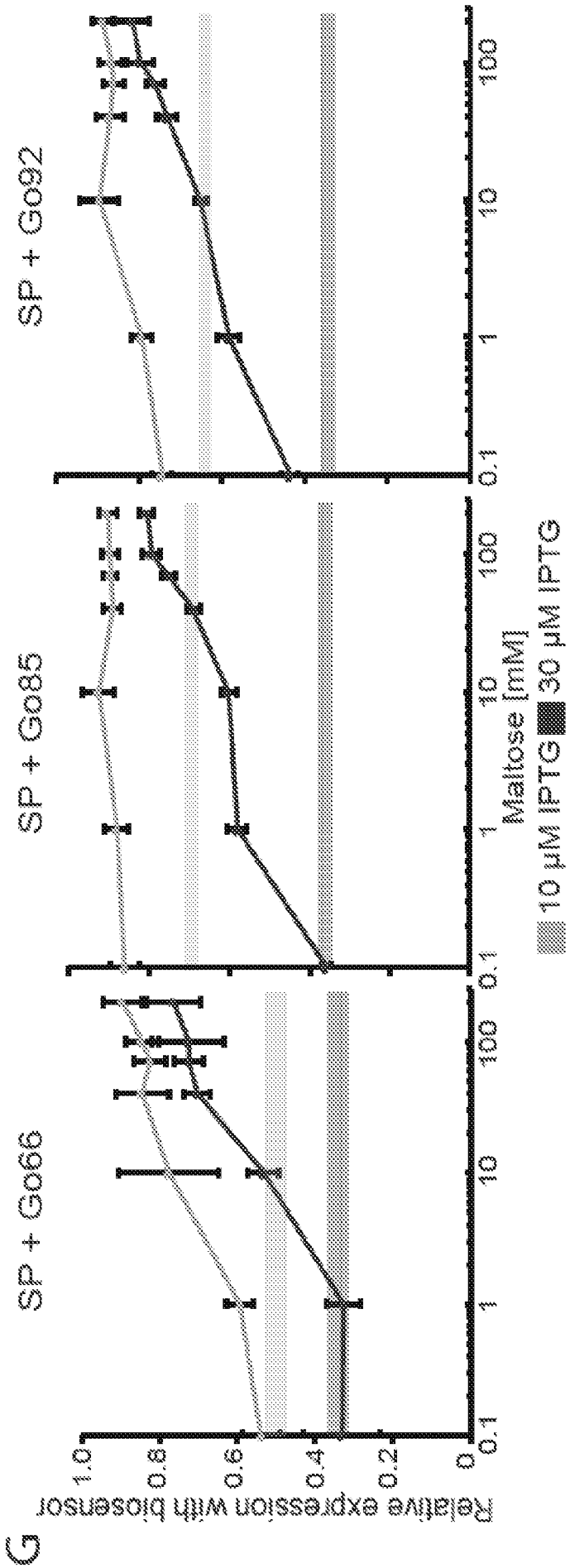


Figure 4 (continued)

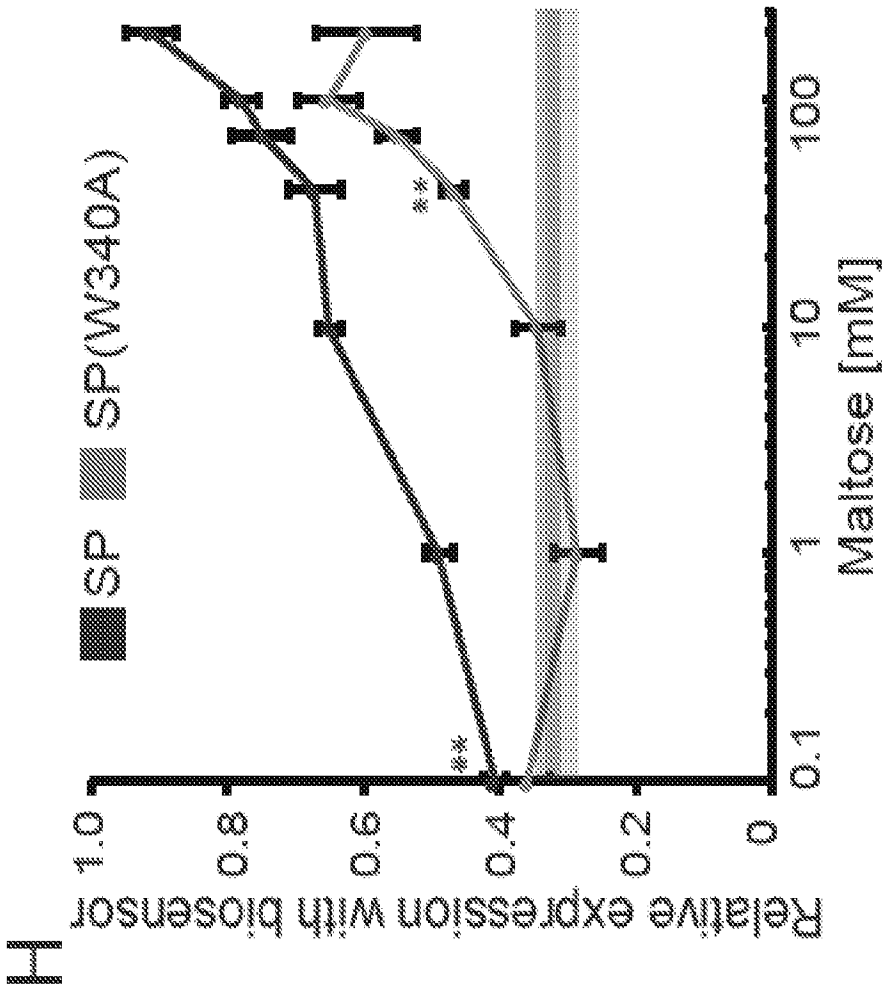


Figure 4 (continued)

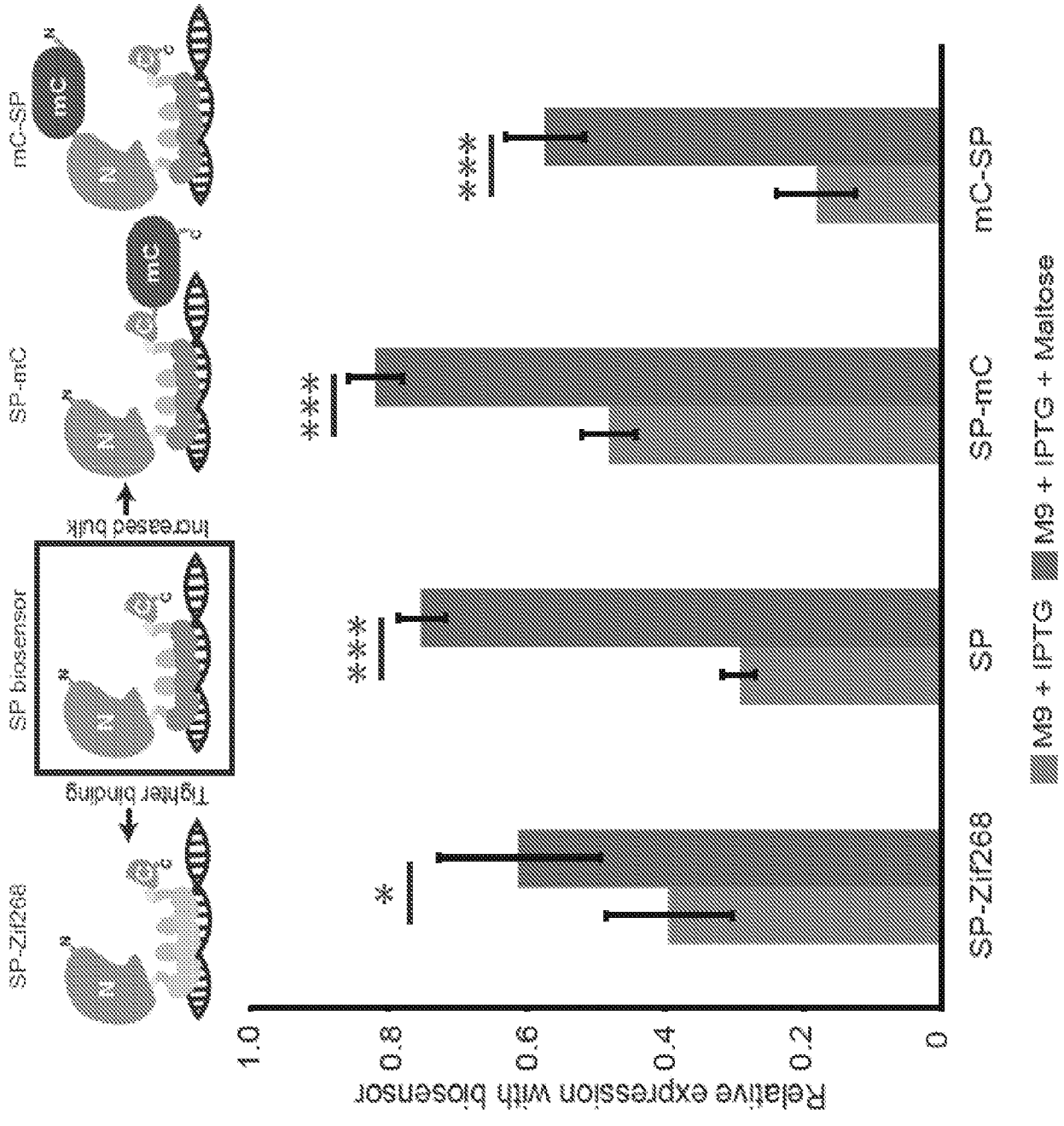

SP-Zif268
Tighter binding
SP biosensor
Increased bulk
SP-mC
mC-SP
Relative expression with biosensor
1.0
0.8
0.6
0.4
0.2
0
*
***
***
***
SP-Zif268
SP
SP-mC
mC-SP
M9 + IPTG
M9 + IPTG + Maltose

Figure 5

A

pAY242 pBAD BCR1
4636 bp
araBAD promoter
BCR1 Zinc...
rbsAY001
rbsAY001
L3S3P11
L3S3P42
KanR RSR
T0
T1
1000
1500
2000
2500
3000
3500
4000
4500
500

Figure 6

B

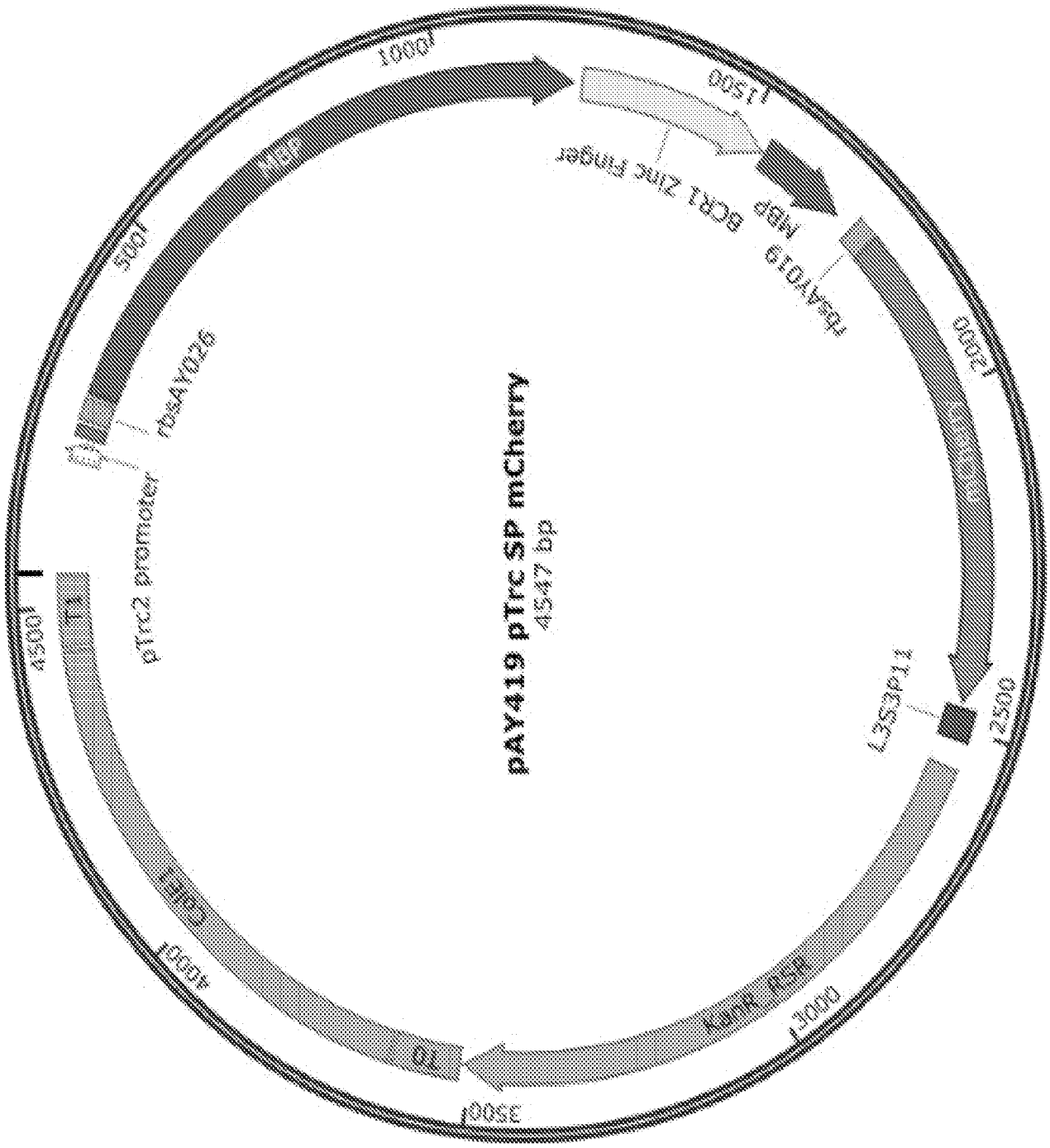


Figure 6 (continued)

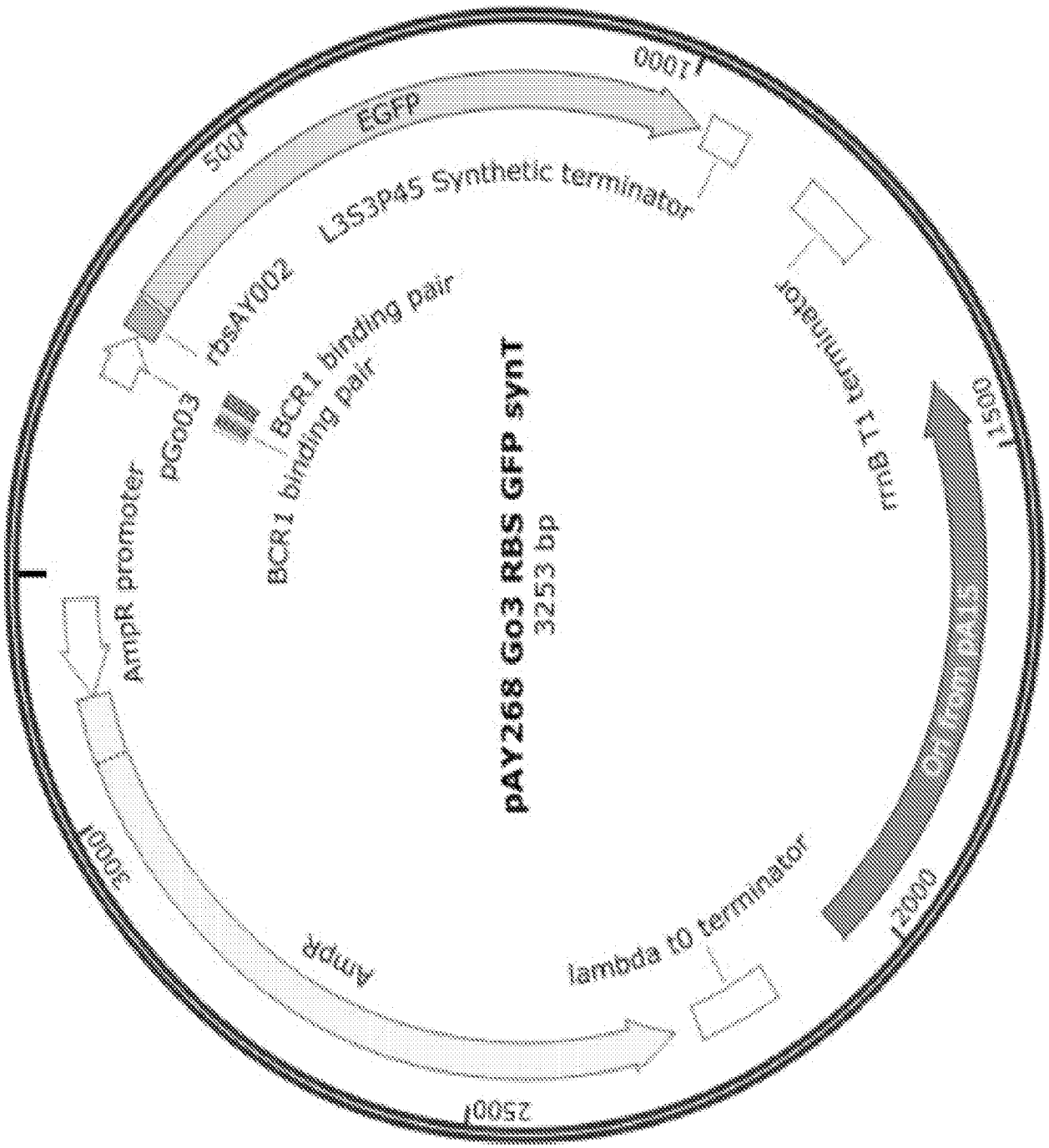


Figure 6 (continued)

Promoter feature	Sequence and coloring
-10 Box	TATAAT
-35 Box	TTGACA
BCR-ABL1 binding site	gcagaagcc
BCR-ABL1 1st finger	gca
BCR-ABL1 2nd finger	gaa
BCR-ABL1 3rd finger	gcc
Spacer sequence	nnnnn

pGoXX	Promoter Sequence 5'-3'	pGoXX	Promoter sequence 5'-3'	pGoXX	Promoter sequence 5'-3'	pGoXX	Promoter sequence 5'-3'
No Sites		Go20	See SEQ ID NO:18	Go45	See SEQ ID NO:35	Go68	See SEQ ID NO:52
Go03	See SEQ ID NO:2	Go21	See SEQ ID NO:19	Go46	See SEQ ID NO:36	Go69	See SEQ ID NO:53
Go04	See SEQ ID NO:3	Go22	See SEQ ID NO:20	Go49	See SEQ ID NO:37	Go70	See SEQ ID NO:54
Go05	See SEQ ID NO:4	Go23	See SEQ ID NO:21	Go50	See SEQ ID NO:38	Go71	See SEQ ID NO:55
Go06	See SEQ ID NO:5	Go24	See SEQ ID NO:22	Go51	See SEQ ID NO:39	Go72	See SEQ ID NO:56
Go07	See SEQ ID NO:6	Go25	See SEQ ID NO:23	Go56	See SEQ ID NO:40	Go73	See SEQ ID NO:57
Go08	See SEQ ID NO:7	Go26	See SEQ ID NO:24	Go57	See SEQ ID NO:41	Go74	See SEQ ID NO:58
Go10	See SEQ ID NO:8	Go29	See SEQ ID NO:25	Go58	See SEQ ID NO:42	Go75	See SEQ ID NO:59
Go11	See SEQ ID NO:9	Go30	See SEQ ID NO:26	Go59	See SEQ ID NO:43	Go76	See SEQ ID NO:60
Go12	See SEQ ID NO:10	Go31	See SEQ ID NO:27	Go60	See SEQ ID NO:44	Go77	See SEQ ID NO:61
Go13	See SEQ ID NO:11	Go32	See SEQ ID NO:28	Go61	See SEQ ID NO:45	Go78	See SEQ ID NO:62
Go14	See SEQ ID NO:12	Go33	See SEQ ID NO:29	Go62	See SEQ ID NO:46	Go79	See SEQ ID NO:63
Go15	See SEQ ID NO:13	Go35	See SEQ ID NO:30	Go63	See SEQ ID NO:47	Go85	See SEQ ID NO:64
Go16	See SEQ ID NO:14	Go36	See SEQ ID NO:31	Go64	See SEQ ID NO:48	Go88	See SEQ ID NO:65
Go17	See SEQ ID NO:15	Go39	See SEQ ID NO:32	Go65	See SEQ ID NO:49	Go90	See SEQ ID NO:66
Go18	See SEQ ID NO:16	Go40	See SEQ ID NO:33	Go66	See SEQ ID NO:50	Go91	See SEQ ID NO:67
Go19	See SEQ ID NO:17	Go41	See SEQ ID NO:34	Go67	See SEQ ID NO:51	Go92	See SEQ ID NO:68

SUBSTITUTE SHEET (RULE 26)

Figure 7

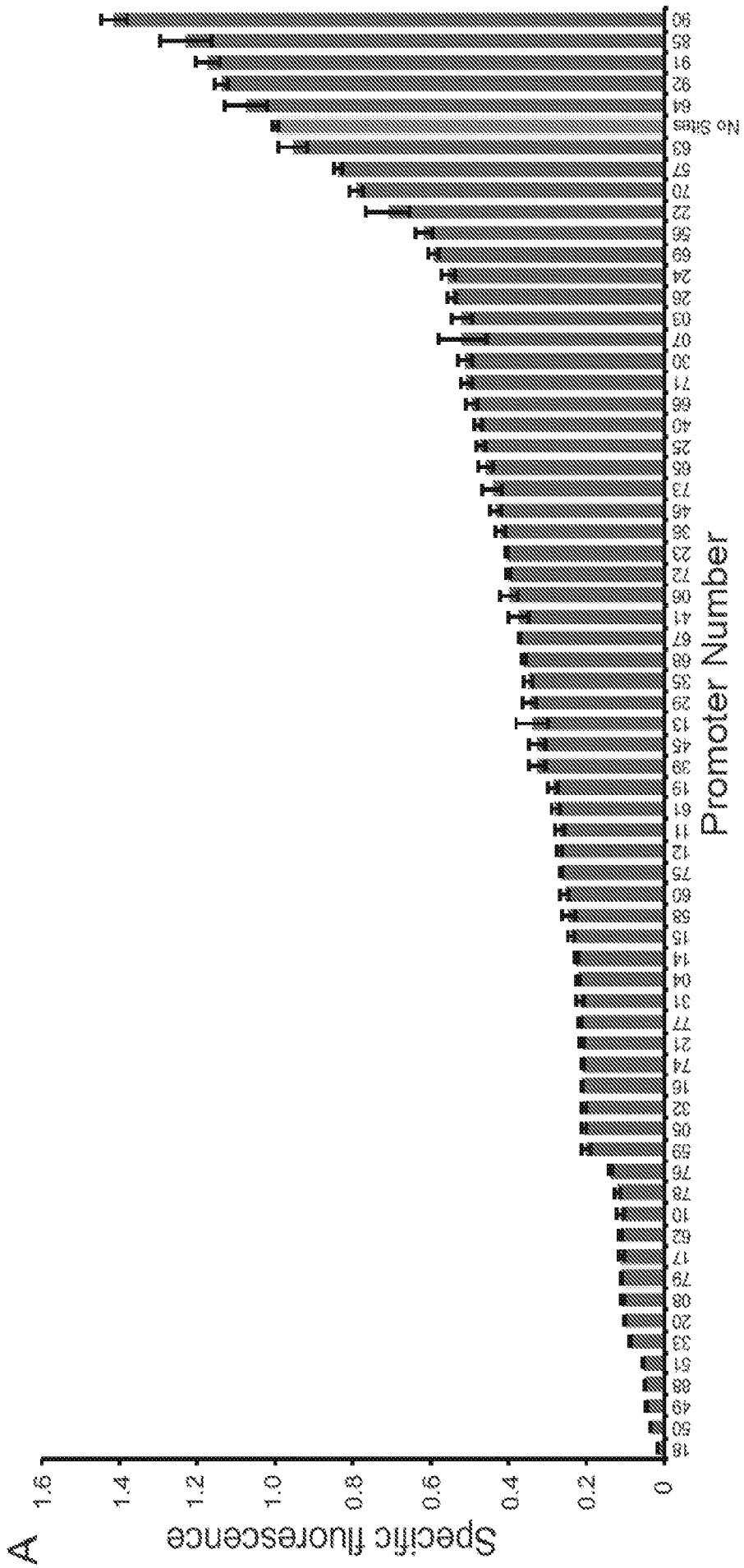

Specific fluorescence
A
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Promoter Number
No Sites

Figure 8

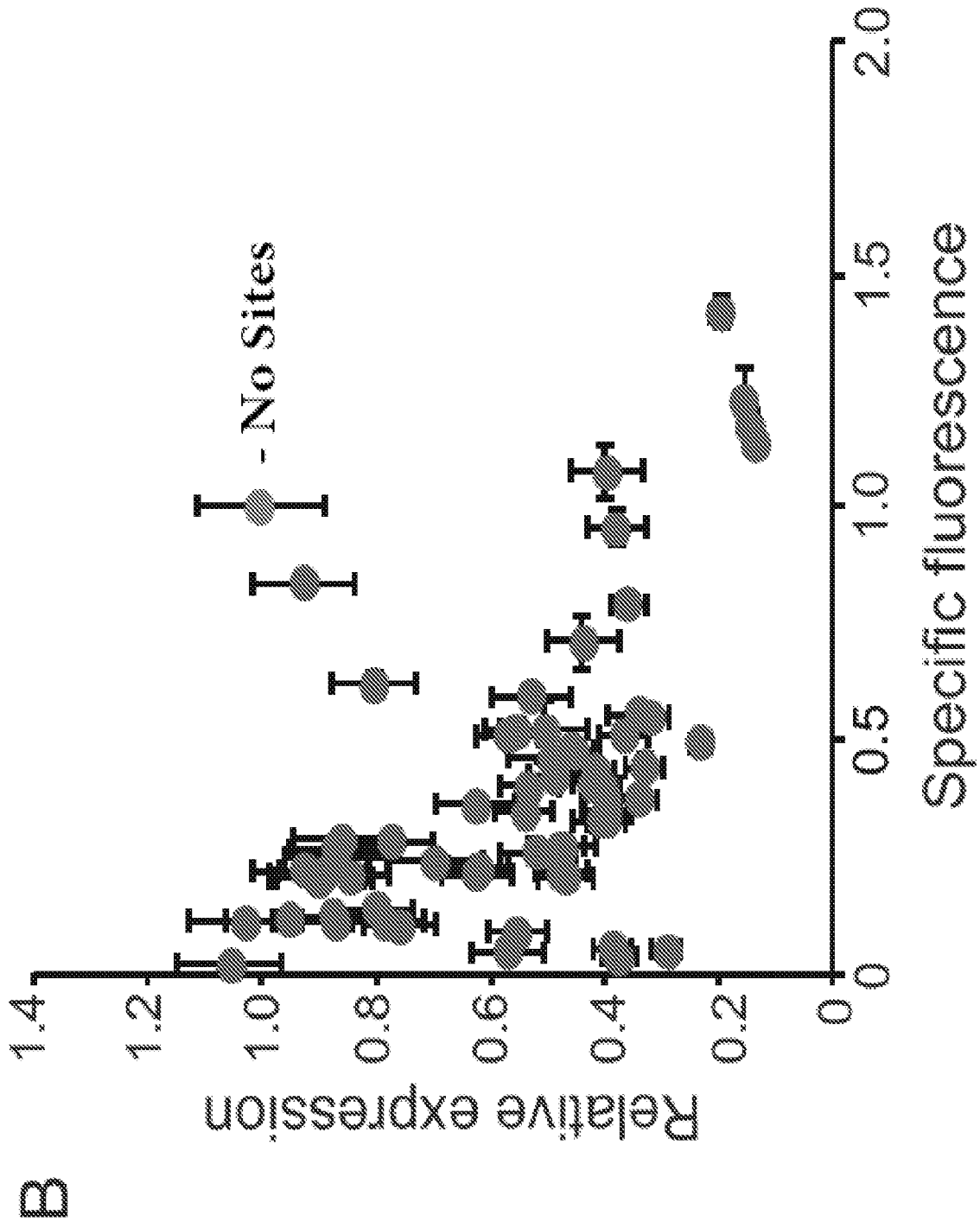


Figure 8 (continued)

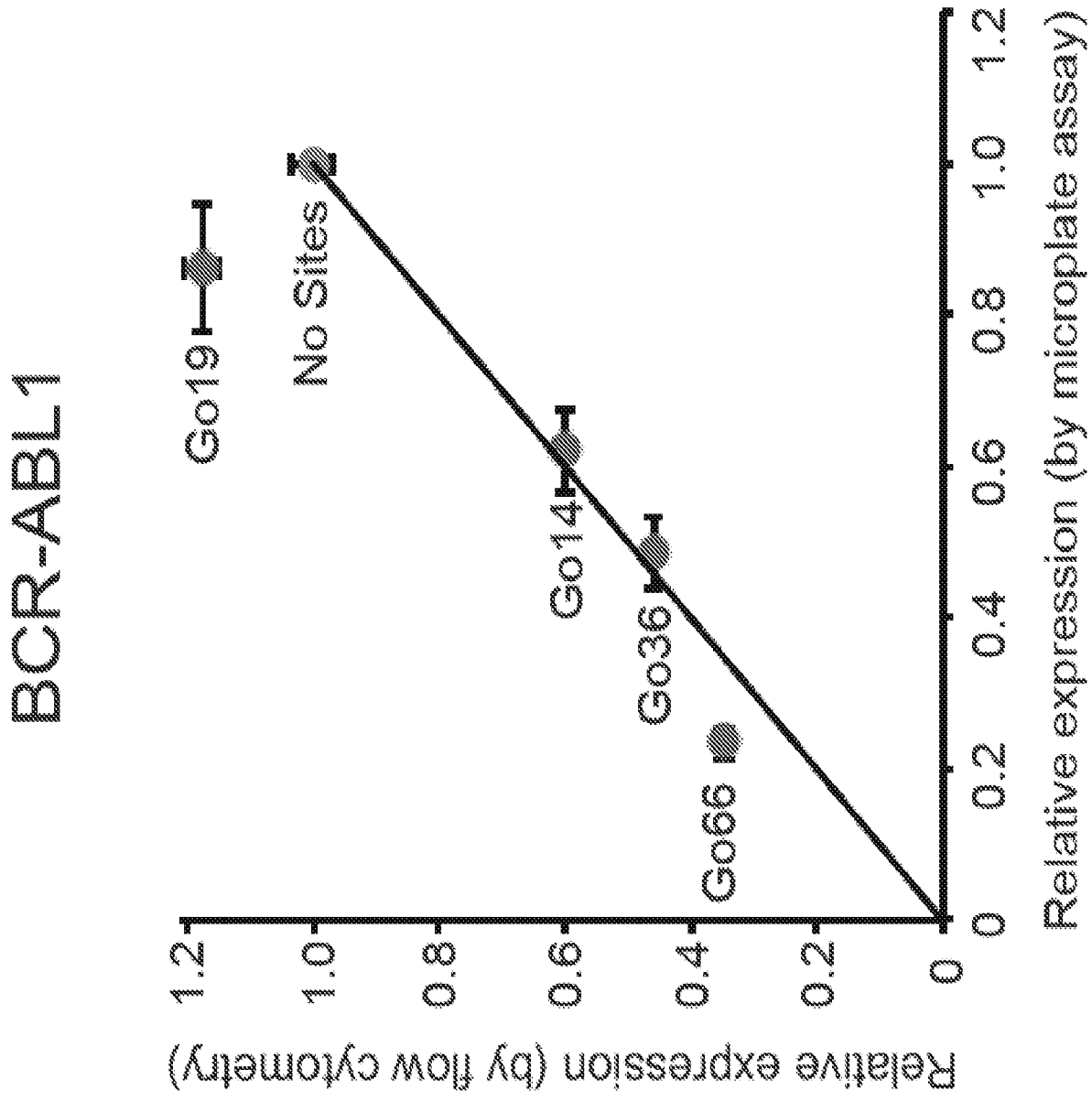

BCR-ABL1
Relative expression (by flow cytometry)
1.2
1.0
0.8
0.6
0.4
0.2
0
Go19
No Sites
Go14
Go36
Go66
0
0.2
0.4
0.6
0.8
1.0
1.2
Relative expression (by microplate assay)

Figure 9

A

Feature Number	Shorthand	Description
1	*BS in core*	Number of binding sites in the core
2	*pairs in core*	Number of pairs in the core
3	*BS upstream*	Number of binding sites upstream of the -35 region
4	*pairs upstream*	Number of pairs upstream of the -35 region
5	*BS downstream*	Number of binding sites downstream of the -10 box
6	*pairs downstream*	Number of pairs downstream of the -10 box
7	*length*	Length of the promoter in base pairs
8	*spacer -10*	Base pairs downstream of the -10 box, before the first binding site
9	*spacer -35*	Base pairs upstream of the -35 region, before the first binding site
10	*spacer inside -10*	Base pairs upstrsam of the -10 box, before the first binding site
11	*spacer inside -35*	Bse pairs downstream of the -35 region, before the first binding site
12	*core middle space*	Non-binding site base pairs in the core
13	*ave down space*	Average number of base pairs between binding sites downstream
14	*ave up space*	Average number of base pairs between binding sites upstream
15	*down length*	Base pairs downstream of the -10 box
16	*up length*	base pairs upstream of the -35 box
17	*lopsidedness*	Absolute value of (binding sites upstream) - (binding sites downstream)

Figure 10

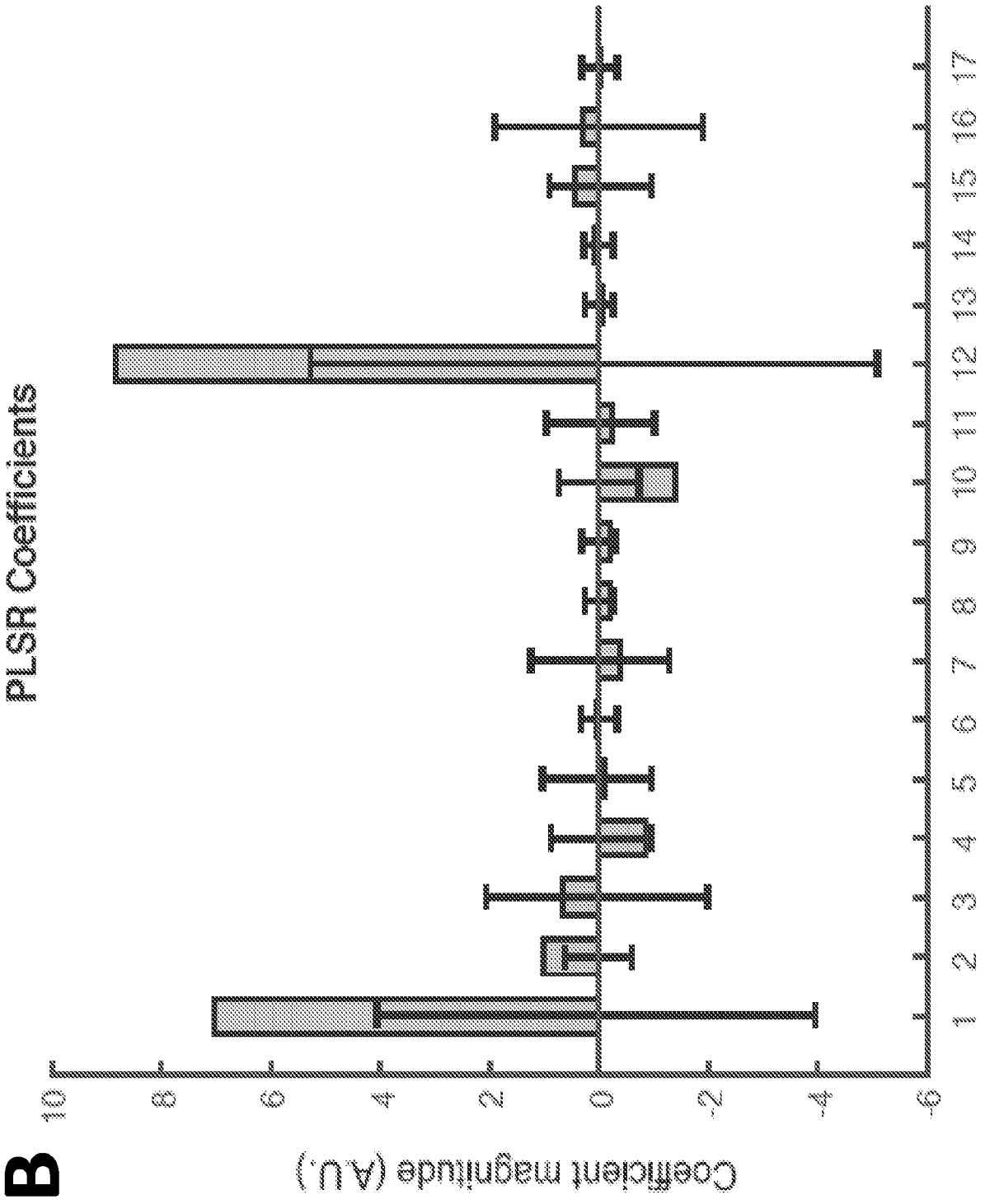


Figure 10 (continued)

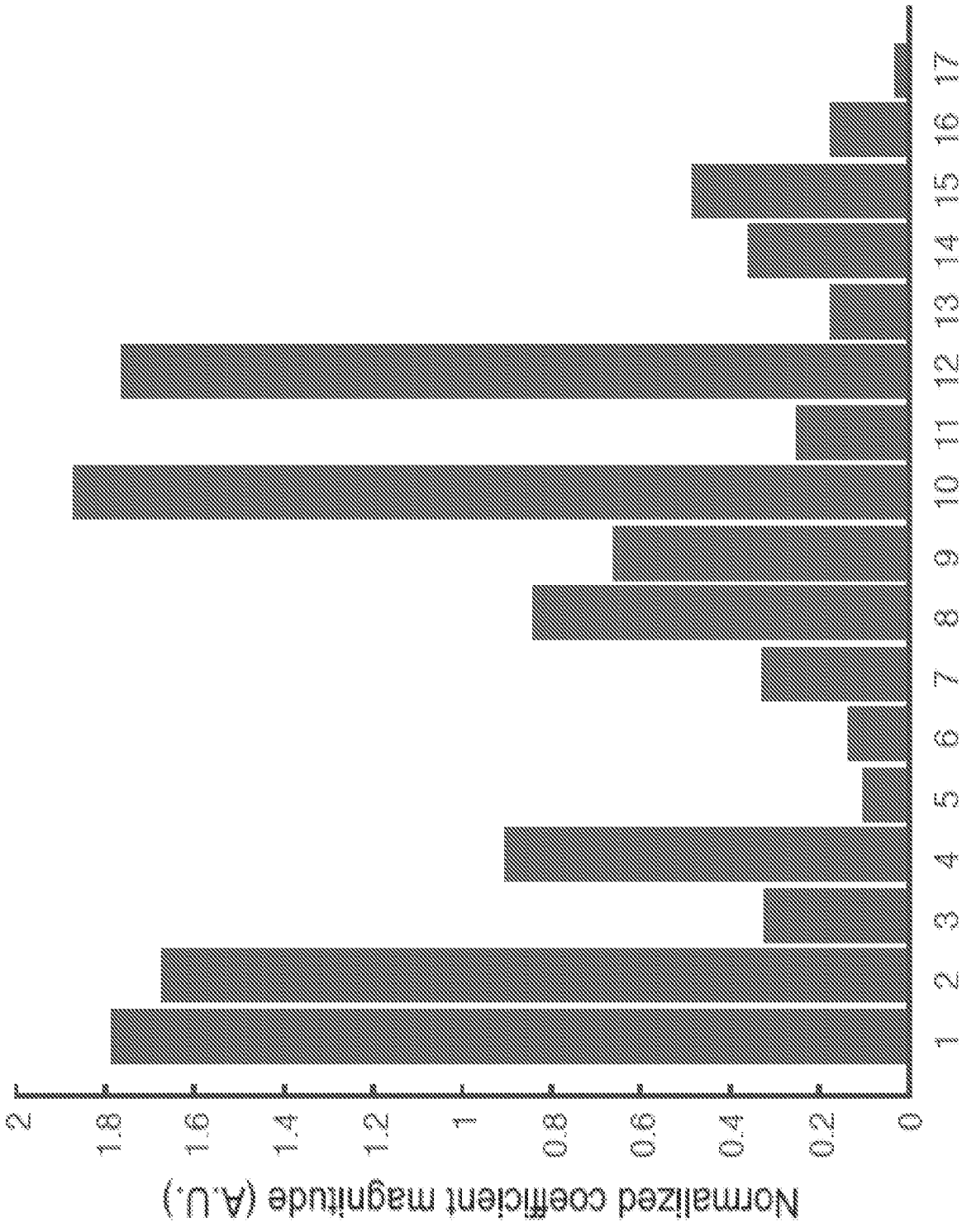


Figure 10 (continued)

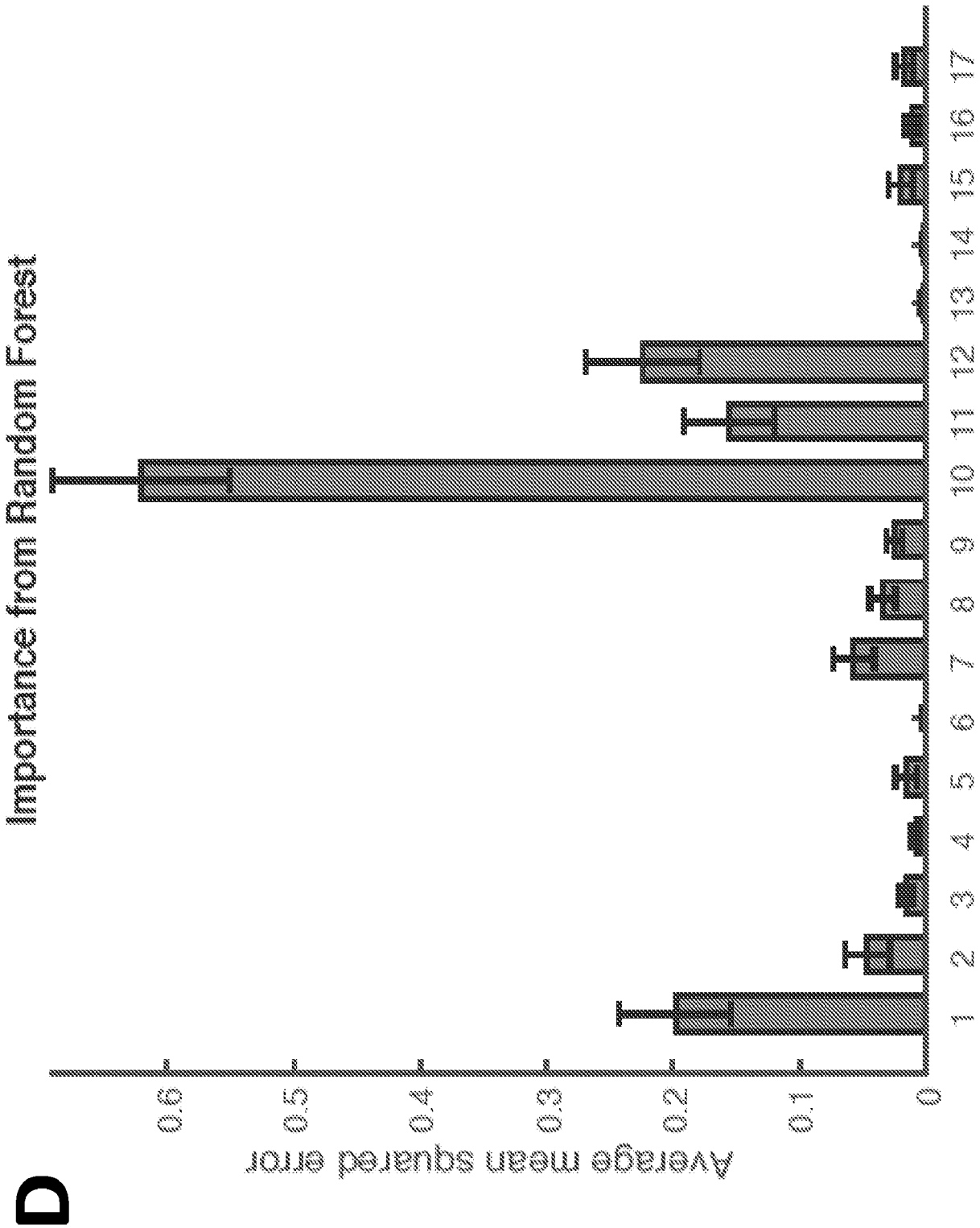


Figure 10 (continued)

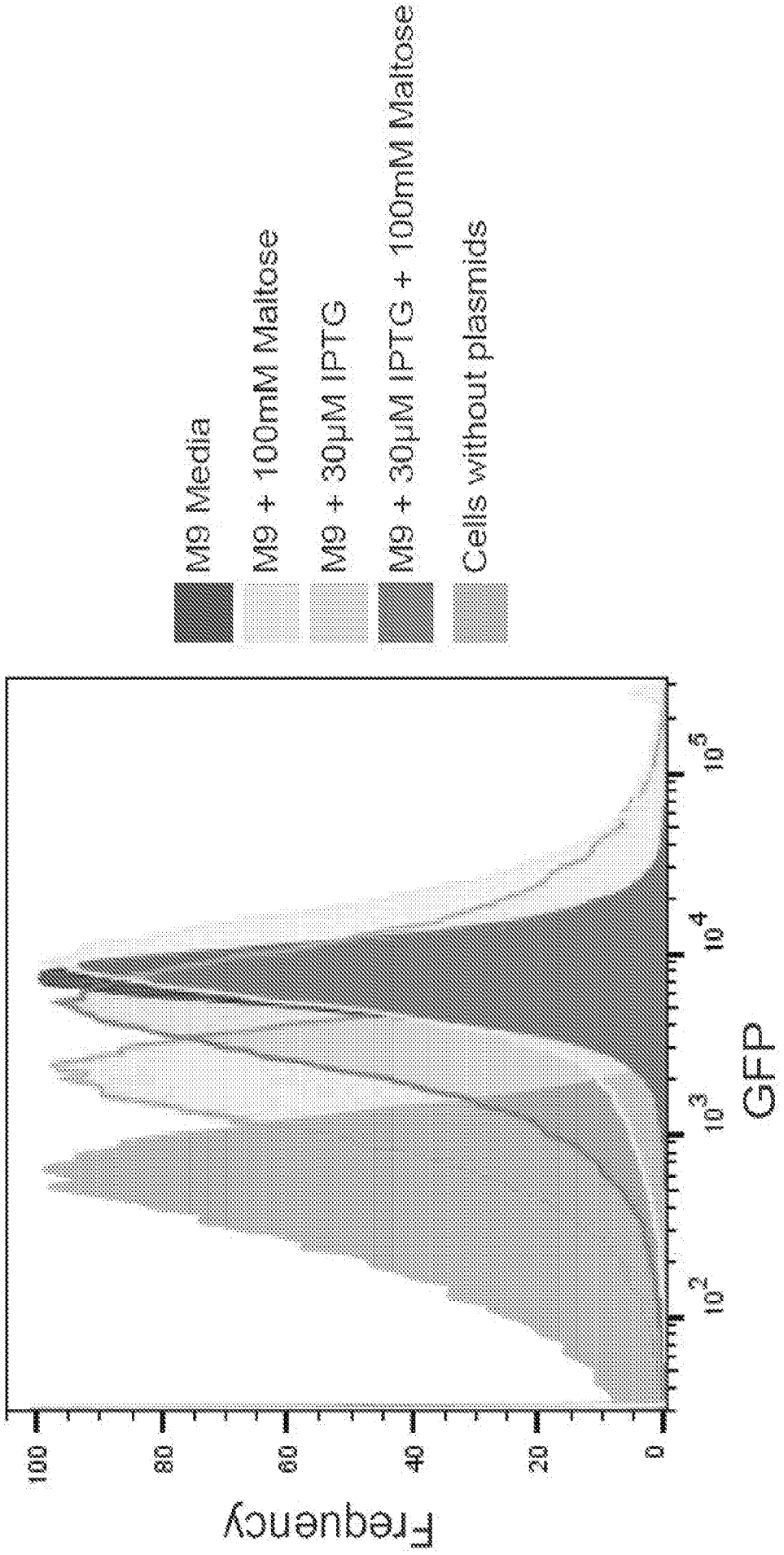

M9 Media
M9 + 100mM Maltose
M9 + 30μM IPTG
M9 + 30μM IPTG + 100mM Maltose
Cells without plasmids
Frequency
100
80
60
40
20
0
10^2
10^3
10^4
10^5
GFP

Figure 11

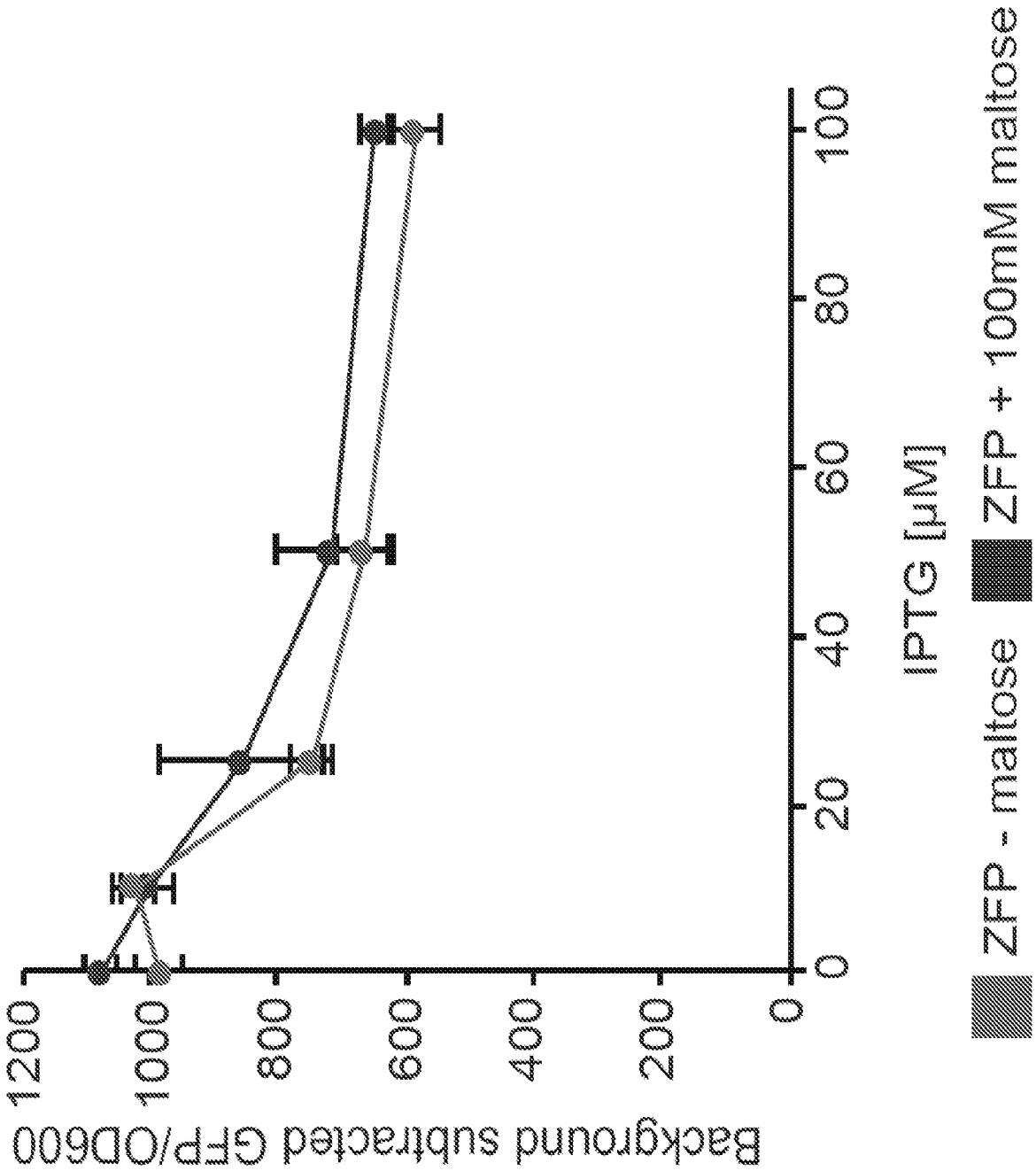

Background subtracted GFP/OD600
1200
1000
800
600
400
200
0
0
20
40
60
80
100
IPTG [µM]
ZFP - maltose
ZFP + 100mM maltose

Figure 12

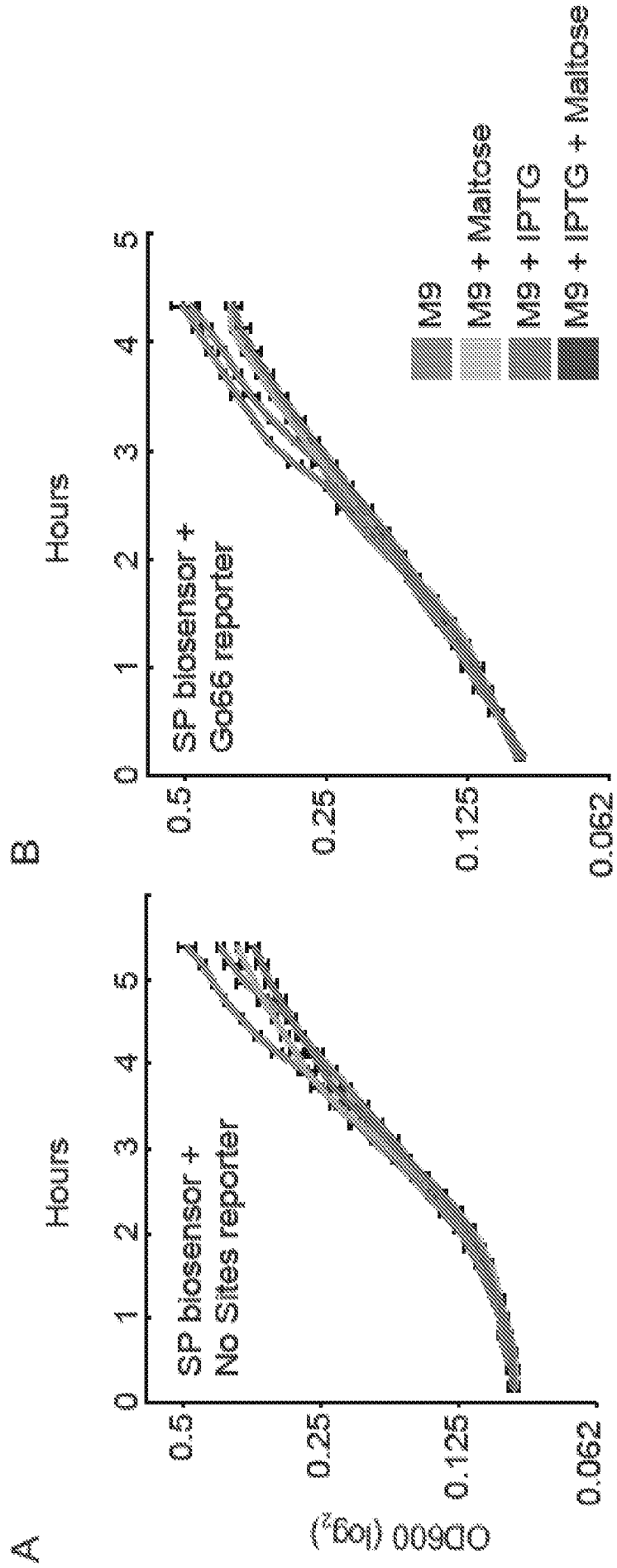

A
B
Hours
OD600 (log2)
SP biosensor +
No Sites reporter
SP biosensor +
G066 reporter
M9
M9 + Maltose
M9 + IPTG
M9 + IPTG + Maltose
0
1
2
3
4
5
0.5
0.25
0.125
0.062

Figure 13

Biosensor	SP	SP-Zif	SP-mC	mC-SP
Fold repression	4.1 ± 0.1	2.4 ± 0.4	3.5 ± 0.1	6.0 ± 0.4
Fold alleviation	3.0 ± 0.1	1.8 ± 0.3	2.0 ± 0.1	3.8 ± 0.3

Figure 14

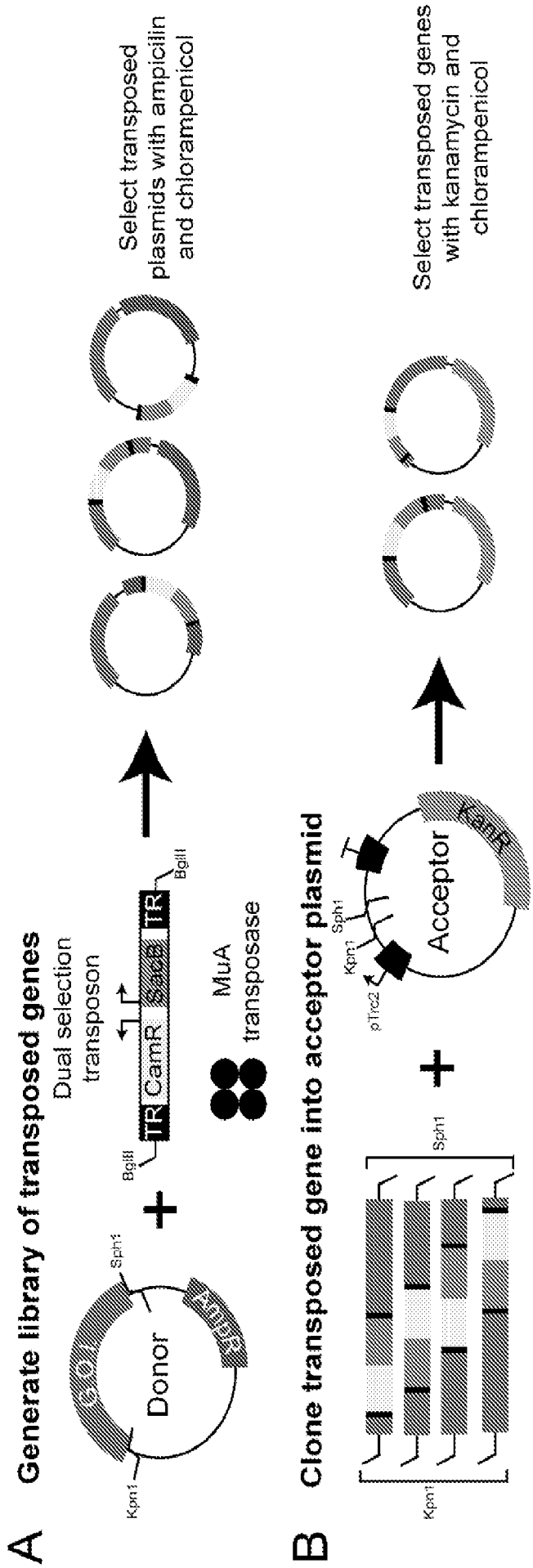

A
Generate library of transposed genes
Donor
Kpn1
Sph1
AmpR
BglII
TR
CamR
SacB
TR
BglII
Dual selection transposon
MuA transposase
Select transposed plasmids with ampicilin and chlorampenicol
B
Clone transposed gene into acceptor plasmid
Kpn1
Sph1
pTrc2
Kpn1
Sph1
Acceptor
KanR
Select transposed genes with kanamycin and chlorampenicol

Figure 15

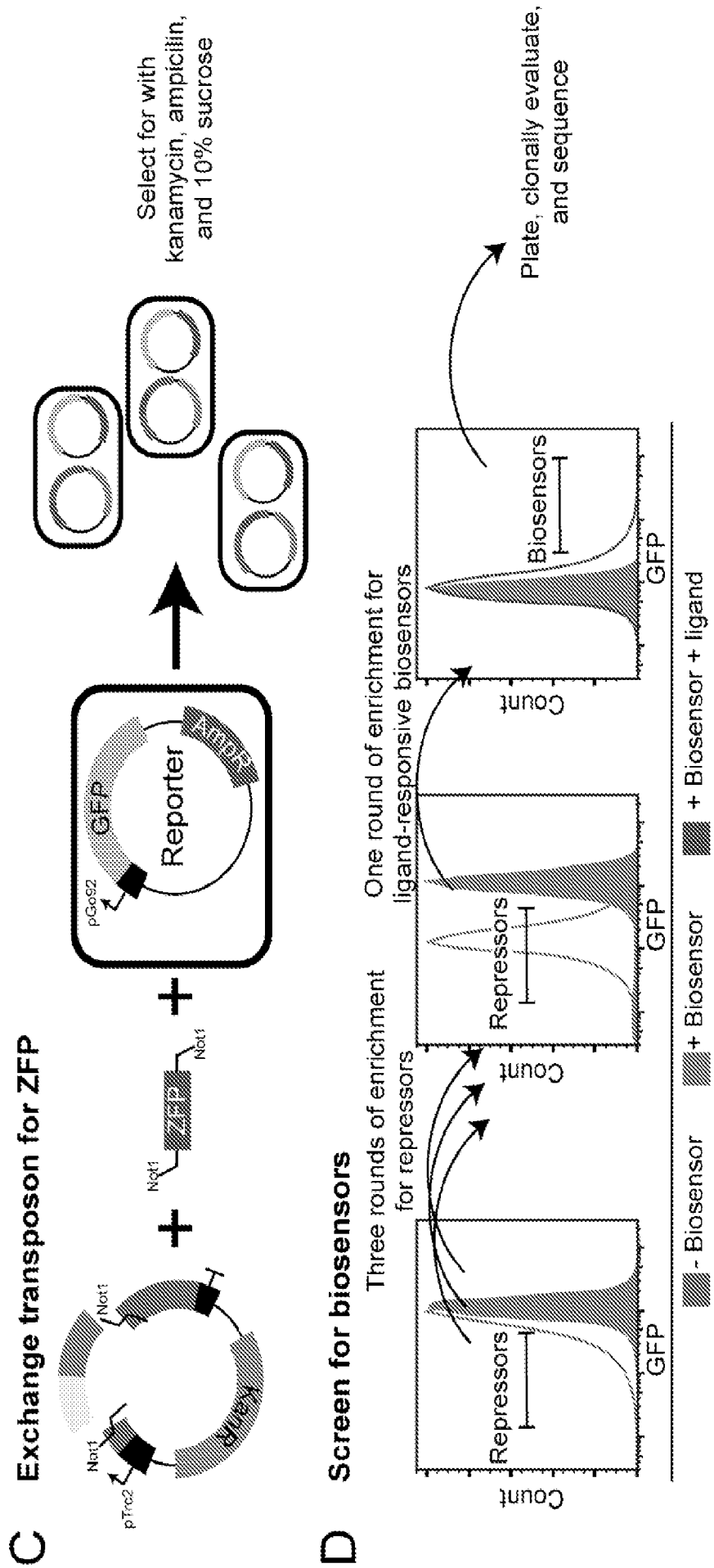


Figure 15 (continued)

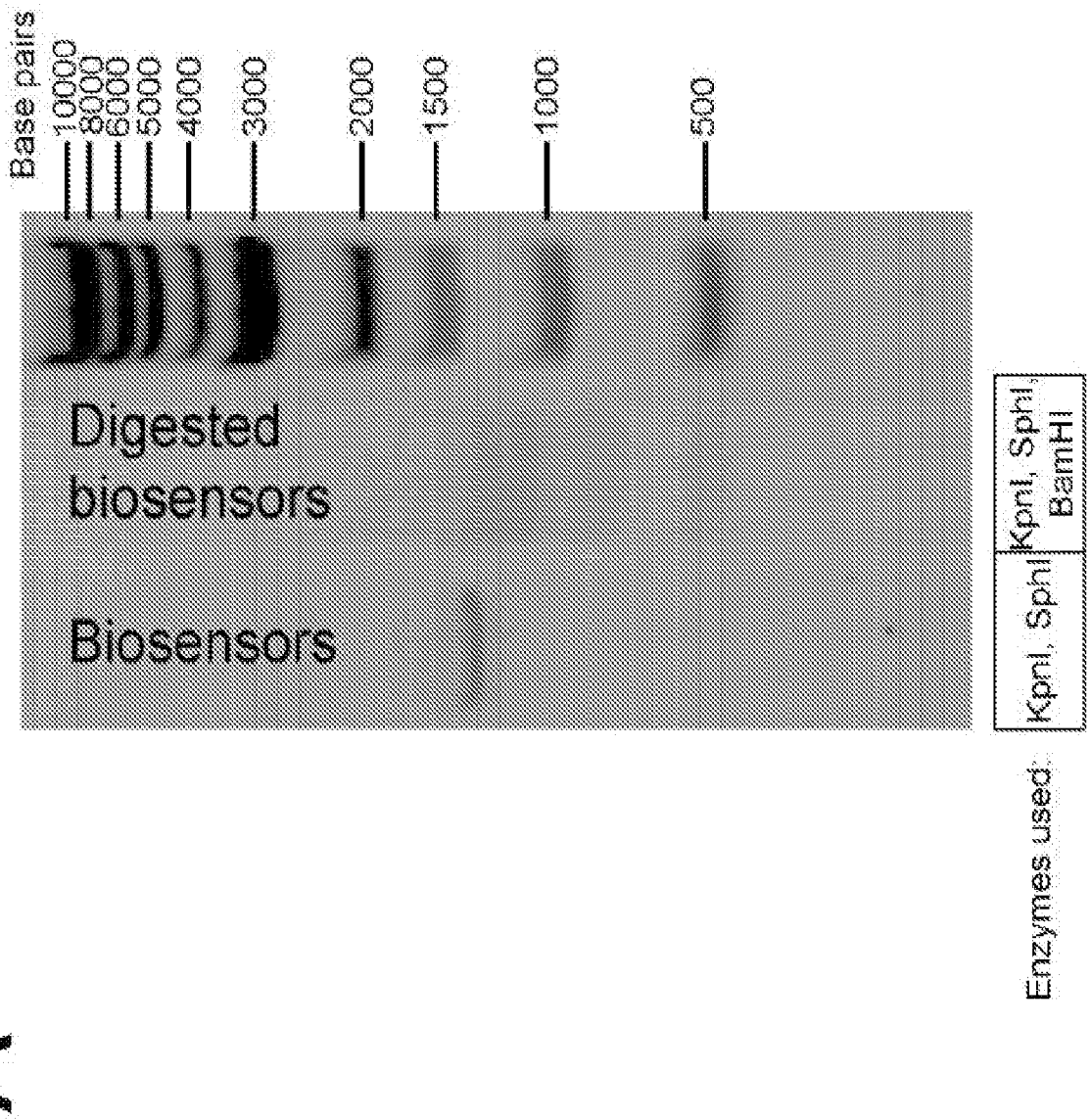

A
Base pairs
10000
8000
6000
5000
4000
3000
2000
1500
1000
500
Digested biosensors
Biosensors
Enzymes used:
KpnI, SphI
KpnI, SphI, BamHI

Figure 16

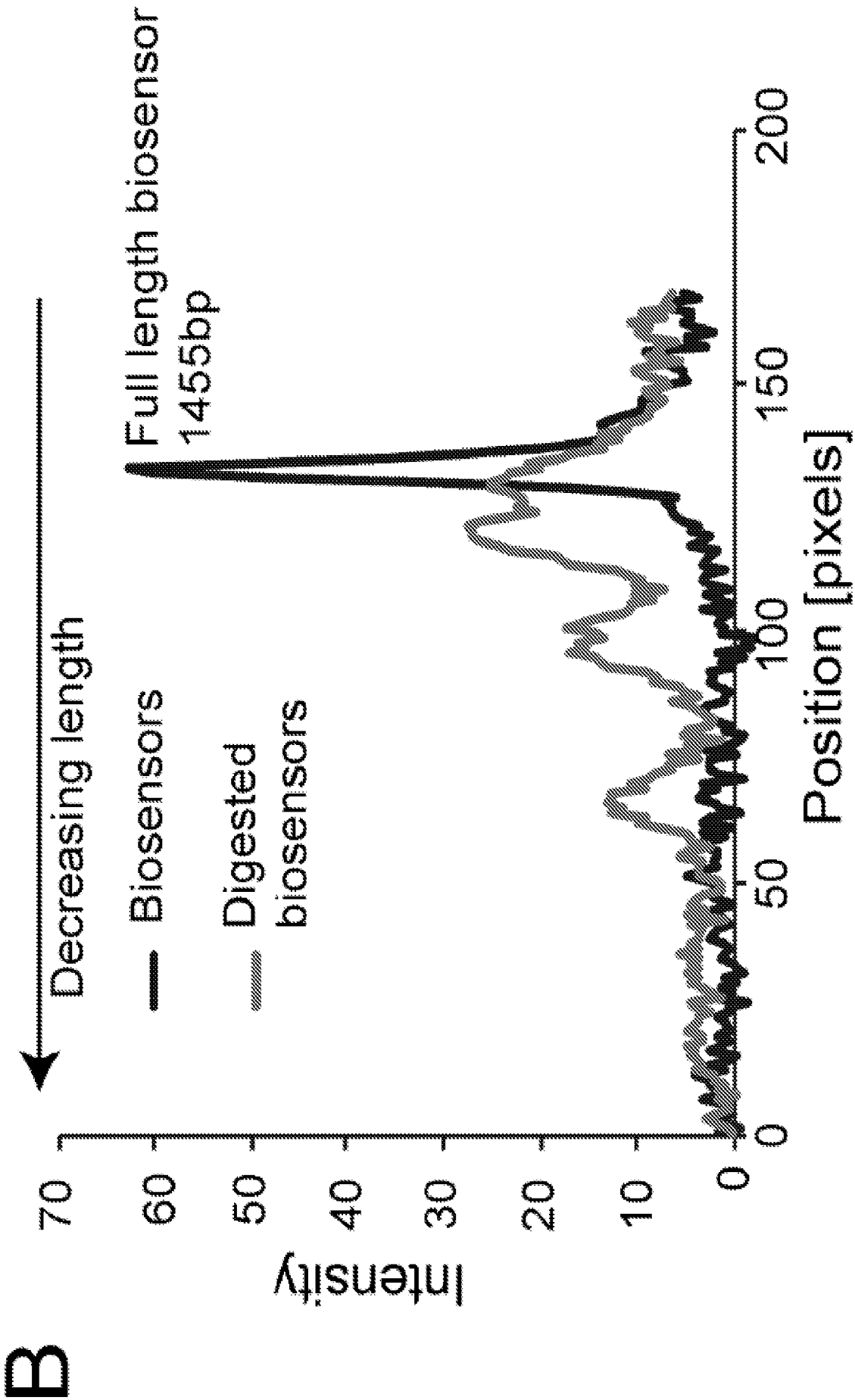


Figure 16 (continued)

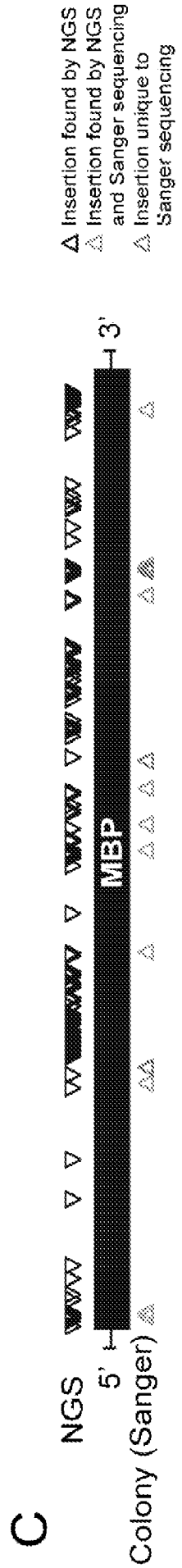


Figure 16 (continued)

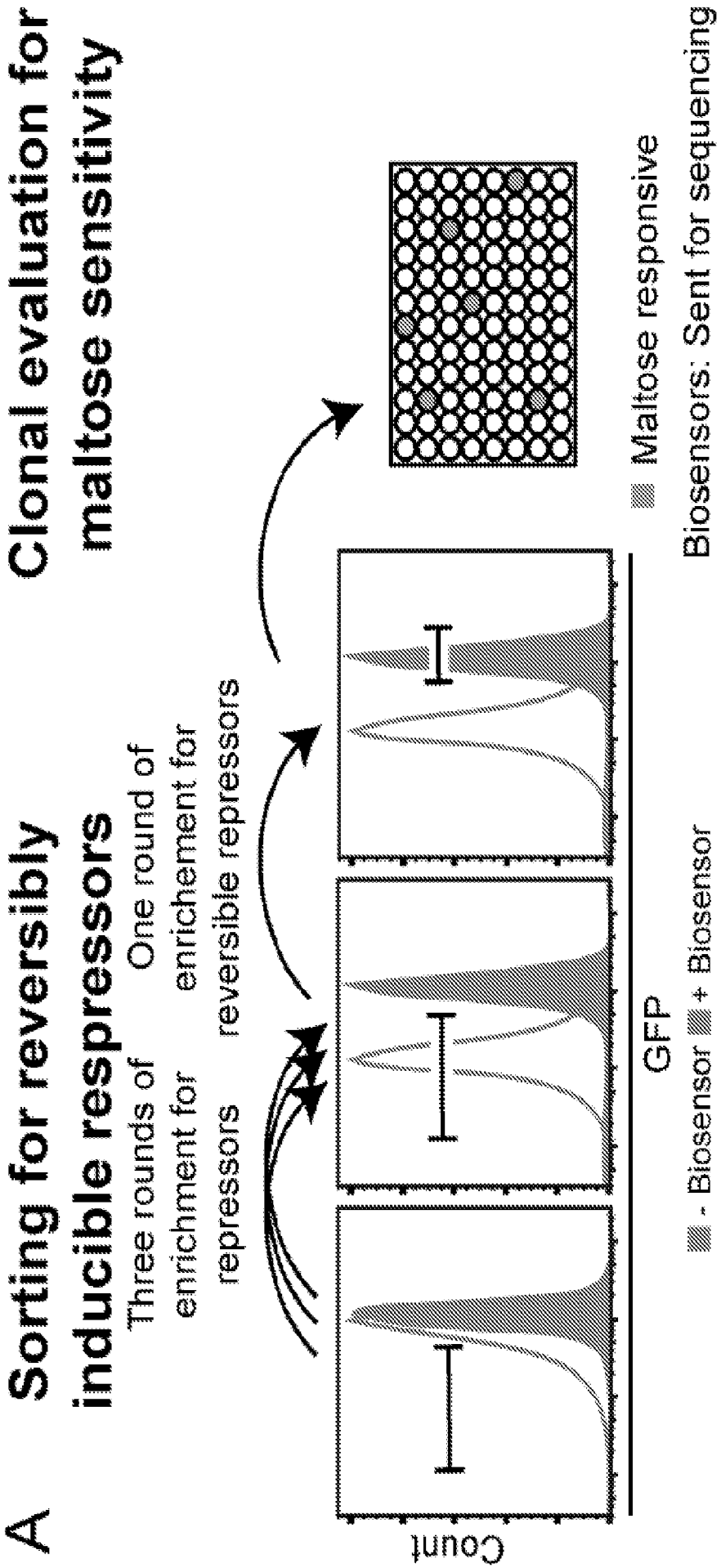

A
Sorting for reversibly inducible respressors
Three rounds of enrichment for repressors
One round of enrichment for reversible repressors
Clonal evaluation for maltose sensitivity
Count
GFP
- Biosensor + Biosensor
Repressors: No more than 1% of the "ON" poulation
Reversible repressors: repressors that express GFP in the absence of IPTG
Maltose responsive
Biosensors: Sent for sequencing

Figure 17

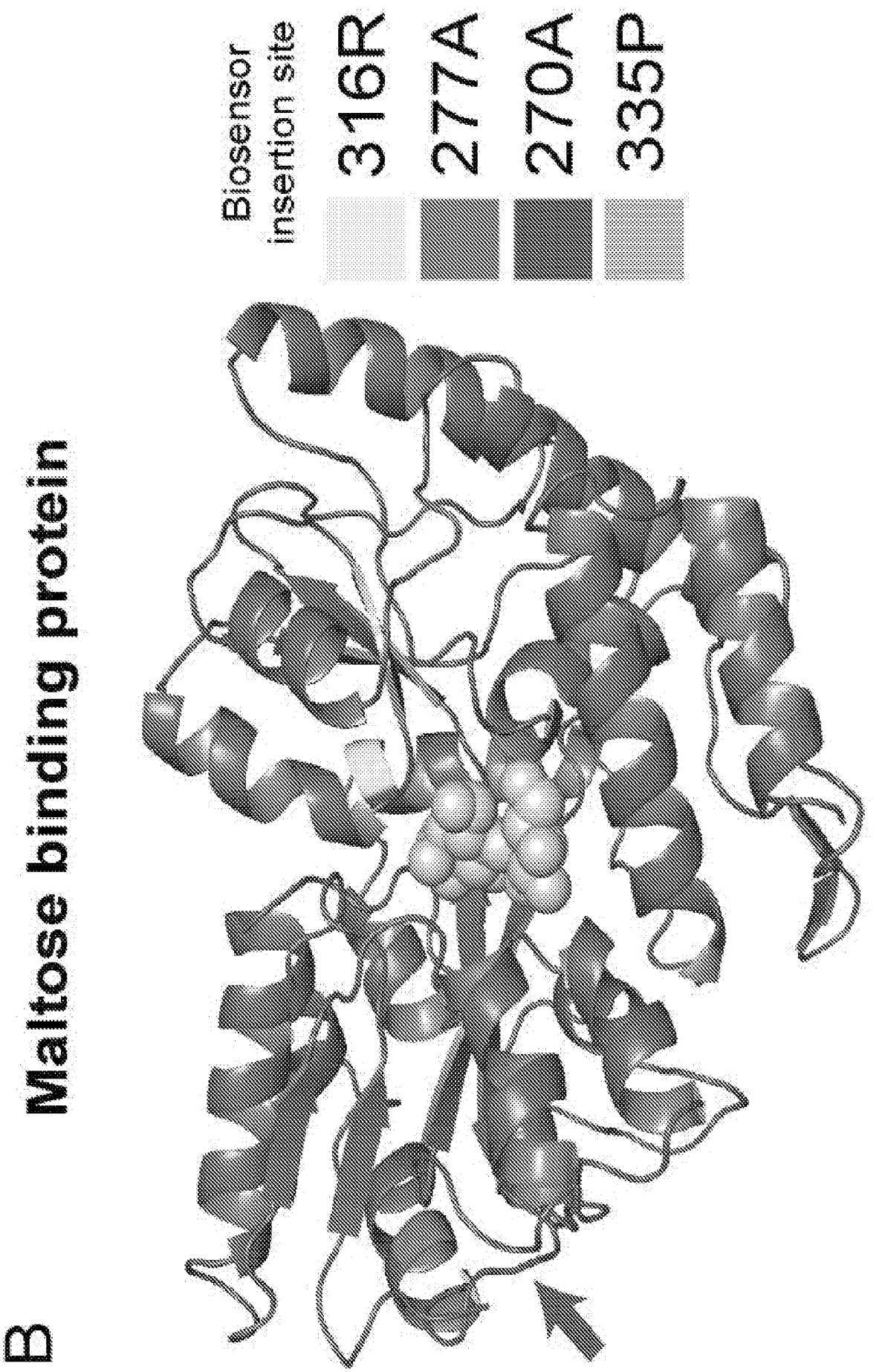


Figure 17 (continued)

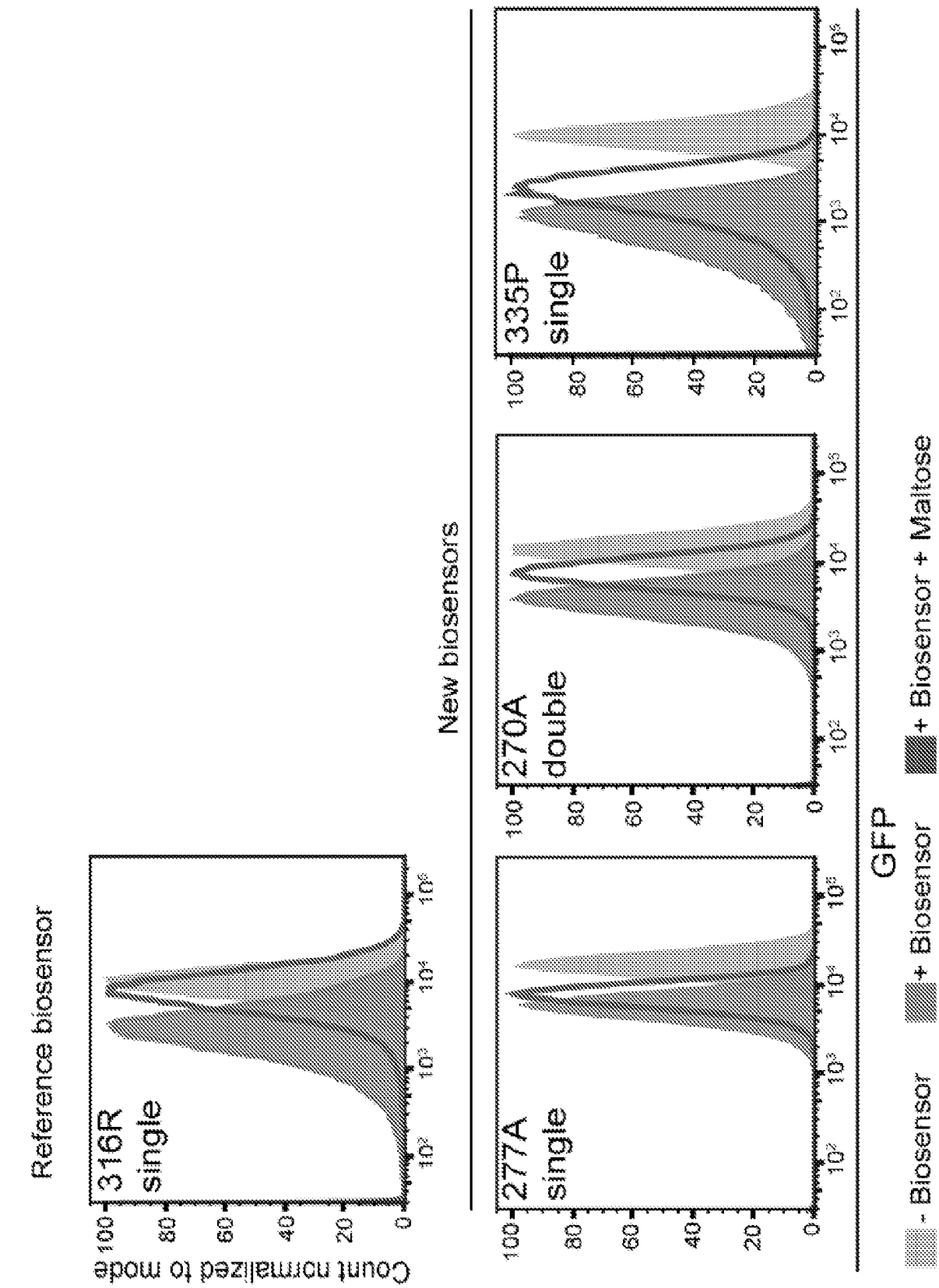


Figure 17 (continued)

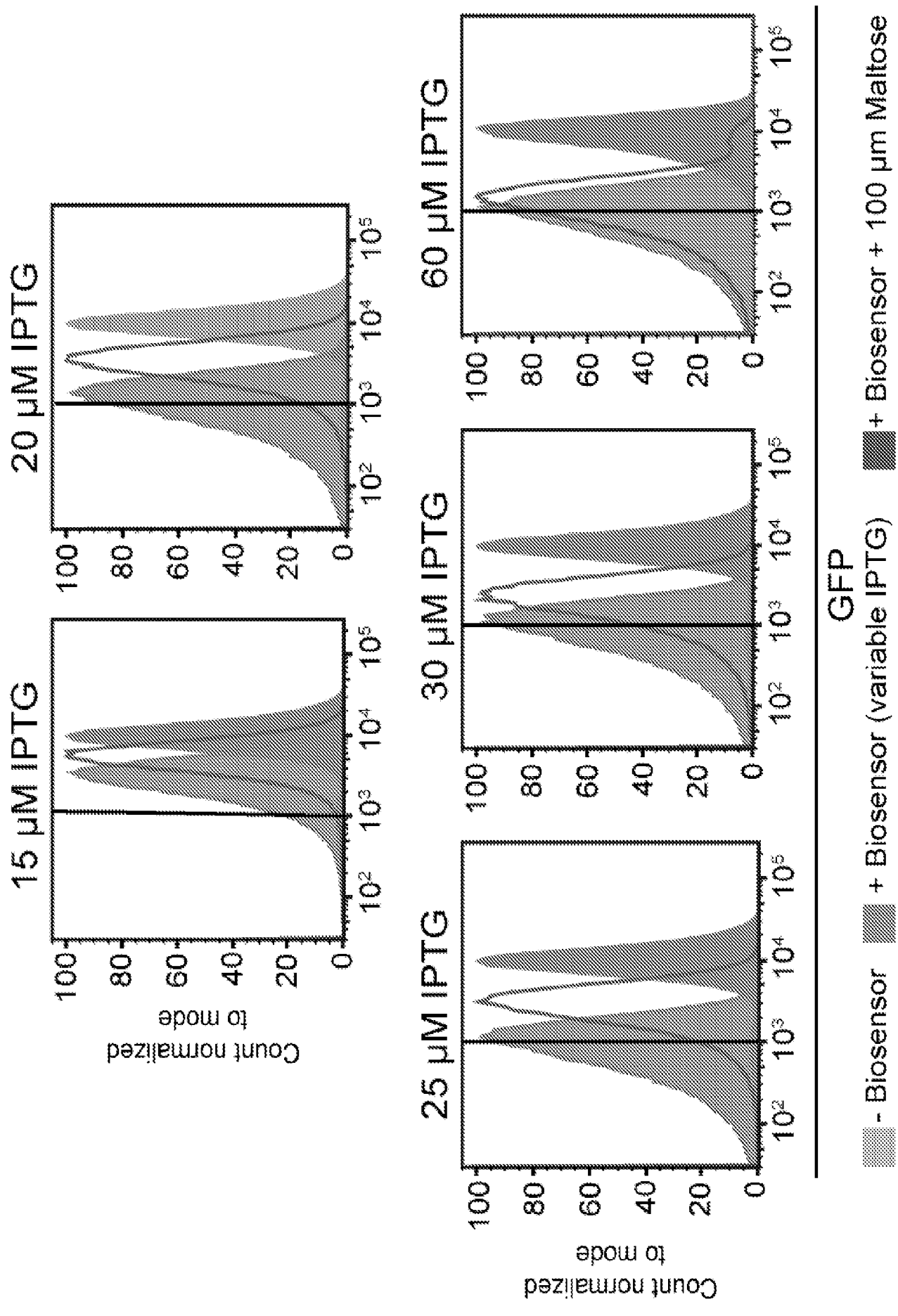

15 µM IPTG
20 µM IPTG
25 µM IPTG
30 µM IPTG
60 µM IPTG
Count normalized to mode
GFP
- Biosensor
+ Biosensor (variable IPTG)
+ Biosensor + 100 µm Maltose

Figure 18

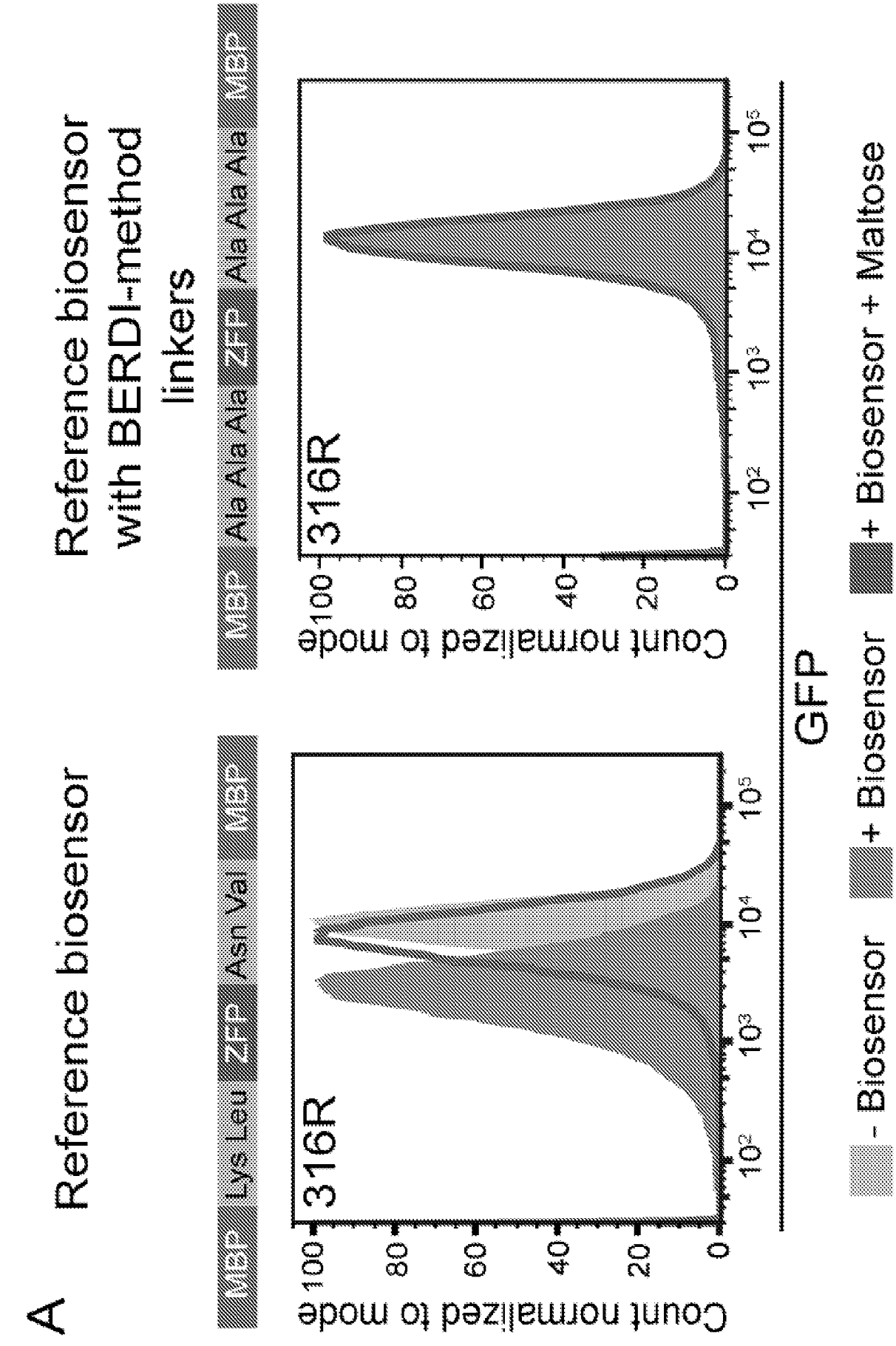

A
Reference biosensor
Reference biosensor with BERDI-method linkers
MBP Lys Leu ZFP Asn Val MBP
MBP Ala Ala Ala ZFP Ala Ala Ala MBP
316R
316R
Count normalized to mode
Count normalized to mode
0
20
40
60
80
100
10^2
10^3
10^4
10^5
GFP
- Biosensor
+ Biosensor
+ Biosensor + Maltose

Figure 19

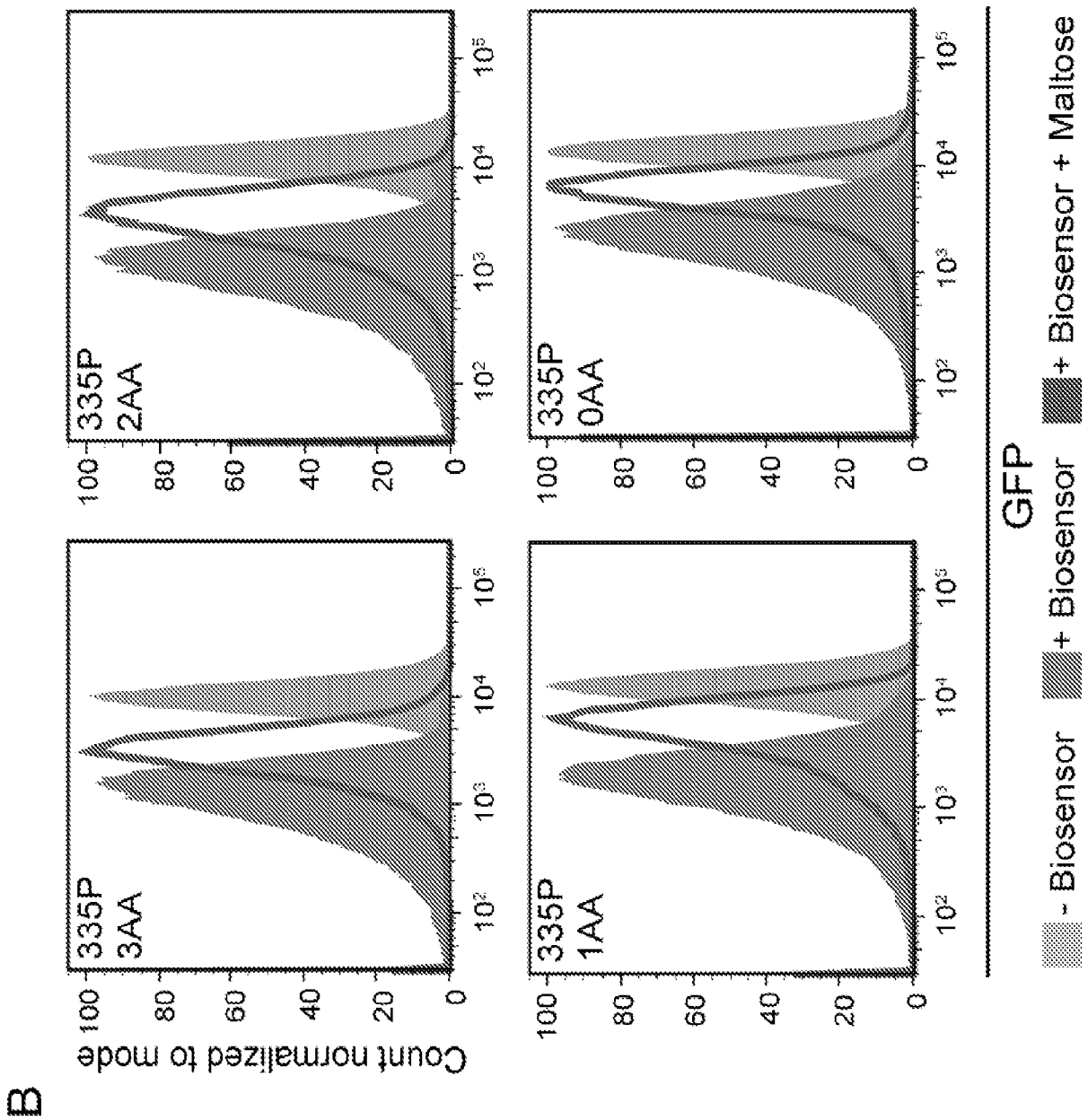


Figure 19 (continued)

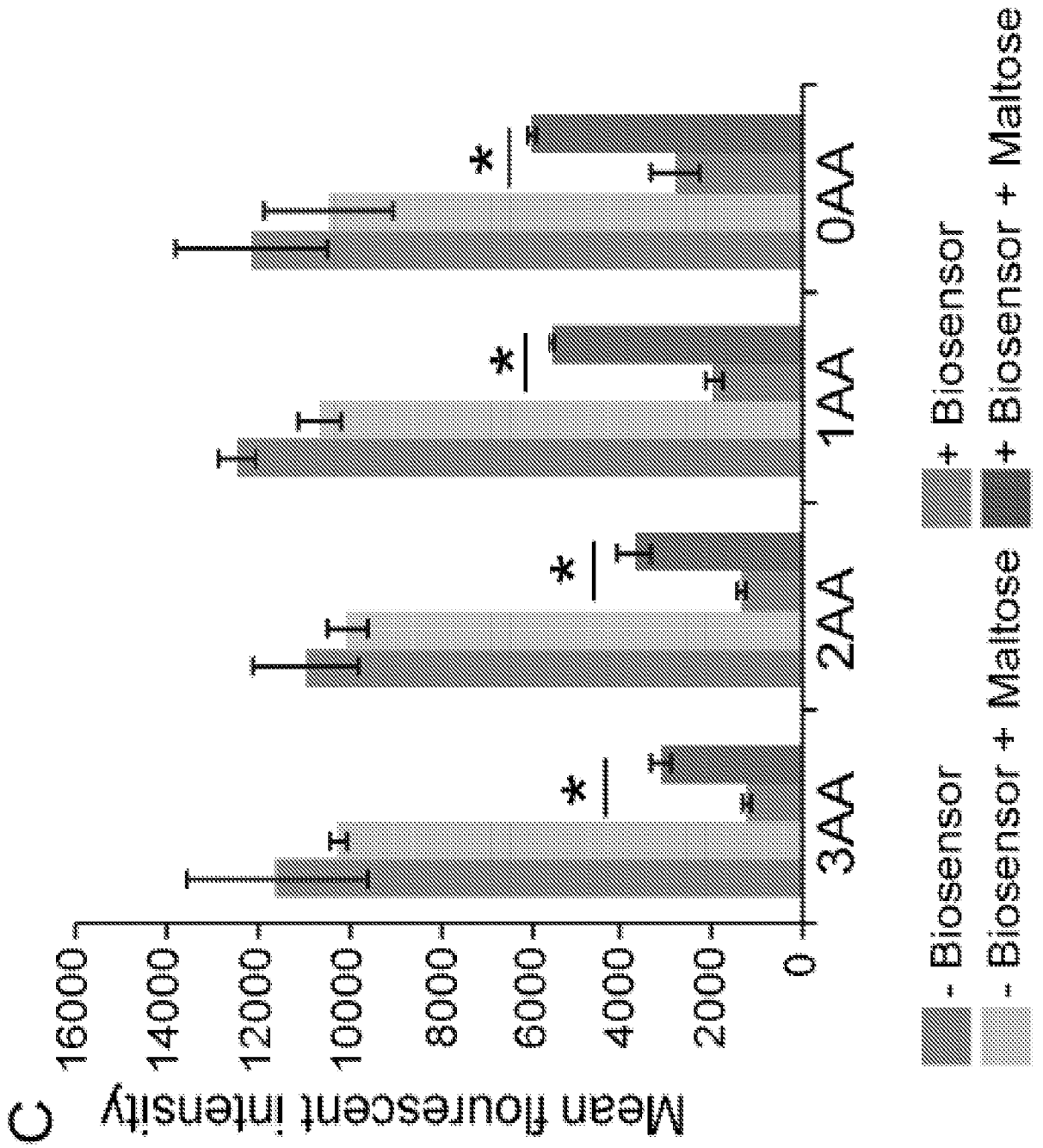


Figure 19 (continued)

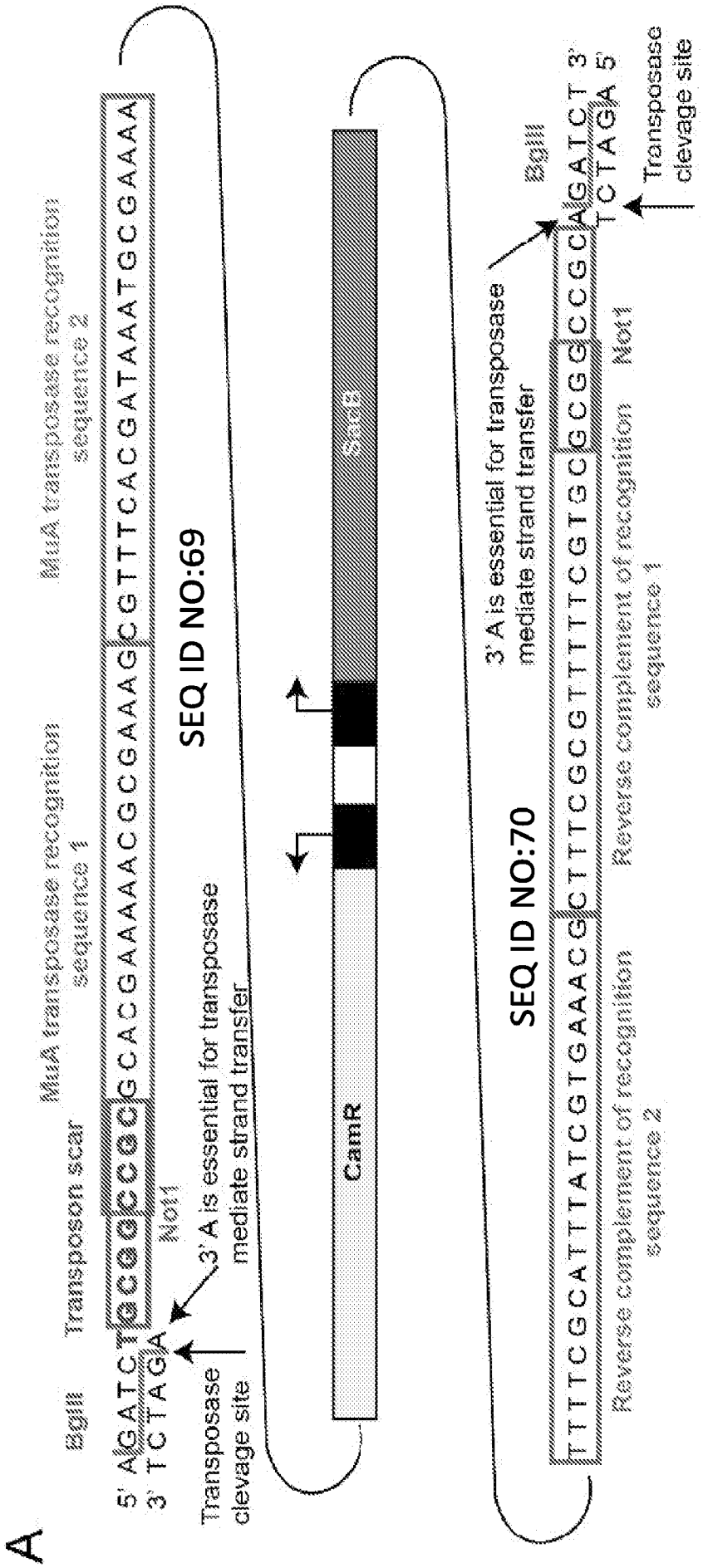

A
BglII
Transposon scar
MuA transposase recognition sequence 1
MuA transposase recognition sequence 2
5' AGATCT
3' TCTAGA
NotI
3' A is essential for transposase mediate strand transfer
Transposase clevage site
SEQ ID NO:69
CamR
SEQ ID NO:70
BglII
Transposase clevage site
Reverse complement of recognition sequence 1
Reverse complement of recognition sequence 2

Figure 20

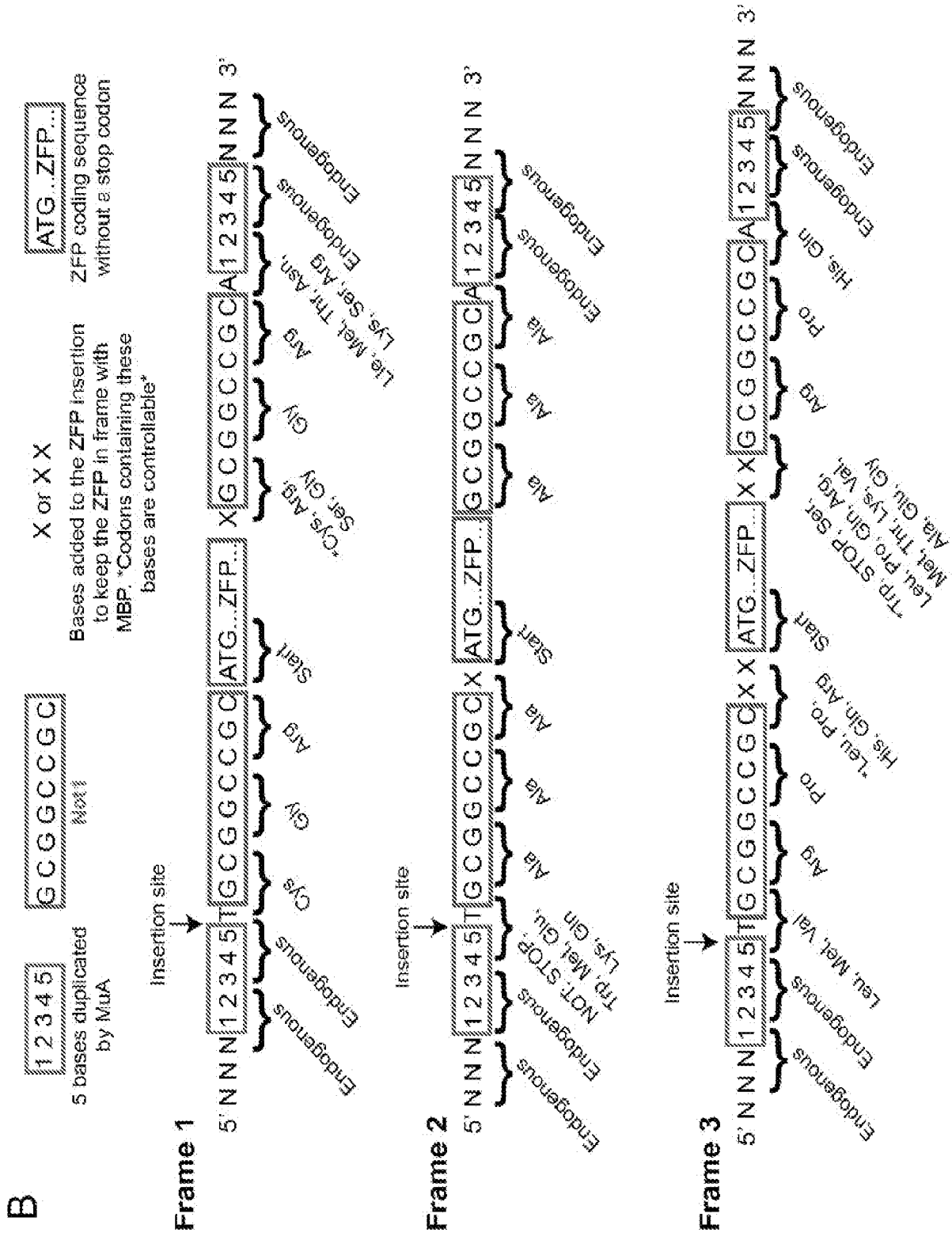


Figure 20 (continued)

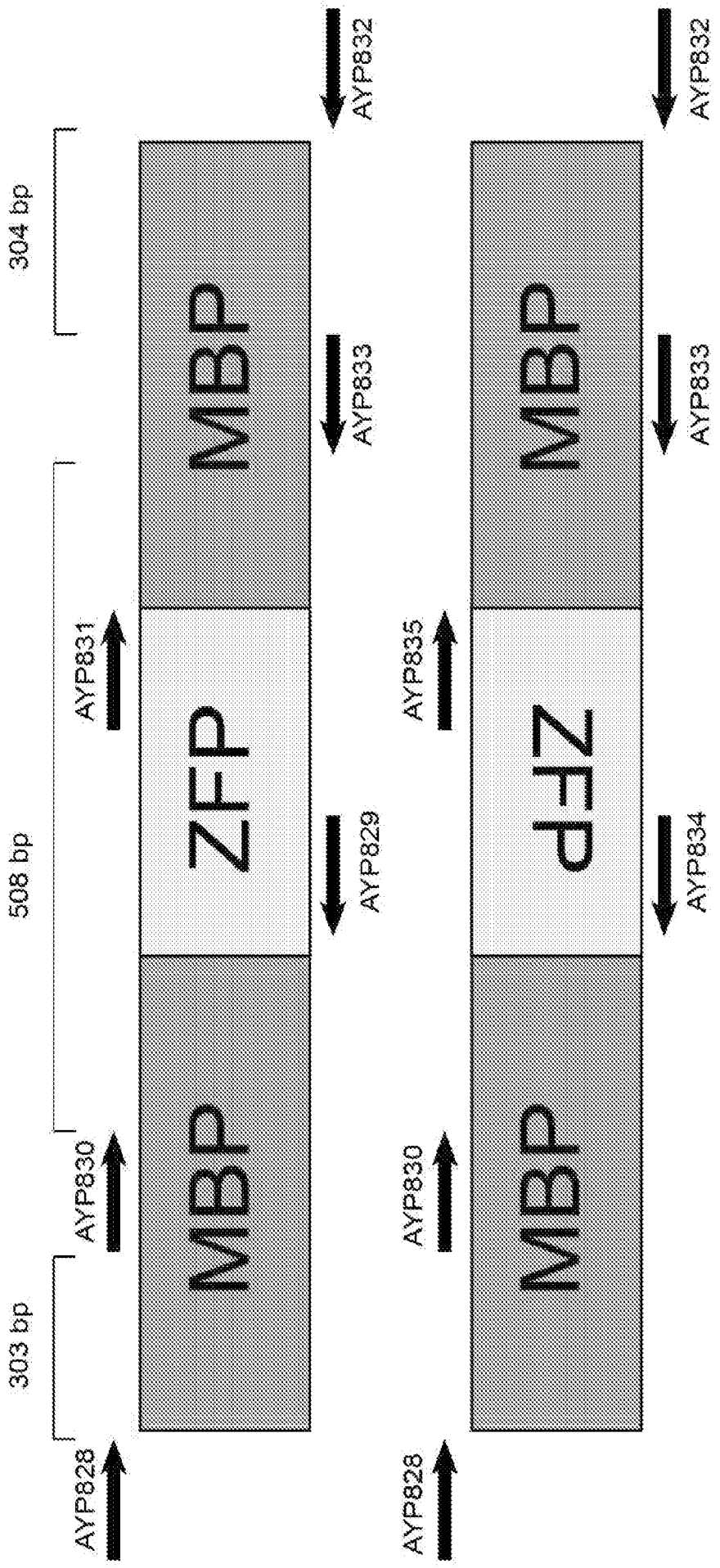

304 bp
508 bp
303 bp
AYP828
AYP830
AYP831
AYP829
AYP832
AYP833
MBP
ZFP
MBP
AYP828
AYP830
AYP835
AYP834
AYP832
AYP833
MBP
ZFP

Figure 21

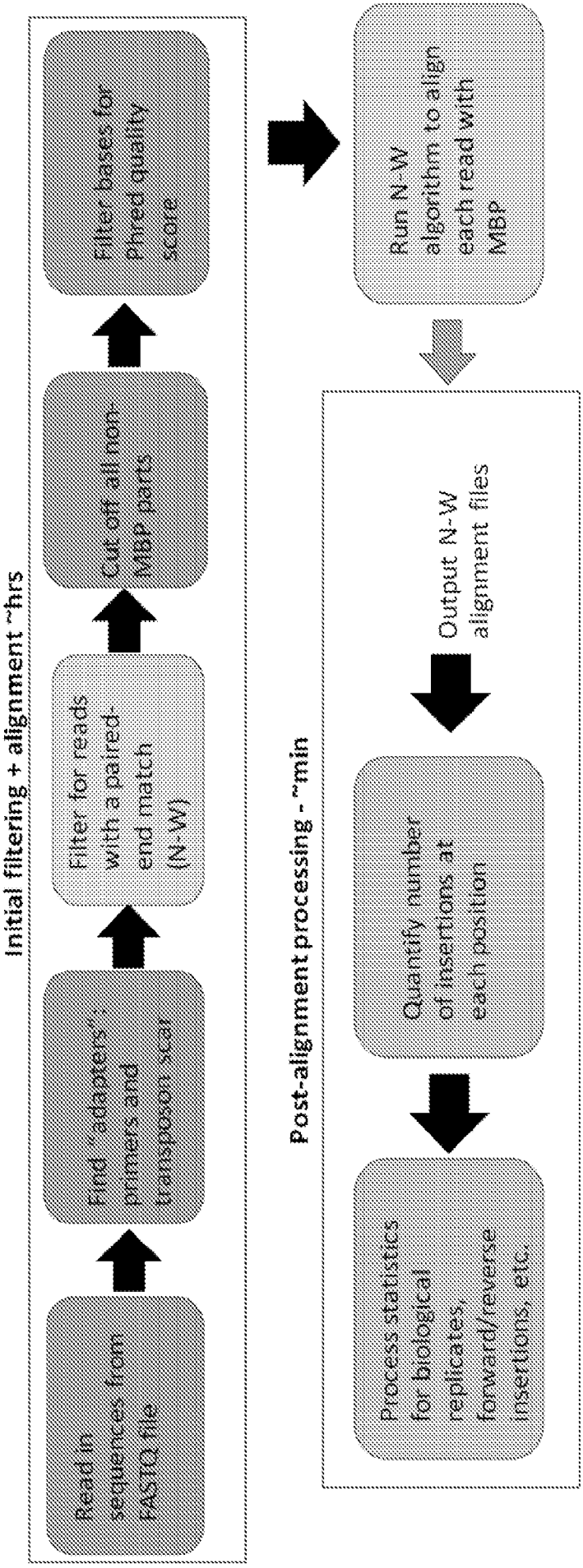

Initial filtering + alignment ~hrs
Read in sequences from FASTQ file
Find "adapters", primers and transposon scar
Filter for reads with a paired-end match (N-W)
Cut off all non-MBP parts
Filter bases for Phred quality score
Run N-W algorithm to align each read with MBP
Post-alignment processing - ~min
Output N-W alignment files
Quantify number of insertions at each position
Process statistics for biological replicates, forward/reverse insertions, etc.

Figure 22

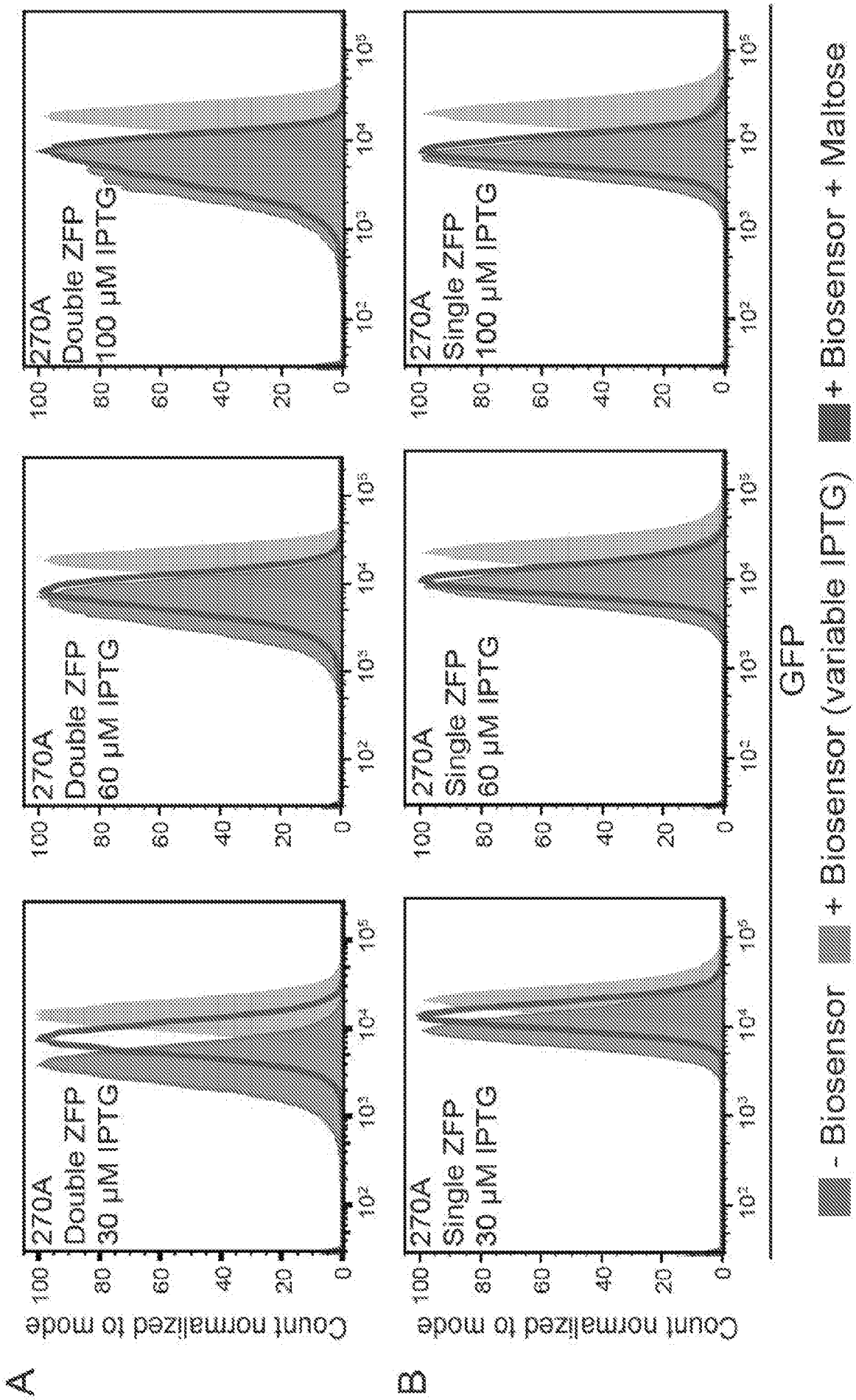

A
B
Count normalized to mode
270A
Double ZFP
30 µM IPTG
60 µM IPTG
100 µM IPTG
Single ZFP
GFP
- Biosensor
+ Biosensor (variable IPTG)
+ Biosensor + Maltose

Figure 23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2017/056933

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/6897 *(2018.01)*
G01N 33/53 *(2006.01)*
G01N 33/566 *(2006.01)*
C07K 14/00 *(2006.01)*

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/6897, 1/68, G01N 33/53, 33/566, C07K 14/00

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

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

E-Library, Espacenet, PatSearch, PATENTSCOPE, RUPTO, NCBI, EMBL-EBI, Google Scholar, PubMed, USPTO, ScienceDirect

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016/ 0202256 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE et al.) 14.07.2016, abstract, par.[0009], [0064], claims	1-23
A	NADLER D. C. et al. « Rapid construction of metabolite biosensors using domain-insertion profiling». NATURE COMMUNICATIONS, 29.07.2016, 7:12266, DOI: 10.1038/ncomms12266, pp.1-11	1-23
A	FENG J. et al. «A general strategy to construct small molecule biosensors in eukaryotes», eLife. 2015; 4:e10606. DOI: 10.7554/eLife.10606, p.1-23	1-23
A	JEONG J. et al. «Monitoring of conformational change in maltose binding protein using split green fluorescent protein». Biochemical and Biophysical Research Communications, 2006, Vol.339, pp.647–651	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 February 2018 (01.02.2018)	14 February 2018 (14.02.2018)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer S.Il'chenko Telephone No. 495 531 65 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2017/056933

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WEHR M. C. et al. «Split protein biosensor assays in molecular pharmacological studies». Drug Discovery Today, March 2016, Vol.21, no.3, p.415-429	1-23