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(54) Title: *BACILLUS* EXPRESSION SYSTEM

(57) Abstract: An inducible promoter expression system based on the T7 RNA Polymerase (T7 RNAP), lactose repressor (Lad), and a chimeric T7lac promoter (PT7lac), which can be integrated as a single copy into the *B. subtilis* genome. In the absence of IPTG, Lad strongly represses T7RNAP and PT7lac, and expression of an exemplary ORF — here superfolder green fluorescent protein (sfGFP) reporter protein — is undetectable by flow cytometry. Addition of IPTG de-represses PT7lac, and simultaneously induces expression of T7RNAP, resulting in very high sfGFP levels.

BACILLUS EXPRESSION SYSTEM

PRIOR RELATED APPLICATIONS

[0001] This application claims priority to US Serial No. 62/776,132, filed December 6, 2018, and incorporated by reference in its entirety for all purposes.

FEDERALLY SPONSORED RESEARCH STATEMENT

[0002] This invention was made with government support under Grant Nos: R21AI115014 and MCB1616755, awarded by the NIH and NSF, respectively. The government has certain rights in the invention.

FIELD OF THE DISCLOSURE

[0003] This invention provides materials and methods allowing expression of RNA and proteins in *Bacillus*, especially *B. subtilis* and similar species, with a new tightly regulated, integrative and inducible expression system.

BACKGROUND OF THE DISCLOSURE

[0004] *Bacillus subtilis* is a model organism for studies of Gram-positive bacterial biology and systems biology of cellular differentiation, stress responses, and multicellular organization. Additionally, *B. subtilis* is among the most widely used hosts for protein production in the biotechnology industry due to its ability to secrete proteins into the cell medium, its non-pathogenic GRAS (generally recognized as safe) designation, and its high genetic tractability. For example, *B. subtilis* is used for large-scale production of lipases, proteases, and amylases, among other industrially-relevant proteins.

[0005] However, *B. subtilis* work has been limited by a lack of high-quality synthetic biological parts. For example, biotechnology applications require inducible promoters capable of switching between a low production state for early-stage culturing and a high production state that maximizes protein yield during fermentation. Such parts are particularly important when the recombinant protein or metabolic pathway of interest are toxic to the host cells when overproduced.

[0006] Typical inducible promoters in *B. subtilis* have dynamic ranges (ratio of output protein expression in the presence versus absence of inducer) of at most a few hundred. While a *B. subtilis* bacitracin-inducible promoter with a 1,000-fold range has been reported (13), it requires antibiotic selection to maintain a multicopy plasmid, and its activity is transient and shuts down less than two hours after induction, likely due to an endogenous bacitracin stress response. In contrast, for the Gram-negative bacterium *Escherichia coli*, inducible promoters have been engineered with dynamic ranges greater than 1,000 or even 10,000.

[0007] Thus, improved *B. subtilis* inducible promoter systems are greatly needed in the art. The ideal system will be very tightly regulated, result in high levels of protein expression when induced, have 10,000 or even greater fold dynamic range, and ideally be integratable into the genome.

SUMMARY OF THE DISCLOSURE

[0008] Protein expression systems that are activated in response to chemicals such as IPTG, xylose or bacitracin currently exist for *B. subtilis*. However, their fold-activation values are fairly low, and their active output levels are limited by the *B. subtilis* endogenous transcription machinery. On the other hand, a few systems based on the exogenous viral T7 RNA Polymerase (T7 RNAP) have been previously built. While these achieve high expression levels, protein production in the inactive state or "leakiness" is fairly high and therefore fold-activation is still low.

[0009] Here, we have engineered an extraordinarily stringent and strongly inducible protein expression system for *Bacillus*. This system combines the viral T7 RNAP, which produces high amounts of recombinant protein in the active state, with the LacI bacterial transcription factor that inhibits production of both the protein and the viral polymerase, resulting in nearly undetectable output in the inactive state. Our system achieves greater than 10,000-fold or even 20,000-fold activation in response to a chemical inducer. We expect this system to improve yield of existing protein production, especially toxic proteins, or metabolic engineering applications using *B. subtilis* and possibly other *Bacillus* species, and enable synthesis of new proteins and chemicals that were previously unfeasible.

[0010] Our LacI-T7 inducible system comprises the following elements:

- Transcriptional repressor LacI, expressed constitutively.
- The T7 phage RNA polymerase (T7 RNAP), expressed from the *B. subtilis* promoter $P_{hy\text{-}spank}$, which is normally repressed by LacI.
- The hybrid P_{T7lac} promoter, normally repressed by LacI as well, under whose control the recombinant protein of interest or ORF is placed.

[0011] For an integrative sequence, these elements should be flanked by first and second portions of a non-essential portion of the *Bacillus* genome, thereby allowing integration into that genome. The order of parts is typically not critical, but whatever the order, the cassette is flanked by the integrating sequences such that everything there between is integrated into the genome. In addition, sequences to optimize expression of the target ORF can also be included, such as ribosome binding sites, enhancers, terminators, and the like.

[0012] The cassette can be assembled and used as is, or can be formulated as an expression vector—capable of self-reproduction and ORF expression. For an expression vector, additional vector sequences are added, allowing replication in *Bacillus* and/or *E.coli* (e.g., ori), marker genes for selection (e.g., antibiotic resistance), unique restriction endonuclease (RE) sites or the multi-cloning site, shuttle sequences allowing the vector to shuttle e.g., between *E. coli* and *Bacillus* (both ori sequences), or between yeast and *Bacillus* (*Bacillus* ori and autonomously replicating sequence (ARS), a yeast centromere (CEN), and a yeast selectable marker), viral packaging sequences, protein degradation tags, and the like. It may also be possible to formulate the cassette described herein as minivectors, such as those described by Twister® (Houston TX).

[0013] LacI and the $P_{hy\text{-}spank}$ promoter were obtained from pDR111 (ATCC® 53123), a commercially available plasmid for genomic integration in *B. subtilis*. The P_{T7lac} promoter sequence is identical to that included in some of the commercially-available *E. coli* pET plasmids, and was synthesized via oligo annealing and extension. The sequence of the T7 RNAP is identical to that found in the *E. coli* BL21(DE3) strain, and was obtained from an *E. coli* plasmid.

[0014] DNA coding for these elements was assembled, together with a spectinomycin resistance cassette. Flanking sequences for genome integration were added that were homologous to the *B. subtilis amyE* genomic locus, but any non-essential loci could be used. The assembled DNA cassette was then integrated into the *Bacillus* genome via standard *Bacillus* transformation methods.

[0015] In the inactive state, LacI repression of **both** T7 RNAP production and P_{T7lac} ensures very low production of recombinant protein. Upon addition of chemical inducer IPTG to the cell media, LacI-repression is relieved and the T7 RNAP is produced. The T7 RNAP can now transcribe the recombinant gene from the now unrepressed P_{T7lac} promoter.

[0016] To assess the performance of the LacI-T7 system, we placed superfolder GFP (sfGFP) under P_{T7lac} and measured cell fluorescence in the presence or absence of IPTG. This resulted in high levels of fluorescence in the presence of IPTG, but undetectable levels in its absence. Based on estimates of the limit of detection of our flow cytometer instrument, we placed a lower bound on the fold-activation of this system at 20,000. We have also tested the system with other report

[0017] The invention includes any one or more of the following in any combination(s) thereof.:

An inducible and integrative genetic system for *Bacillus subtilis*, said system comprising the following components: a) a LacI repressor constitutively expressed under a first constitutive promoter from *B. subtilis*; b) a T7 phage RNA polymerase (T7 RNAP) expressed from a second *B. subtilis* promoter that is normally repressed by LacI; c) a hybrid output promoter activated by T7RNAP and repressed by LacI; d) part a) being 5' flanked by a first portion of a chromosomal gene from *B. subtilis*, and part c) being 3' flanked by a second portion of said chromosomal gene from *B. subtilis,* so as to allow integration into a genome of *B. subtilis*.
A system as herein described, having at least one open-reading frame under the control of the output promoter.
A system as herein described, having at least 10,000 fold or 20,000 activation (expression of said ORF before and after induction) when induced.
The system can also include other sequences for convenience of use, such as E. coli ori, antibiotic resistance cassette, multiple cloning sites, and the like, and thus be able to shuttle from species to species and/or function as a vector for amplification and cloning purposes. Herein, however, we assembled the components *in vitro* and then used the assembled material for direct integration.
A system as herein described, wherein the LacI was obtained from or is equivalent to the sequences in pDR111.
A system as herein described, wherein said first promoter is a P_{penP} promoter.
A system as herein described, wherein said second promoter is a $P_{hy\text{-}spank}$ promoter.
A system as herein described, wherein the $P_{hy\text{-}spank}$ promoter was obtained from or is

equivalent to the sequences in pDR111.
A system as herein described, wherein the P_{T7lac} promoter sequence was obtained from or is equivalent to *E. coli* pET plasmids.
A system as herein described, wherein the T7 RNAP was obtained from or is equivalent to the *E. coli* BL21(DE3).
A system as herein described, further comprising an antibiotic resistance cassette, e.g., a spectinomycin resistance cassette or others.
A system as herein described, wherein said chromosomal gene from *B. subtilis* is an amyE gene.
An expression system for *Bacillus subtilis*, said system having a structure of FIG. 1A.
An expression system for *Bacillus subtilis*, said system having a sequence of SEQ ID NO 7, or at least 95% identity to same
An inducible and integrative expression system for *Bacillus subtilis*, said system comprising the following components: a) a LacI repressor constitutively expressed under a *B. subtilis* P_{penP} promoter; b) a lacI-repressible T7 phage RNA polymerase (T7 RNAP) expressed from a *B. subtilis* $P_{hy\text{-}spank}$ promoter; c) a hybrid P_{T7lac} promoter regulated by said T7RNAP; d) a multiple cloning site for insertion of an ORF operably linked to said P_{T7lac} promoter; e) part a) being 5' flanked by a first portion of a chromosomal gene from *B. subtilis*, and part d) being 3' flanked by a second portion of said chromosomal gene from *B. subtilis,* so as to allow integration into a genome of *B. subtilis*.
A recombinant *Bacillus,* wherein said *Bacillus* is transformed with a system as herein described, said system having an ORF for a target protein inserted after P_{T7lac}, said *Bacillus* able to express said target protein when induced with IPTG.
A recombinant *Bacillus,* wherein said *Bacillus* is transformed with the system herein described, said system having an ORF for a target protein inserted after P_{T7lac}, said ORF being integrated into a genome of said *Bacillus*, and said *Bacillus* able to express said target protein when induced with IPTG.
A method of transforming *Bacillus subtilis,* comprising introducing the system as herein described to a population of *Bacillus subtilis* under conditions that allow transformation and integration of said system into the genome of said *Bacillus subtilis*, and selecting for transformed and integrated *Bacillus subtilis*.
A method of producing a target protein, comprising introducing an ORF encoding a target protein after P_{T7lac} of the system as herein described, introducing said system and ORF to a population of *Bacillus subtilis* under conditions that allow integration of said ORF into a genome of said *Bacillus subtilis*, and selecting for integrated *Bacillus subtilis,* growing said integrated *Bacillus subtilis* in a growth medium until cells reach near saturation, adding IPTG to said growth medium in an amount sufficient to induce the lac operon, continue culturing said integrated *Bacillus subtilis* until said ORF is expressed, and isolating said target protein from said growth medium or said transformed *Bacillus subtilis* or both.
A *Bacillus* promoter system, comprising an DNA molecule comprising: a) a LacI repressor constitutively expressed under a first constitutive promoter; b) a T7 phage RNA polymerase (T7 RNAP) expressed from a second promoter that is normally repressed by LacI; and c) a third promoter sequence regulated by a T7RNAP that is normally repressed by LacI.
Any promoter system herein, further comprising an open reading frame ("ORF") for a gene of interest inserted after said third promoter sequence, such that said ORF is regulated by said third promoter sequence.
Any promoter system herein, having at least 5,000, 10,000, 15,000 or 20,000 fold activation of expression of said ORF when induced by IPTG.
Any promoter system herein, comprising one or more of the following: a) the LacI is from or is equivalent to the sequences in pDR111 or having at least 95% nucleotide identity to SEQ ID NO 11; or b) the first constitutive promoter is a PpenP promoter or a sequence having at least 95% nucleotide identity to SEQ ID NO 15; or c) the second promoter is a Phy-spank promoter or a sequence having at least 95% nucleotide identity to SEQ ID NO 13; or d) the Phy-spank promoter is from or is equivalent to the sequences in pDR111; or

e) the hybrid PT7lac promoter sequence is from or is equivalent to E. coli pET plasmids; or f) the T7 RNAP is from or is equivalent to the E. coli BL21(DE3); or g) the T7RNAP is a sequence having at least 95% nucleotide identity to SEQ ID NO 12 h) the third promoter is a hybrid PT7lac promoter sequence or a sequence having at least 95% nucleotide identity to SEQ ID NO 14; or i) the first constitutive promoter for the LacI repressor is a promoter from *Bacillus*; or j) the T7RNAP is expressed from a *Bacillus* LacI repressed promoter.
An expression system for *Bacillus*, said system being in a *Bacillus* and having: a) at least 95% nucleotide identity to the sequence of SEQ ID NO 7, said 95% identity excluding an open reading frame (ORF) added thereto; and b) having at least 5,000, 10,000, 15,000 or 20,000 fold more expression of said ORF with IPTG induction than without IPTG induction.
An inducible and integrative expression system for *Bacillus subtilis*, said system comprising an expression cassette with the following structure: a) a first portion of a chromosomal sequence from B. subtilis at a 5' end of said expression cassette; b) a second portion of said chromosomal sequence at a 3' end of said expression cassette; c) a LacI repressor constitutively expressed under a B. subtilis PpenP promoter within said expression cassette; d) a lacI-repressible T7 phage RNA polymerase (T7 RNAP) expressed from a B. subtilis Phy-spank promoter within said expression cassette; e) a hybrid promoter activated by T7RNAP and repressed by LacI that controls expression of an ORF.
Any system herein, further comprising a cassette comprising an antibiotic resistance gene or a spectinomycin resistance gene.
Any system herein, further comprising a cassette comprising a 5' chromosomal sequence from the *Bacillus* amyE gene and a 3' chromosomal sequence from the *Bacillus* amyE gene to allow homologous recombination into a genome of *Bacillus*.
Any promoter system herein, further comprising one or more *B. subtilis*-specific ribosome-binding sites (RBSs) placed to stimulate translation from mRNAs expressed from the first, second or third promoters and/or comprising one or more *B. subtilis*-specific ribosome-binding sites (RBSs) placed to stimulate translation from mRNAs expressed from PT7lac, Phy-spank, or PpenP promoters, or any combination thereof
Any system herein, said cassette further comprising a self-cleaving ribozyme placed after PT7lac to improve output production.
Any system herein, further comprising a multiple cloning site after said third promoter (for insertion of the ORF).
Any system herein, wherein said *Bacillus* is a *Bacillus* subtilis.
Any system herein, wherein the promoter system is an inducible promoter system or is an inducible by IPTG.
Any system herein, wherein said ORF encodes an output mRNA, that may or may not encode a protein.
Any system herein, comprising an ORF for a target protein and being capable of a 10,000 fold increase in expression of said ORF when induced with IPTG as compared to without IPTG.
A vector comprising any system herein, said vector being self-reproduceable in a *Bacillus*, Escherichia or yeast.
Any vector herein producing at least 10,000 fold more expression of said ORF in a *Bacillus* transformed with said vector and induced with IPTG induction, as compared to without IPTG.
A recombinant *Bacillus*, wherein said *Bacillus* is transformed with any system herein, said system being integrated or not.
A method of transforming *Bacillus subtilis*, comprising introducing any system herein to a population of Bacillus subtilis under conditions that allow transformation and integration of said system into a chromosome of said *Bacillus subtilis*, and selecting for transformed and integrated *Bacillus subtilis*.
A method of producing a target protein, comprising introducing any system herein to a

population of *Bacillus subtilis* under conditions that allow integration of said expression cassette into a genome of said *Bacillus subtilis*, and selecting for integrated *Bacillus subtilis*, growing said integrated *Bacillus subtilis* in a growth medium until cells reach near saturation, adding IPTG to said growth medium in an amount sufficient to induce said lac operon, continuing culturing said integrated *Bacillus subtilis* until said ORF is expressed, and isolating said target protein from said growth medium or said transformed *Bacillus subtilis* or both.

[0018] As used herein "recombinant" or "engineered" is relating to, derived from, or containing genetically engineered material. In other words, the genome was intentionally manipulated by humans in some way.

[0019] "Expression vectors" are used in accordance with the art-accepted definition of a plasmid, virus, cosmid, or other propagatable sequence designed for protein expression in cells. There are thousands of such vectors commercially available, and typically each has an origin of replication (ori); a multiple cloning site; a selectable marker; ribosome binding sites; a promoter and often enhancers; and the needed termination sequences. Most expression vectors are inducible, although constitutive expression vectors also exist and either can be used.

[0020] As used herein, "inducible" means that gene expression can be controlled by the hand-of-man, by adding e.g., a ligand to induce expression from an inducible promoter. Exemplary inducible promoters include the lac promoter, inducible by isopropylthio-β-D-galactopyranoside (IPTG), the yeast AOX1 promoter inducible with methanol, the strong LAC4 promoter inducible with lactate, and the like. Low level of constitutive protein synthesis may occur even in expression vectors with tightly controlled promoters. "Constitutive" means there is always expression from that promoter. P_{veg} is an example of same.

[0021] As used herein, "integrative" or "integratable" means the nucleic acid has the ability to integrate into a *B. subtilis* or equivalent genome.

[0022] As used herein, an "integrated sequence" means the sequence has been integrated into the host genome, as opposed to being maintained on an expression vector or as a separate integration cassette. It will still be expressible, either inducibly or constitutively. Herein, we are more interested in tightly regulated and highly

activatable inducible expression vectors and/or cassettes that are integrative or are integrated.

[0023] As used herein, "operatively connected" or "operatively coupled" with respect to DNA sequences means that the oligonucleotide segments are connected in such a way as to allow the DNA (or RNA or protein derived therefrom) to be functional in a cell. Typically, this means e.g., the correct spacing, essential regulatory sequences, and reading frame (if applicable) are maintained.

[0024] As used herein, "homolog" means an enzyme with at least 40% identity to one of the listed sequences and also having the same general catalytic activity, although kinetic parameters of the reactions can of course vary. While higher identity (60%, 70%, 80%) and the like may be preferred, it is typical for bacterial sequences to diverge significantly (40-60% identity), yet still be identifiable as homologs, while mammalian species tend to diverge much less (80-90% identity). Unless specified otherwise, any reference to an enzyme herein also includes its homologs that catalyze the same reaction.

[0025] As used herein, references to cells or bacteria or strains and all such similar designations include progeny thereof. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations that have been added to the parent. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

[0026] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims or the specification means one or more than one, unless the context dictates otherwise.

[0027] The term "about" means the stated value plus or minus the margin of error of measurement or plus or minus 10% if no method of measurement is indicated.

[0028] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or if the alternatives are mutually exclusive.

[0029] The terms "comprise", "have", "include" and "contain" (and their variants) are open-ended linking verbs and allow the addition of other elements when used in a claim.

[0030] The phrase "consisting of" is closed, and excludes all additional elements.

[0031] The phrase "consisting essentially of" excludes additional material elements, but allows the inclusions of non-material elements that do not substantially change the nature of the invention.

[0032] The following abbreviations are used herein:

ABBREVIATION	TERM
CDS	Protein coding sequence. Equivalent to an ORF.
GFP	Green fluorescent protein
IM	Integration module
IPTG	Isopropyl β-D-1-thiogalactopyranoside. When IPTG is added, the lac repressor is inactivated, allowing the T7 polymerase to be used and therefore the protein of interest will be expressed.
LacI	Lactose operon repressor, e.g. P03023
LacI-T7	LacI operatively coupled to the T7 phage RNA polymerase
ORF	Open-reading frame
$P_{hy\text{-}spank}$	Promoter hyper spank
P_{penP}	Promoter from the penicillinase gene (penP) of *Bacillus licheniformis*.
RBS	Ribosome binding site
sfGFP	Superfolder GFP. A constitutively fluorescent green fluorescent protein, published in 2005.
T7 RNAP	T7 phage RNA polymerase, e.g., P00573

DESCRIPTION OF FIGURES

[0033] **FIG 1AF**. LacI-T7 design and performance.

[0034] **FIG. 1A** Genetic device schematic of LacI-T7 with regulatory interactions shown.

[0035] **FIG. 1B** sfGFP fluorescence from LacI-T7 and Phy-spank in the absence or presence of IPTG. Fluorescence is shown in calibrated Molecules of Equivalent Fluorescein (MEFL) units. Bars represent the mean of three experimental replicates run on separate days. Dots show the values of the individual replicates.

[0036] **FIG. 1C-D** Representative flow cytometry histograms of autofluorescence control (*B. subtilis* lacking any *sfgfp* gene), and those with *sfgfp* under either LacI-T7 (**1C**) or $P_{hy\text{-}spank}$ (**1D**), in the presence or absence of IPTG.

[0037] **FIG. 1E** sfGFP Fluorescence from LacI-T7 and $P_{hy\text{-}spank}$ as a function of IPTG concentration.

[0038] **FIG. 1F** sfGFP fluorescence from LacI-T7 and $P_{hy\text{-}spank}$ after addition of saturating IPTG. Dots and error bars show the mean and standard deviation, respectively, of three experiments run on separate days. Error bars are, in most cases, smaller than the size of the dots, and thus not visible. Black lines represent model fits.

[0039] **FIG. 2**: T7lac promoter (P_{T7lac}) and placing of the gene of interest. P_{T7lac} is highlighted with underline. Ribosome binding site is highlighted with double underline. The start codon (ATG) of the gene to be expressed is shown in bold. SEQ ID NO 1.

[0040] **FIG. 3:** Promoter hyper spank SEQ ID NO 2.

[0041] **FIG. 4.** T7 RNAP SEQ ID NO 3.

[0042] **FIG. 5**: Promoter $_{penP}$ SEQ ID NO 4.

[0043] **FIG. 6.** *LacI-T7-sfgfp*. SEQ ID NO 5.

[0044] **FIG. 7:** Compete expression system sequence SEQ ID NO 6.

[0045] **FIG. 8** Complete Vector without sfGFP SEQ ID NO 7.

[0046] **FIG. 9.** Variant LacI-T7-sfgfp SEQ ID NO 8.

[0047] **FIG. 10:** Compete Vector with sfGFP Variant SEQ ID NO 9.

[0048] **FIG. 11.** Growth curves of LacI-T7 strains. LacI-T7-*sfgfp* (A) and LacI-T7-lacZ (B) strains were grown in the absence or presence of IPTG, and the OD600 of each culture was measured at the indicated timepoints. Dots indicate individual OD600 measurements. Continuous lines indicate best fits to an exponential growth model. Estimated doubling times and uncertainties (standard error) are indicated next to the corresponding growth curves.

[0049] **FIG. 12** SDS-PAGE and immunoblot analysis of sfGFP expression from Phy-spank and LacI-T7. (A) Whole cell extracts of wild-type, Phy-spank-sfgfp and LacI-T7-*sfgfp* strains grown in the absence or presence of IPTG were analyzed by SDS-PAGE and stained with Coomassie brilliant-blue . The arrow on the right points to the expected sfGFP location. (B) sfGFP expression was further examined by immunoblot analysis using polyclonal anti-GFP antibodies. (C) As a loading control, expression of the constitutively expressed σA protein was examined by immunoblotting with anti-σA antibodies.

[0050] **FIG. 13.** Performance of LacI-T7 measured via β-galactosidase. (A) Device schematic of the LacI-T7 system expressing lacZ. (B) β-galactosidase activity in the absence or presence of IPTG. Bars show the mean of three experiments run on separate days. Dots show values of individual experimental replicates.

[0051] **Table 1.** Data.

[0052] **Table 2.** Various sequences SEQ ID NO 10-22.

DETAILED DESCRIPTION

[0053] In more detail, we have engineered a stringent (i.e. non-leaky) and highly-inducible LacI-T7 promoter system for *B. subtilis*. Our system utilizes the hybrid P_{T7lac} promoter to express a gene of interest, and the IPTG-inducible promoter $P_{hy\text{-}spank}$ to express the T7 RNA Polymerase (T7 RNAP) (**FIG. 1A**).

[0054] In the absence of the IPTG inducer, the repressor lacI is active and both T7 RNAP and P_{T7lac} are thereby repressed. This dual repression minimizes leaky expression of the gene of interest. Thus, the system is very stringent, with little to no

detectable leaking. Upon addition of IPTG, LacI activity is inhibited, and the newly produced T7 RNAP strongly transcribes the gene of interest from the now de-repressed P_{T7lac} (**FIG. 1A**).

[0055] Our LacI-T7 design is conceptually similar to some variants of the commercial *E. coli* pET expression system, where IPTG also induces both expression of T7 RNAP and de-repression of P_{T7lac}. However, our system uses *Bacillus* or *B. subtilis*-specific promoters (other than P_{T7lac}) and ribosome-binding sites (RBSs). Additionally, while a few *B. subtilis* gene expression systems based on T7 RNAP have been previously reported, they use the LacI-independent P_{T7} instead of P_{T7lac}, resulting in dynamic ranges of less than 50.

[0056] To validate our design, we placed a *sfgfp* reporter gene[22] with a codon-optimized N-terminal fragment (*sfgfp**) under control of LacI-T7, the self-cleaving ribozyme RiboJ, and RBS MF001, and integrated it into the non-essential *amyE* locus of the *B. subtilis* genome as a single copy (**FIG. 1A**).

[0057] We utilized flow cytometry to characterize sfGFP fluorescence levels in the absence and presence of IPTG. We found that, in the absence of inducer, sfGFP fluorescence equals 17.9 ± 4.7 molecules of equivalent fluorescein (MEFL) (**FIG. 1B** and **C**). In contrast, sfGFP levels in the presence of IPTG are 432,000 ± 20,000 MEFL (**FIG. 1B** and **C**), resulting in a dynamic range of 25,300 ± 6,900. Remarkably, only a small growth slowdown was observed under inducing conditions (cell division time: 25.15 ± 0.46 minutes without IPTG, 30.2 ± 1.1 minutes with IPTG) (not shown). Polyacrylamide gel electrophoresis (PAGE) analysis of total cellular protein indicated that sfGFP is the mostly highly expressed protein in the cell in the presence of IPTG (not shown).

[0058] To validate that this high dynamic range is preserved when expressing a different gene of interest, we placed the *lacZ* reporter gene under LacI-T7 (**FIG. 13**) and measured β-galactosidase activity in the absence and presence of IPTG. This resulted in a dynamic range of 11,000 ± 1,200 (**FIG. 11-12**). The difference between this value and the one obtained with sfGFP might be explained by uncertainty in reporter measurements under non-inducing conditions, which result in signals that are close to background. Again, we observed only a mild growth slowdown (cell division

time: 23.84 ± 0.22 minutes without IPTG, 28.49 ± 0.98 minutes with IPTG). We conclude that LacI-T7 can regulate expression of several genes with a dynamic range greater than 10,000-fold with little toxicity.

[0059] $P_{hy\text{-}spank}$ is a variant of the IPTG-inducible promoter P_{spac} optimized for higher expression and dynamic range, and is perhaps the most widely used *B. subtilis* inducible promoter system. We constructed a second *B. subtilis* strain wherein *sfgfp** was expressed under the control of $P_{hy\text{-}spank}$, RiboJ, and RBS MF001 to compare its performance to that of LacI-T7. We found that $P_{hy\text{-}spank}$ exhibits much greater leakiness (565 ± 18 MEFL) and a lower maximal output (160,000 ± 14,000 MEFL) than LacI-T7, resulting in a dynamic range of only 282 ± 18 (**FIG. 1B**, **D**).

[0060] Next, we measured the steady state transfer function of both systems by growing the corresponding strains under different concentrations of IPTG. In both cases, sfGFP fluorescence increases as a function of IPTG concentration in a manner well-approximated by a Hill function (**FIG. 1E**). Remarkably, both systems exhibit similar IPTG detection thresholds (50% activation concentrations: LacI-T7: 54.1 ± 1.6 μM, $P_{hy\text{-}spank}$: 50.2 ± 1.5 μM). On the other hand, the Hill coefficient of the LacI-T7 system is larger (3.377 ± 0.051, compared to 2.120 ± 0.033 for $P_{hy\text{-}spank}$), indicating that protein expression is more sensitive to changes in IPTG levels in the responsive range of the system.

[0061] Finally, we characterized the response dynamics of both systems after an instantaneous addition of saturating IPTG (**FIG. 1F**). As expected, both responses show an exponential-like increase until saturation. However, LacI-T7 responds slightly slower ($t_{1/2}$ = 82.1 ± 6.5 min for LacI-T7, 62.1 ± 1.1 min for $P_{hy\text{-}spank}$), consistent with the need to produce an intermediate protein (T7RNAP) before expression of the reporter gene. In conclusion, LacI-T7 exhibits lower leakiness, higher maximal expression output, similar sensitivity to inducer, and much higher dynamic range than the widely used $P_{hy\text{-}spank}$ system, albeit with a slightly slower response time.

[0062] The superior performance of LacI-T7 appears to arise from its unique design features. First, high maximal expression results from the use of T7 RNAP, a strong viral RNA polymerase which is capable of re-directing all bacterial resources towards

expression of a single output gene. In contrast, previous expression systems based on endogenous promoters are limited by the native transcriptional machinery and are subject to competition with other endogenous promoters. Second, leaky expression in the absence of inducer is reduced via the dual repression activity of LacI. In *E. coli*, a similar design has been shown to reduce basal expression by more than an order of magnitude compared to an unmodified P_{T7} output promoter.

[0063] LacI-T7 should be useful in quantitative studies of *B. subtilis* biology. In particular, its stable single-copy chromosomal location and its low leakiness are desirable for analyses of ultrasensitive or excitable networks where low amounts of excess protein can cause cells to undergo dramatically different differentiation programs.

[0064] We also expect LacI-T7 to be useful for heterologous protein expression applications. High expression from P_{T7lac} should enable high yields of both cytoplasmic and secreted proteins. Additionally, low leakiness in the absence of inducer should allow for fast initial cell growth, even with potentially toxic proteins. Furthermore, LacI-T7 is integrated into the *B. subtilis* genome, and thus will not suffer from plasmid instability issues or require strong selective pressure to be maintained. Because it relies on the orthogonal T7 polymerase, LacI-T7 could also be ported to other industrially-relevant *Bacillus* species or strains with little additional work.

DNA ASSEMBLY AND CLONING

[0065] All cloning and experiments were performed in *B. subtilis* strain PY79. Primers were ordered from Integrated DNA Technologies, Inc. $P_{hy\text{-}spank}$ was amplified from integration plasmid pDR111. P_{T7lac} was constructed via oligo annealing and extension. Synthetic RBS MF001 was obtained from integration plasmid pMF35. Genomic homology fragments required for chromosomal integration were amplified from the purified genome of *B. subtilis* PY79.

[0066] All systems were built as linear double-stranded integration module (IM) or cassette, as we have previously described. Our IMs contain the DNA of interest and a selection marker flanked by 1.5kb-long sequences homologous to the *amyE* locus of the *B. subtilis* genome where chromosomal integration via double crossover occurs.

IMs were assembled from PCR-amplified parts using Golden Gate. The resulting Golden Gate product was amplified using NEB Phusion DNA Polymerase and gel purified to obtain the IM. 500 ng was transformed into competent *B. subtilis* using standard transformation methods.

[0067] The transformants were plated on selective media. Colonies were picked the next day and grown in LB media at 37°C and 250 RPM for a few hours. Finally, freezer stocks were prepared with 700 µL culture and 300 µL 60% glycerol, and stored at -80°C. This method avoids sub-cloning of integration plasmids in *E. coli*, as long as enough PCR-amplified DNA can be obtained. The complete sequences of all IMs constructed in this study can be found in GenBank via the following accession numbers: $P_{hy\text{-}spank}$-*sfgfp*: MN005205, LacI-T7-*sfgfp*: MN005204, LacI-T7-*lacZ*: MN005206.

[0068] For DNA sequence verification, an overnight LB culture was grown from a freezer stock, and 2 µL saturated culture was used as template for a 50 µL PCR reaction, either with Taq or Phusion DNA Polymerase. PCR products obtained in this fashion were gel-purified and sent for sequence verification to Genewiz, Inc.

MEDIA AND EXPERIMENTAL PROTOCOLS:

[0069] We used a modified M9 medium for all experiments. 1L 5xM9 salts at pH ~ 6.8 were prepared with 64 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 9.2 mL 6M HCl, and up to 1 L dH_2O. For 1 L M9, we used 200 mL 5x M9 salts, 20 mL 10% casamino acids, 6.67 mL 60% glycerol, 1 mL 50 mM $FeCl_3$/100 mM $C_6H_8O_7$ solution, 2 mL 50 mM $MnSO_4$, 2 mL 1M $MgSO_4$, 100 µL 1M $CaCl_2$, and dH_2O up to 1 L.

[0070] For each experiment, an overnight LB culture was started from the freezer stock of each relevant strain. The next day, saturated cultures (OD_{600} ~ 3) were diluted 10^5-fold in M9. Media was distributed in culture tubes (3 mL per tube), inoculated with the appropriate inducers (0 µM or 500 µM IPTG), and incubated in a shaker operating at 250 rpm and 37°C, until the OD_{600} reached between 0.08 and 0.15 (around 6 hours). Culture tubes were then transferred to ice. 100 µL of each sample was transferred to a flow cytometry tube containing 1 mL PBS for measurement.

FLOW CYTOMETRY ANALYSIS

[0071] The sfGFP fluorescence distribution of each culture was measured using a BD FACScan flow cytometer with an excitation source of 488 nm and an emission window of 510/21 nm. 30,000 events were collected per sample. A suspension of calibration beads (Spherotech® RCP-30-5A) in PBS was measured with each experiment. After data acquisition, raw .fcs flow cytometry files were processed using FlowCal.

[0072] Cell populations were gated by forward scatter/side scatter density (not shown) retaining 50% of the total number of events. Next, fluorescence of each gated event in arbitrary units was converted into standardized MEFL values using the calibration bead data. The total cellular fluorescence of each culture sample was then obtained by calculating the median MEFL fluorescence of all gated events in that sample. Finally, the reported sfGFP fluorescence values were obtained by subtracting the total cellular fluorescence of a wild-type PY79 sample measured the same day from each sample's total cellular fluorescence. Numerical sfGFP fluorescence values of every sample and replicate can be found in **Table 1**.

CODON OPTIMIZATION OF *SFGFP*

[0073] Codon optimization of the N-terminal sequence of the *sfgfp* ORF was performed to decrease secondary structure with the RBS and increase translation efficiency. To do so, for each of the first 15 codons of the original *sfgfp* sequence, a synonymous codon was chosen to reduce GC and increase AU content, with A preferred over U, with no regard for codon frequency. These changes were confirmed to increase the mRNA secondary structure free energy (and thus decrease secondary structure stability) via Nupack, by using the sequence from the transcription start site up to the 90^{th} nucleotide residue of the ORF. The complete optimized *sfgfp** sequence can be found in **Table 2**.

[0074] Statistical methods are not reproduced herein, but can be found in Castillo (2019).

[0075] Each of the following references is incorporated by reference herein in its entirety for all purposes:

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[0090] Studier, F.W. & Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol., 189, 113–130.

[0091] Castillo-Hair, Sebastian M., et al. "An Engineered B. subtilis Inducible Promoter System with over 10 000-Fold Dynamic Range." ACS Synthetic Biology, 8, no. 7 (2019) American Chemical Society: 1673-1678.

TABLE 1

Strain	IPTG (μM)	Replicate	sfGFP Fluorescence (MEFL)
Physpank-sfgfp	0	1	12.6247004
Physpank-sfgfp	0	2	8.483803292
Physpank-sfgfp	0	3	7.244700498
Physpank-sfgfp	500	1	2429.998994
Physpank-sfgfp	500	2	2611.038636
Physpank-sfgfp	500	3	2068.52592
LacI-T7-sfgfp	0	1	-11.70963282
LacI-T7-sfgfp	0	2	-0.006598202
LacI-T7-sfgfp	0	3	-2.802232792
LacI-T7-sfgfp	500	1	108557.0156
LacI-T7-sfgfp	500	2	113360.4879
LacI-T7-sfgfp	500	3	115850.8173

TABLE 2: VARIOUS SEQUENCES			
Name	**Type**	**Description**	**SEQUENCE**
sfgfp	CDS	Superfolder green fluorescent protein SEQ ID NO 10	ATGCGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCT ATTCTGGTGGAACTGGATGGTGATGTCAACGGTCATAAG TTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAAT GGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAA CTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACT TATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATG AAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGC TATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGGC ACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGAT ACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTT AAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATAC AATTTTAACAGCCACAATGTTTACATCACCGCCGATAAA CAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCAC AACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTAC CAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTG CCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCT AAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTG GAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGAT GAACTGTACAAATGATGA
lacI	CDS	Lactose repressor SEQ ID NO 11	ATGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCC GGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAG GCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAA GCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTG GCACAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGC GTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAA ATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGT GCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTC GAAGCCTGTAAAACGGCGGTGCACAATCTTCTCGCGCAA CGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGAC CAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTT CCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATC AACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTG GGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATC GCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGT CTGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCAAT CAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGT GCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAAT GAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGAT CAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCC GGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATAC GACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTA ACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACC AGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCG GTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAA AGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCT CCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGA CAGGTTTCCCGACTGGAAAGCGGGCAGTGA
t7rnap	CDS	T7 RNA Polymerase SEQ ID NO 12	ATGAACACGATTAACATCGCTAAGAACGACTTCTCTGAC ATCGAACTGGCTGCTATCCCGTTCAACACTCTGGCTGAC CATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTT GAGCATGAGTCTTACGAGATGGGTGAAGCACGCTTCCGC

TABLE 2: VARIOUS SEQUENCES			
Name	**Type**	**Description**	**SEQUENCE**
			AAGATGTTTGAGCGTCAACTTAAAGCTGGTGAGGTTGCG GATAACGCTGCCGCCAAGCCTCTCATCACTACCCTACTC CCTAAGATGATTGCACGCATCAACGACTGGTTTGAGGAA GTGAAAGCTAAGCGCGGCAAGCGCCCGACAGCCTTCCAG TTCCTGCAAGAAATCAAGCCGGAAGCCGTAGCGTACATC ACCATTAAGACCACTCTGGCTTGCCTAACCAGTGCTGAC AATACAACCGTTCAGGCTGTAGCAAGCGCAATCGGTCGG GCCATTGAGGACGAGGCTCGCTTCGGTCGTATCCGTGAC CTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACAA CTCAACAAGCGCGTAGGGCACGTCTACAAGAAAGCATTT ATGCAAGTTGTCGAGGCTGACATGCTCTCTAAGGGTCTA CTCGGTGGCGAGGCGTGGTCTTCGTGGCATAAGGAAGAC TCTATTCATGTAGGAGTACGCTGCATCGAGATGCTCATT GAGTCAACCGGAATGGTTAGCTTACACCGCCAAAATGCT GGCGTAGTAGGTCAAGACTCTGAGACTATCGAACTCGCA CCTGAATACGCTGAGGCTATCGCAACCCGTGCAGGTGCG CTGGCTGGCATCTCTCCGATGTTCCAACCTTGCGTAGTT CCTCCTAAGCCGTGGACTGGCATTACTGGTGGTGGCTAT TGGGCTAACGGTCGTCGTCCTCTGGCGCTGGTGCGTACT CACAGTAAGAAAGCACTGATGCGCTACGAAGACGTTTAC ATGCCTGAGGTGTACAAAGCGATTAACATTGCGCAAAAC ACCGCATGGAAAATCAACAAGAAAGTCCTAGCGGTCGCC AACGTAATCACCAAGTGGAAGCATTGTCCGGTCGAGGAC ATCCCTGCGATTGAGCGTGAAGAACTCCCGATGAAACCG GAAGACATCGACATGAATCCTGAGGCTCTCACCGCGTGG AAACGTGCTGCCGCTGCTGTGTACCGCAAGGACAAGGCT CGCAAGTCTCGCCGTATCAGCCTTGAGTTCATGCTTGAG CAAGCCAATAAGTTTGCTAACCATAAGGCCATCTGGTTC CCTTACAACATGGACTGGCGCGGTCGTGTTTACGCTGTG TCAATGTTCAACCCGCAAGGTAACGATATGACCAAAGGA CTGCTTACGCTGGCGAAAGGTAAACCAATCGGTAAGGAA GGTTACTACTGGCTGAAAATCCACGGTGCAAACTGTGCG GGTGTCGATAAGGTTCCGTTCCCTGAGCGCATCAAGTTC ATTGAGGAAAACCACGAGAACATCATGGCTTGCGCTAAG TCTCCACTGGAGAACACTTGGTGGGCTGAGCAAGATTCT CCGTTCTGCTTCCTTGCGTTCTGCTTTGAGTACGCTGGG GTACAGCACCACGGCCTGAGCTATAACTGCTCCCTTCCG CTGGCGTTTGACGGGTCTTGCTCTGGCATCCAGCACTTC TCCGCGATGCTCCGAGATGAGGTAGGTGGTCGCGCGGTT AACTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGGG ATTGTTGCTAAGAAAGTCAACGAGATTCTACAAGCAGAC GCAATCAATGGGACCGATAACGAAGTAGTTACCGTGACC GATGAGAACACTGGTGAAATCTCTGAGAAAGTCAAGCTG GGCACTAAGGCACTGGCTGGTCAATGGCTGGCTTACGGT GTTACTCGCAGTGTGACTAAGCGTTCAGTCATGACGCTG GCTTACGGGTCCAAAGAGTTCGGCTTCCGTCAACAAGTG CTGGAAGATACCATTCAGCCAGCTATTGATTCCGGCAAG GGTCTGATGTTCACTCAGCCGAATCAGGCTGCTGGATAC ATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGACGGTG GTAGCTGCGGTTGAAGCAATGAACTGGCTTAAGTCTGCT GCTAAGCTGCTGGCTGCTGAGGTCAAAGATAAGAAGACT GGAGAGATTCTTCGCAAGCGTTGCGCTGTGCATTGGGTA ACTCCTGATGGTTTCCCTGTGTGGCAGGAATACAAGAAG

TABLE 2: VARIOUS SEQUENCES			
Name	**Type**	**Description**	**SEQUENCE**
			CCTATTCAGACGCGCTTGAACCTGATGTTCCTCGGTCAG TTCCGCTTACAGCCTACCATTAACACCAACAAAGATAGC GAGATTGATGCACACAAACAGGAGTCTGGTATCGCTCCT AACTTTGTACACAGCCAAGACGGTAGCCACCTTCGTAAG ACTGTAGTGTGGGCACACGAGAAGTACGGAATCGAATCT TTTGCACTGATTCACGACTCCTTCGGTACCATTCCGGCT GACGCTGCGAACCTGTTCAAAGCAGTGCGCGAAACTATG GTTGACACATATGAGTCTTGTGATGTACTGGCTGATTTC TACGACCAGTTCGCTGACCAGTTGCACGAGTCTCAATTG GACAAAATGCCAGCACTTCCGGCTAAAGGTAACTTGAAC CTCCGTGACATCTTAGAGTCGGACTTCGCGTTCGCGTAA
Phy-spank	Promoter	IPTG-inducible promoter SEQ ID NO 13	GGTAAATGTGAGCACTCACAATTCATTTTGCAAAAGTTG TTGACTTTATCTACAAGGTGTGGCATAATGTGTGTAATT GTGAGCGGATAACAATTAAGCTTAGTCGACA
PT7lac	Promoter	Hybrid T7 promoter with lac operators SEQ ID NO 14	TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACA ATTCCCCT
PpenP	Promoter	Promoter driving expression of lacI SEQ ID NO 15	CGGTGGAAACGAGGTCATCATTTCCTTCCGAAAAAACGG TTGCATTTAAATCTTACATATGTAATACTTTCAAAGACT ACATTTGTAAGATTTG
MF001	RBS	Synthetic RBS in pMF35 SEQ ID NO 16	AAGCTTACATAAGGAGGAACTACT
MF002	RBS	Slightly modified MF001 SEQ ID NO 17	GCTAGCACATAAGGAGGAACTACT
penP	RBS	RBS driving expression of lacI SEQ ID NO 18	TTCAAACGGAGGGAGACGATTTTG
B0015	Terminator	Terminator SEQ ID NO 19	CCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACT GGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTC TCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTT TCTGCGTTTATA
sfGFP	CDS	Codon optimized sfGFP SEQ ID NO 20.	ATGAGAAAAGGAGAAGAATTATTTACAGGAGTTGTTCCA ATTTTAGTGGAACTGGATGGTGATGTCAACGGTCATAAG TTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAAT GGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAA CTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACT TATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATG AAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGC TATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGGC ACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGAT ACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTT AAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATAC AATTTTAACAGCCACAATGTTTACATCACCGCCGATAAA CAAAAAAATGGCATTAAAGCGAATTTTAAAATTCGCCAC

TABLE 2: VARIOUS SEQUENCES			
Name	**Type**	**Description**	**SEQUENCE**
			AACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTAC CAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTG CCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCT AAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTG GAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGAT GAACTGTACAAATAA
lacZ	CDS	β-galactosidase enzyme SEQ ID NO 21	ATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAA CGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAAT CGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAAT AGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTG CGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCG GCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGAT CTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTGG CAGATGCACGGTTACGATGCGCCCATCTACACCAACGTG ACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACG GAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTT GATGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTATT TTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAAC GGGCGCTGGGTCGGTTACGGCCAGGACAGTCGTTTGCCG TCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGAGAA AACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGC AGTTATCTGGAAGATCAGGATATGTGGCGGATGAGCGGC ATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACA CAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGAT GATTTCAGCCGCGCTGTACTGGAGGCTGAAGTTCAGATG TGCGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCT TTATGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCG CCTTTCGGCGGTGAAATTATCGATGAGCGTGGTGGTTAT GCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCG AAACTGTGGAGCGCCGAAATCCCGAATCTCTATCGTGCG GTGGTTGAACTGCACACCGCCGACGGCACGCTGATTGAA GCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATT GAAAATGGTCTGCTGCTGCTGAACGGCAAGCCGTTGCTG ATTCGAGGCGTTAACCGTCACGAGCATCATCCTCTGCAT GGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATC CTGCTGATGAAGCAGAACAACTTTAACGCCGTGCGCTGT TCGCATTATCCGAACCATCCGCTGTGGTACACGCTGTGC GACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATT GAAACCCACGGCATGGTGCCAATGAATCGTCTGACCGAT GATCCGCGCTGGCTACCGGCGATGAGCGAACGCGTAACG CGAATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATC ATCTGGTCGCTGGGGAATGAATCAGGCCACGGCGCTAAT CACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCT TCCCGCCCGGTGCAGTATGAAGGCGGCGGAGCCGACACC ACGGCCACCGATATTATTTGCCCGATGTACGCGCGCGTG GATGAAGACCAGCCCTTCCCGGCTGTGCCGAAATGGTCC ATCAAAAAATGGCTTTCGCTACCTGGAGAGACGCGCCCG CTGATCCTTTGCGAATACGCCCACGCGATGGGTAACAGT CTTGGCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAG TATCCCCGTTTACAGGGCGGCTTCGTCTGGGACTGGGTG GATCAGTCGCTGATTAAATATGATGAAAACGGCAACCCG TGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAAC GATCGCCAGTTCTGTATGAACGGTCTGGTCTTTGCCGAC

TABLE 2: VARIOUS SEQUENCES			
Name	**Type**	**Description**	**SEQUENCE**
			CGCACGCCGCATCCAGCGCTGACGGAAGCAAAACACCAG CAGCAGTTTTTCCAGTTCCGTTTATCCGGGCAAACCATC GAAGTGACCAGCGAATACCTGTTCCGTCATAGCGATAAC GAGCTCCTGCACTGGATGGTGGCGCTGGATGGTAAGCCG CTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCCACAA GGTAAACAGTTGATTGAACTGCCTGAACTACCGCAGCCG GAGAGCGCCGGGCAACTCTGGCTCACAGTACGCGTAGTG CAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATC AGCGCCTGGCAGCAGTGGCGTCTGGCGGAAAACCTCAGT GTGACGCTCCCCGCCGCGTCCCACGCCATCCCGCATCTG ACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAAT AAGCGTTGGCAATTTAACCGCCAGTCAGGCTTTCTTTCA CAGATGTGGATTGGCGATAAAAAACAACTGCTGACGCCG CTGCGCGATCAGTTCACCCGTGCACCGCTGGATAACGAC ATTGGCGTAAGTGAAGCGACCCGCATTGACCCTAACGCC TGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCC GAAGCAGCGTTGTTGCAGTGCACGGCAGATACACTTGCT GATGCGGTGCTGATTACGACCGCTCACGCGTGGCAGCAT CAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGG ATTGATGGTAGTGGTCAAATGGCGATTACCGTTGATGTT GAAGTGGCGAGCGATACACCGCATCCGGCGCGGATTGGC CTGAACTGCCAGCTGGCGCAGGTAGCAGAGCGGGTAAAC TGGCTCGGATTAGGGCCGCAAGAAAACTATCCCGACCGC CTTACTGCCGCCTGTTTTGACCGCTGGGATCTGCCATTG TCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAAC GGTCTGCGCTGCGGGACGCGCGAATTGAATTATGGCCCA CACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGCCGC TACAGTCAACAGCAACTGATGGAAACCAGCCATCGCCAT CTGCTGCACGCGGAAGAAGGCACATGGCTGAATATCGAC GGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGC CCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGC TACCATTACCAGTTGGTCTGGTGTCAAAAATAA
RiboJ	Non-coding RNA	Self-cleaving ribozyme SEQ ID NO 22	AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGT GAGGACGAAACAGCCTCTACAAATAATTTTGTTTAA

CLAIMS

1) A *Bacillus* promoter system, comprising one or more DNA molecules comprising:

a) a LacI repressor constitutively expressed under a first constitutive promoter;

b) a T7 phage RNA polymerase (T7 RNAP) expressed from a second promoter that is normally repressed by LacI; and

c) a third promoter sequence regulated by a T7RNAP that is normally repressed by LacI.

2) The promoter system of claim 1, further comprising an open reading frame ("ORF") for a gene of interest inserted after said third promoter sequence, such that said ORF is regulated by said third promoter sequence.

3) The promoter system of claims 1-2, having at least 5,000, 10,000, 15,000 or 20,000 fold activation of expression of said ORF when induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG).

4) The promoter system of claims 1-3, comprising one or more of the following:

a) the LacI is from or is equivalent to the sequences in pDR111 or having at least 95% nucleotide identity to SEQ ID NO 11; or

b) the first constitutive promoter is a P_{penP} promoter or a sequence having at least 95% nucleotide identity to SEQ ID NO 15; or

c) the second promoter is a $P_{hy\text{-}spank}$ promoter or a sequence having at least 95% nucleotide identity to SEQ ID NO 13; or

d) the $P_{hy\text{-}spank}$ promoter is from or is equivalent to the sequences in pDR111; or

e) the hybrid P_{T7lac} promoter sequence is from or is equivalent to *E. coli* pET plasmids; or

f) the T7 RNAP is from or is equivalent to the *E. coli* BL21(DE3); or

g) the T7RNAP is a sequence having at least 95% nucleotide identity to SEQ ID NO 12

h) the third promoter is a hybrid P_{T7lac} promoter sequence or a sequence having at least 95% nucleotide identity to SEQ ID NO 14; or

i) the first constitutive promoter for the LacI repressor is a promoter from *Bacillus*; or

j) the T7RNAP is expressed from a *Bacillus* LacI repressed promoter.

5) The promoter system of claims 1-4, further comprising an antibiotic resistance gene.

6) The promoter system of claims 1-4, wherein parts a-c are in a single DNA cassette and further comprising a spectinomycin resistance gene within said cassette.

7) The promoter system of claims 1-6, wherein parts a-c are in a single DNA cassette comprising a 5' chromosomal sequence from a *Bacillus* amyE gene at a first end of said cassette and a 3' chromosomal sequence from said *Bacillus* amyE gene at a second end of said cassette, thereby allowing homologous recombination into a genome of *Bacillus*.

8) The promoter system of claims 1-7, further comprising one or more *B. subtilis*-specific ribosome-binding sites (RBSs) placed to stimulate translation from mRNAs expressed from the first, second or third promoters.

9) The promoter system of claims 1-8, wherein said *Bacillus* is a *Bacillus subtilis*.

10) The promoter system of claim 9, further comprising one or more *B. subtilis*-specific ribosome-binding sites (RBSs) placed to stimulate translation from mRNAs expressed from P_{T7lac}, $P_{hy\text{-}spank}$, or P_{penP} promoters, or any combination thereof.

11) The promoter system of claims 1-10, said cassette further comprising a self-cleaving ribozyme placed after P_{T7lac} to improve output production.

12) The promoter system of claims 1-11, further comprising a multiple cloning site after said third promoter.

13) The promoter system of claims 1-12, wherein the promoter system is an inducible

promoter system.

14) The promoter system of claim 13, wherein said promoter system is inducible by IPTG.

15) An expression system for *Bacillus*, said system being in a *Bacillus* and having:

a) at least 95% nucleotide identity to the sequence of SEQ ID NO 7, said 95% identity excluding an open reading frame (ORF) added thereto; and

b) having at least 5,000, 10,000, 15,000 or 20,000 fold more expression of said ORF with Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction than without IPTG induction.

16) An inducible and integrative expression system for *Bacillus subtilis*, said system comprising an expression cassette with the following structure:

a) a first portion of a chromosomal sequence from *B. subtilis* at a 5' end of said expression cassette;

b) a second portion of said chromosomal sequence at a 3' end of said expression cassette;

c) a LacI repressor constitutively expressed under a *B. subtilis* P_{penP} promoter within said expression cassette;

d) a lacI-repressible T7 phage RNA polymerase (T7 RNAP) expressed from a *B. subtilis* $P_{hy\text{-}spank}$ promoter within said expression cassette;

e) a hybrid promoter activated by T7RNAP and repressed by LacI that controls expression of an open reading frame (ORF).

17) The system of claim 16, wherein said ORF encodes an output mRNA, that may or may not encode a protein.

18) The system of claim 16, comprising an ORF for a target protein and being capable of a 10,000 fold increase in expression of said ORF when induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) as compared to without IPTG.

19) A vector comprising the system of claims 16-18, said vector being self-reproduceable

in a *Bacillus*, *Escherichia* or yeast.

20) A vector comprising the system of claims 16-18, said vector producing at least 10,000 fold more expression of said ORF in a *Bacillus* transformed with said vector and induced with IPTG induction, as compared to without IPTG.

21) A recombinant *Bacillus,* wherein said *Bacillus* is transformed with the system of claims 16-18.

22) A recombinant *Bacillus,* wherein said *Bacillus* is transformed with the system of claims 16-18, said expression cassette being integrated into a chromosome of said *Bacillus*.

23) A method of transforming *Bacillus subtilis,* comprising introducing the system of claims 16-18 to a population of *Bacillus subtilis* under conditions that allow transformation and integration of said system into a chromosome of said *Bacillus subtilis*, and selecting for transformed and integrated *Bacillus subtilis.*

24) A method of producing a target protein, comprising introducing the system of claims 16-18 to a population of *Bacillus subtilis* under conditions that allow integration of said expression cassette into a genome of said *Bacillus subtilis*, and selecting for integrated *Bacillus subtilis,* growing said integrated *Bacillus subtilis* in a growth medium until cells reach near saturation, adding IPTG to said growth medium in an amount sufficient to induce said lac operon, continuing culturing said integrated *Bacillus subtilis* until said ORF is expressed, and isolating said target protein from said growth medium or said transformed *Bacillus subtilis* or both.

25) A DNA molecule comprising SEQ ID NO. 7 plus an added open reading frame (ORF).

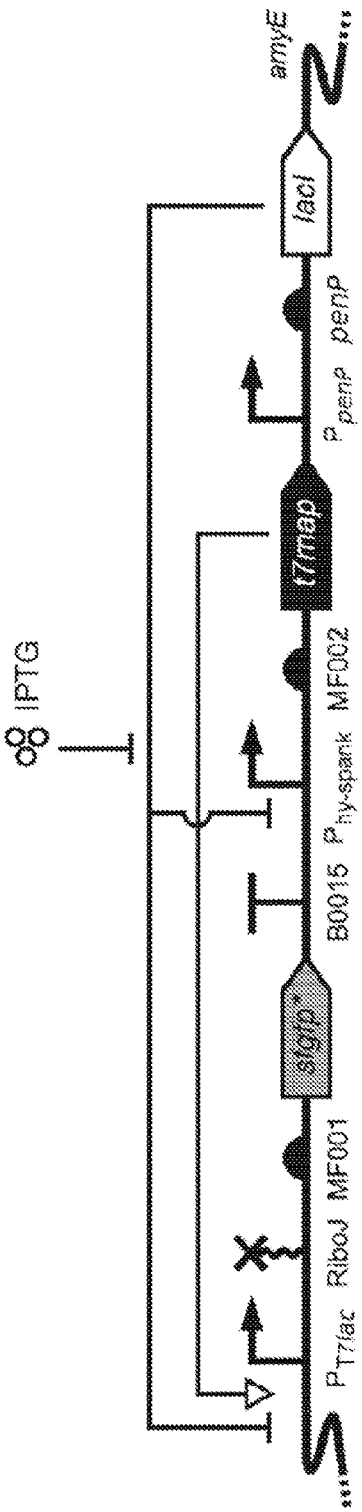
IPTG
amyE
lacI
P penP penP
tHmap
B0015 P hy-spank MF002
sfgfp
P T7lac RiboJ MF001

FIGURE 1A

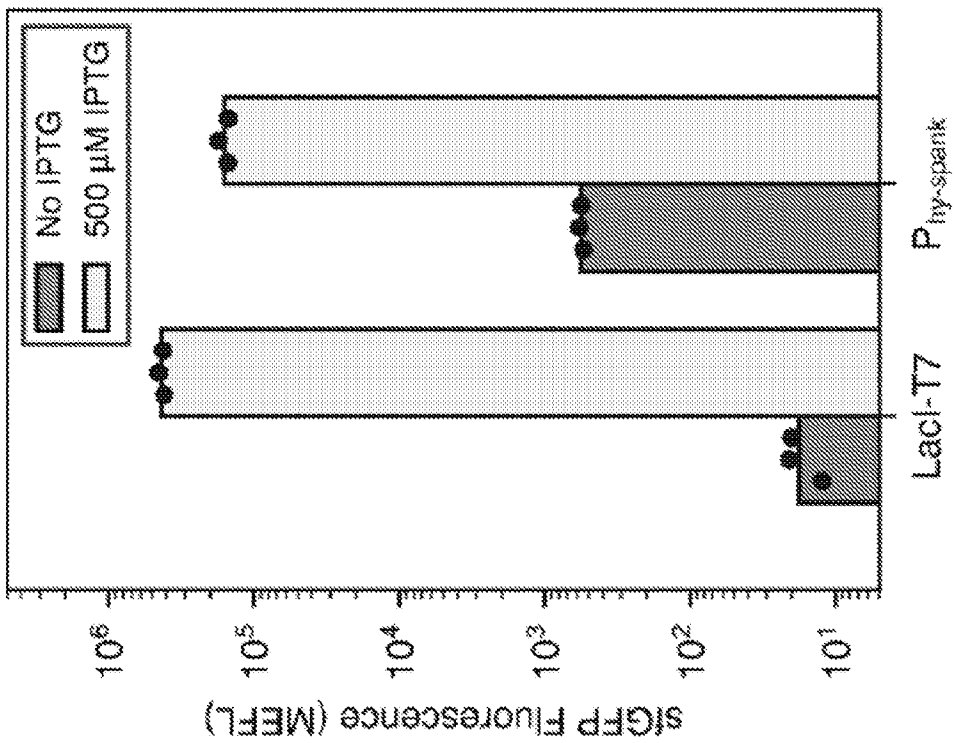
No IPTG
500 µM IPTG
sfGFP Fluorescence (MEFL)
10^6
10^5
10^4
10^3
10^2
10^1
LacI-T7
$P_{hy\text{-}spank}$

FIGURE 1B

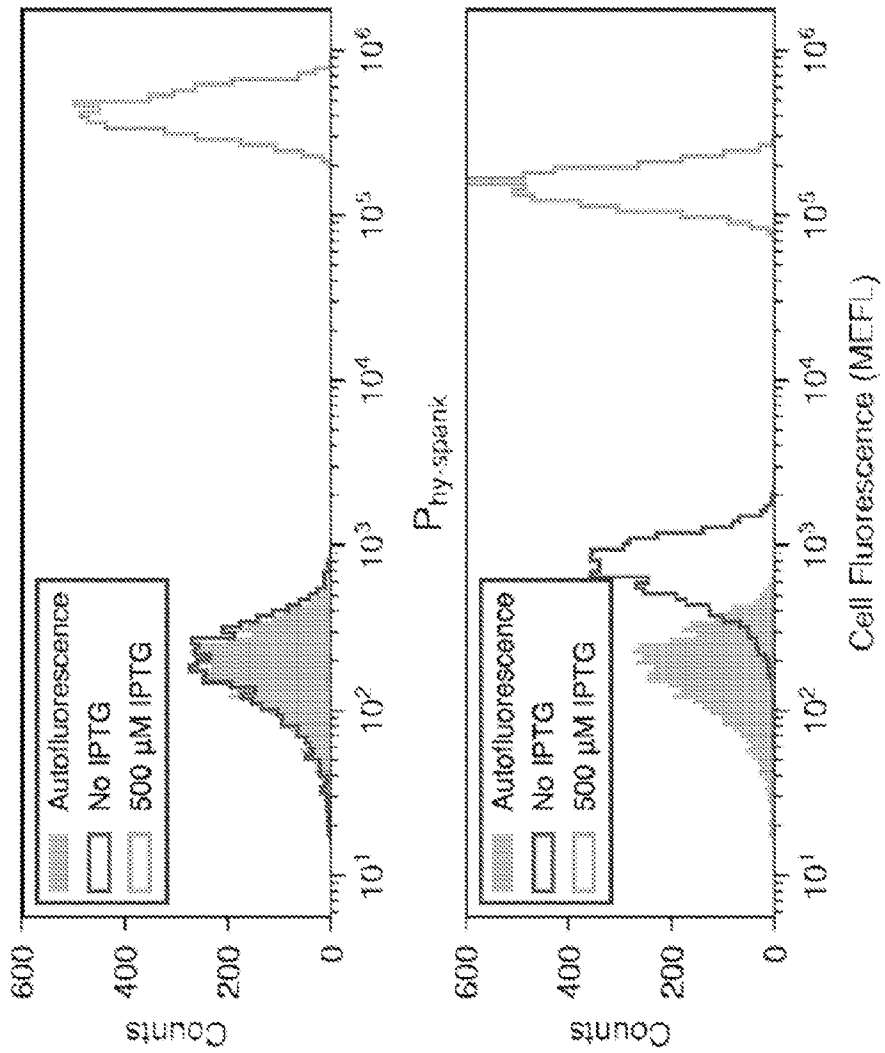
LacI-T7
Autofluorescence
No IPTG
500 µM IPTG
Counts
600
400
200
0
10^1
10^2
10^3
10^4
10^5
10^6
$P_{hy\text{-}spank}$
Cell Fluorescence (MEFL)

FIGURE 1C-D

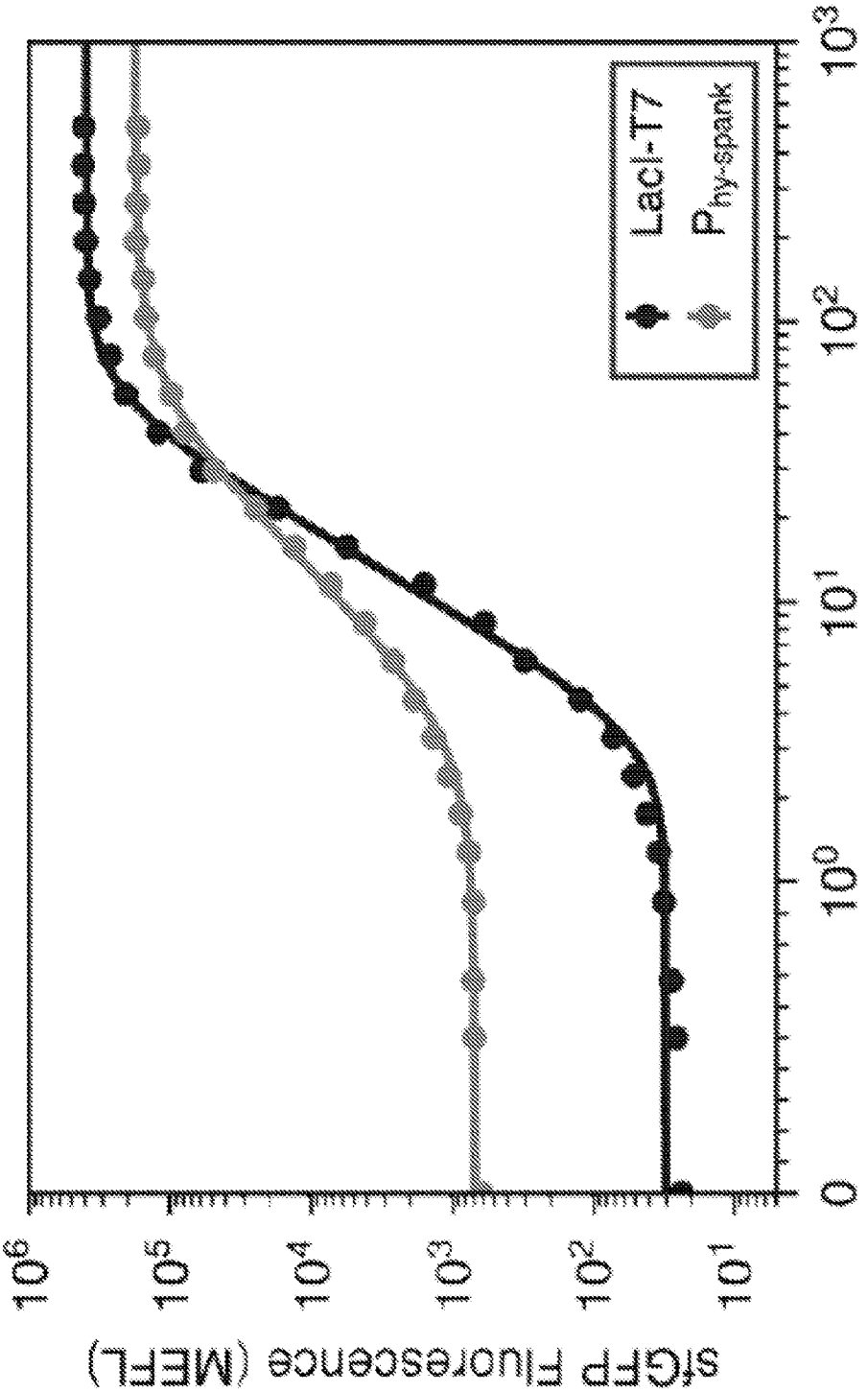
sfGFP Fluorescence (MEFL)
10^6
10^5
10^4
10^3
10^2
10^1
0
10^0
10^1
10^2
10^3
IPTG (μM)
LacI-T7
$P_{hy\text{-}spank}$

FIGURE 1E

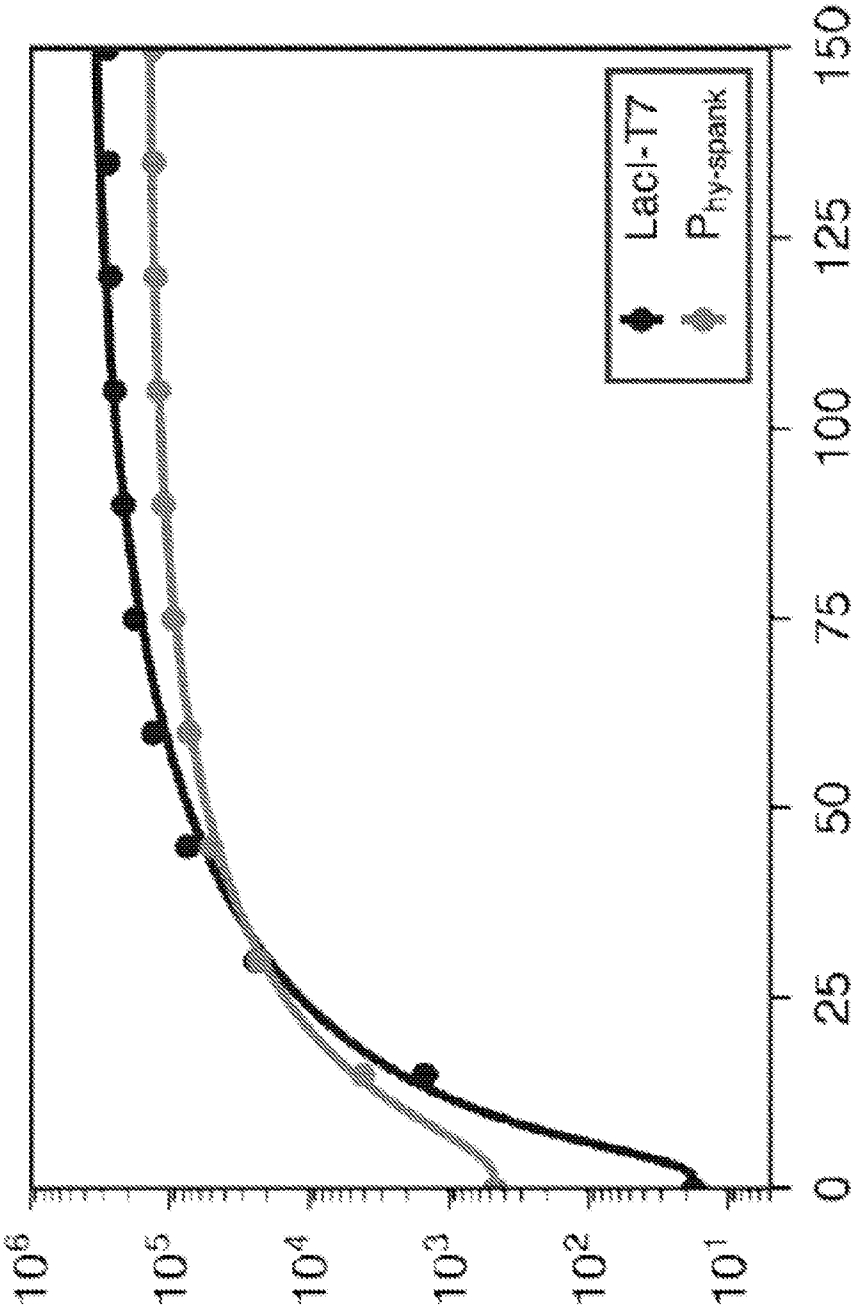
LacI-T7
$P_{hy\text{-}spank}$
Time (min)
0
25
50
75
100
125
150
10^1
10^2
10^3
10^4
10^5
10^6

FIGURE 1F

FIGURE 2: T7lac promoter (P_{T7lac}) and placing of the gene of interest SEQ ID NO 1

TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGA
CGAAACAGCCTCTACAAATAATTTTGTTTAAAAGCTTACATAAGGAGGAACTACT**ATG**

FIGURE 3: Promoter hyper spank SEQ ID NO 2

GGTAAATGTGAGCACTCACAATTCATTTTGCAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTGTGTAATTGTGAGCGGATAACAATTA
AGCTTAGTCGACA

FIGURE 4. T7 RNAP SEQ ID NO 3

MNTINIAKND FSDIELAAIP FNTLADHYGE RLAREQLALE HESYEMGEAR FRKMFERQLK AGEVADNAAA KPLITTLLPK MIARINDWFE EVKAKRGKRP
TAFQFLQEIK PEAVAYITIK TTLACLTSAD NTTVQAVASA IGRAIEDEAR FGRIRDLEAK HFKKNVEEQL NKRVGHVYKK AFMQVVEADM LSKGLLGGEA
WSSWHKEDSI HVGVRCIEML IESTGMVSLH RQNAGVVGQD SETIELAPEY AEAIATRAGA LAGISPMFQP CVVPPKPWTG ITGGGYWANG RRPLALVRTH
SKKALMRYED VYMPEVYKAI NIAQNTAWKI NKKVLAVANV ITKWKHCPVE DIPAIEREEL PMKPEDIDMN PEALTAWKRA AAAVYRKDKA RKSRRISLEF
MLEQANKFAN HKAIWFPYNM DWRGRVYAVS MFNPQGNDMT KGLLTLAKGK
PIGKEGYYWL KIHGANCAGV DKVPFPERIK FIEENHENIM ACAKSPLENT WWAEQDSPFC FLAFCFEYAG VQHHGLSYNC SLPLAFDGSC SGIQHFSAML
RDEVGGRAVN LLPSETVQDI YGIVAKKVNE ILQADAINGT DNEVVTVTDE NTGEISEKVK LGTKALAGQW LAYGVTRSVT KRSVMTLAYG SKEFGFRQQV
LEDTIQPAID SGKGLMFTQP NQAAGYMAKL IWESVSVTVV AAVEAMNWLK SAAKLLAAEV KDKKTGEILR KRCAVHWVTP DGFPVWQEYK KPIQTRLNLM
FLGQFRLQPT INTNKDSEID AHKQESGIAP NFVHSQDGSH LRKTVVWAHE KYGIESFALI HDSFGTIPAD AANLFKAVRE TMVDTYESCD VLADFYDQFA
DQLHESQLDK MPALPAKGNL NLRDILESDF AFA

FIGURE 5: PpenP SEQ ID NO 4

CGGTGGAAACGAGGTCATCATTTCCTTCCGAAAAAACGGTTGCATTTAAATCTTACATATGTAATACTTTCAAAGACTACATTTGTAAGATTTG

FIGURE 6. *LacI-T7-sfgfp.* SEQ ID NO 5

TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAAGCTTACATAAGGAGGAACTACTATGCGTAAAGGCGAAGAGCT
GTTCACTGGTGTCGTCCCTATTCTGGTGAACTGGATGGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAA
TGGTAAACTGACGCTGAAGTTCATCTGTACTACCTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTT
TGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGA
CGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAA
TATCCTGGGCCATAAGCTGGAATACAATTTTACATCACCGCCGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAAT
TCGCCACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCA
CTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCA
TGGTATGGATGAACTGTACAAATGATGATAATAATCTAGACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATC
TGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATACGTTTCGGTGATGAAGATCTTCC
CGATGATTAATTAATTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATGCAGCAATGGCAAGAACGTTGCTCGAGGGTAAATGTGAGCACTCACA
ATTCATTTTGCAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTGTGAGCGGATAACAATTAAGCTTAGTCGACAGCTAGCA
CATAAGGAGGAACTACTATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAACTGGCTGCTATCCCGTTCAACACTCTGGCTGACC
ATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTTGAGCATGAGTCTTACGAGATGGGTGAAGCACGCTTCCGCAAGATGTTTGAGCGTCAAC
TTAAAGCTGGTGAGGTTGCGGATAACGCTGCCGCCAAGCCTCTCATCACTACCCTACTCCCTAAGATGATTGCACGCATCAACGACTGGTTTGAGG
AAGTGAAAGCTAAGCGCGGCAAGCGCCCGACAGCCTTCCAGTTCCTGCAAGAAATCAAGCCGGAAGCCGTAGCGTACATCACCATTAAGACCACTC
TGGCTTGCCTAACCAGTGCTGACAATACAACCGTTCAGGCTGTAGCAAGCGCAATCGGTCGGGCCATTGAGGACGAGGCTCGCTTCGGTCGTATCC
GTGACCTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACAACTCAACAAGCGCGTAGGGCACGTCTACAAGAAAGCATTTATGCAAGTTGTCG
AGGCTGACATGCTCTCTTAAGGGTCTACTCGGTGGCGAGGCGTGGTCTTCGTGGCATAAGGAAGACTCTATTCATGTAGGAGTACGCCTGCATCGAGA
TGCTCATTGAGTCAACCGGAATGGTTAGCTTACACCGCCAAAATGCTGGCGTAGTAGGTCAAGACTCTGAGACTATCGAACTCGCACCTGAATACG
CTGAGGCTATCGCAACCCGTGCAGGTGCGCTGGCTGGCATCTCTCCGATGTTCCAACCTTGCGTAGTTCCTCCTAAGCCGTGGACTGGCATTACTG
GTGGTGGCTATTGGGCTAACGGTCGTCCTCTGGCGCTGGTGCGTACTCACAGTAAGAAAGCACTGATGCGCTACGAAGACGTTTACATGCCTG
AGGTGTACAAAGCGATTAACATTGCGCAAAACACCGCATGGAAAATCAACAAGAAAGTCCTAGCGGTCGCCAACGTAATCACCAAGTGGAAGCATT
GTCCGGTCGAGGACATCCCTGCGATTGAGCGTGAAGAACTCCCGATGAAACCGGAAGACATCGACATGAATCCTGAGGCTCTCACCGCGTGGAAAC
GTGCTGCCGCTGCTGTGTACCGCAAGGACAAGGCTCGCAAGTCTCGCCGTATCAGCCTTGAGTTCATGCTTGAGCAAGCCAATAAGTTTGCTAACC
ATAAGGCCATCTGGTTCCCTTACAACATGGCACTGGCGCGGTCGTGTTTACGCTGTGTCAATGTTCAACCCGCAAGGTAACGATATGACCAAAGGAC
TGCTTACGCTGGCGAAAGGTAAACCAATCGGTAAGGAAGGTTACTACTGGCTGAAAATCCACGGTGCAAACTGTGCGGGTGTCGATAAGGTTCCGT
TCCCTGAGCGCATCAAGTTCATTGAGGAAAACCACGAGAACATCATGGCTTGCGCTAAGTCTCCACTGGAGAACACTTGGTGGGCTGAGCAAGATT
CTCCGTTCTGCTTCCTTGCGTTCTTTGAGTACGCTGGGGTACAGCACCACGGCCTGAGCTATAACTGCTCCCTTCCGCTGGCGTTTGACGGGT
CTTGCTCTGGCATCCAGCACTTCTCCGCGATGCTCCGAGATGAGGTAGGTGGTCGCGCGGTTAA

FIGURE 6. *(cont)*

CTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGGGATTGTTGCTAAGAAAGTCAACGAGATTCTACAAGCAGACGCAATCAATGGGACCGATAA
CGAAGTAGTTACCGTGACCGATGAGAACACTGGTGAAAATCTCTGAGAAAGTCAAGCTAAGGCACTGGCTGGTCAATGGCCTGGCTTACGG
TGTTACTCGCAGTGTGACTAAGCGTTCAGTCATGACGCTGGCTTACGGGTCCAAAGAGTTCGGCTTCCGTCAACAAGTGCTGGAAGATACCATTCA
GCCAGCTATTGATTCCGGCAAGGGTCTGATGTTCACTCAGCCGAATCAGGCTGCTGGATACATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGAC
GGTGGTAGCTGCGGTTGAAGCAATGAACTGGCTTAAGTCTGCTGCTAAGCTGCTGGCTGAGGTCAAAGATAAGAAGACTGGAGAGATTCTTCG
CAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGGTTTCCCTGTGTGGCAGGAATACAAGAAGCCTATTCAGACGCGCTTGAACCTGATGTTCCT
CGGTCAGTTCCGCTTACAGCCTACCATTAACACCAACAAAGATAGCGAGATTGATGCACACACAAACAGGAGTCTGGTATCGCTCCTAACTTTGTACA
CAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAGTGTGGGCACACGAGAAGTACGGAATCGAATCTTTGCACTGATTCACGACTCCTTCGGTAC
CATTCCGGCTGACGCTGCGAACCTGTTCAAAGCAGTGCGCGAAACTATGGTTGACACATATGAGTCTTGTGATGTACTGGCTGATTTCTACGACCA
GTTCGCTGACCAGTTGCACGAGTCTCAATTGGACAAAATGCCAGCACTTCCGGCTAAAGGTAACTTGAACCTCCGTGACATCTTAGAGTCGGACTT
CGCGTTCGCGTAAGCATGCAAGCTAATTCGGTGGAAACGAGGTCATCATTCCTTCCGAAAAAACGGTTGCATTTAAATCTTACATATGTAATACT
TTCAAAGACTACATTTGTAAGATTTGATGTTTGAGTCGGCTGAAAGATCGTACGTACCAATTATTGTTTCGTGATTGTTCAAGCCATAACACTGTA
GGGATAGTGGAAAGAGTGCTTCATCTGGTTACGATCAATAATTCAAACGGAGGAGACGATTTTGATGAAACCAGTAACGTTATACGATGTC
GCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCG
ATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTG
CACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCGAA
GCCTGTAAACGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAA
GCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCCGACTG
GGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGG
CATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTG
AATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCGATTACCGAGTCCGGGCTGCGCGTTGGT
GCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGGGG
CAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACC
CTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGA

FIGURE 7: Compete Vector with sfGFP SEQ ID NO 6

GTTCAGCTCAGTGATACCTGCGATCCCTCCGCGATTCACGACAGCCGGCACACCGATGTACACGTCATCTGCACCGTATTGCCCGTCCAAATATGT
GCTGACAGTTAATATGCTGTTTTCATTATGAAGAATGGCTTTTGTAATGCGAGCAAGACTCATCGCAACCCCATAATAAGTCGCGCCTTTTTCTC
AATGATATGGTAAGCTGCGTTTTTCACATCTACAATTTGGTCCAGCTCCTCTTGTTTGTACGCATCGTTTTTCTCAACGAGTTCACTGACCGG
CACACCGCCGACATTCGCGTGGCTCCAAACAGGAAGCTCTGTGTCGCCGTGCTCTCCGATAATATGCGCGTGTACGTTTTGAGGCGCTGCGCCAAA
GTATTCGCTCAGCATGAAACGGAATCTCGCAGAATCAAGTGTTGTGCCGCTTCCAATCACCCGCTCTTTGGCAGGCCGCTGAATTTCCATGTTGC
GTAAGTCAGGATATCAACCGGATTTGTCGCGACTAAGAAAATGCCGTCGTCAAATCCGCTCGCCATGACTTCACTAACGATGCCTTTGAAAATCTTCAA
GTTCTTTTCTACTAATTCAAGGCGTGTCTCACCAGGTTTTTGGTTTGCTCCGGCGCAAATGCAGACAATATCAGCATCCTTGCAGTCTTCATATGT
TCCGTAAGATGTTTTGACCGGTTGTGGCGCAAACGCCTTTCCGTGGTTTAAATCCATCACATCGCCCCATTGCTTTTCTTTATTTACATCAATGAC
CACAAGCTCATCTGTGATTCCTTGGTTAATTAACGCAAATGCATAACTGCTTCCAACAAAACCCGCTCCGATTAAAGCTACTTTATTTACATGTTT
GTTCATCATTAATCATCCTTCCAGGGTATGTCTTTTGTTTGTGAAGTATTTCACATTTATATTGTGCAACACTTCACACAAACT
TTTGCAAGAGAAAAGTTTTGTCTGATTTATGAACAAAAAGAAACCATCATTGATGGTTTCTTTCGGTAAGTCCCGTCTAGCCTTGCCCTCAATGG
GGAAGAGAACCGCTTAAGCCCGAGTCATTATAAACCATTTAGCACGTAATCAAAGCCAGGCTGATTCTGACCGGGCACTTGGGCGCTGCCATTA
TTAAAAATCACTTTTGCGTTGGTTGTATCCGTGTCCGCAGGCAGCGTCAGCGTGTAAATTCCGTCTGCATTTTTAGTCATTGGTTTCCAGGCCAA
GATCCGGTCAATTCAATTACTCGGCTCCCATCATGTTTATAGATATAAGCATTTACCTGGCTCCAATGATTCGGATTTTGATAGCCGATGGTTTTG
GCCGACGCTGGATCTCTTTTAACAAAACTGTATTTCTCGGTCCTCGTTACACCATCACTGTTCGTTCCTTTTAACATGATGGTGTATGTTTTGCCA
AATGGATCTCCTTTTCCGATTGTGAATTGATCTCCATCCTTAAACGCCGTCTCTGGTCCATTATTGATTTGATAAACGGCTTTTGTTGTATTCGCA
TCTGCACGCAAGGTAATCGTCAGTTGATCATTGAAAGAATGTGTTACACCTGTTTTGTAATTCTCAAGGAAAACATGAGGCGCTTTTGCAATATCA
TCAGGATAAAGCACAGCTACAGACCTGGCATTGATCGTGCCTGTTCACTTGAAATGAACCCGCTCCAGCTTTATTGTCATAC
CTGCCATCAGGCAATTTTGTTGCCGTATTGATAGAGACAGAGGATGAACCTGCATTTGCCAGCACAACGCCATGTGAGCCGCGCTGATTCATAAAT
ATCTGGTTGTTTCCATTCGGGTTCGAGAGTTCCTCAGCCATCACATTGTGAAATCTATTGACCGCAGTGATAGCCTGATCTTCAAAT
AAAGCACTCCCGCGATCGCCTATTTGGCTTTTCCCCGGGAACCTCACACCATTTCCGCCTCCCTCAGGTCTGGAAAGAAAGAGGCGTACTGCCT
GAACGAGAAGCTATCACCGCCCAGCCTAAACGGATATCATCATCGCTCATCCATGTCGAGATCCCCCCTATGCAAGGGTTTATTGTTTCTAAAATC
TGATTACCAATTAGAATGAATATTTCCCAAATATTAAATAATAAACACAAAAAAATTGAAAAAAGTGTTTCCACCATTTTTTCAATTTTTTATAAT
TTTTTTAATCTGTTATTTAAATAGTTTATAGTTTACATTTTCATTAGTCCATTCAATATTCTCTCCAAGATAACTACGAACTGCTAACAAA
ATTCTCTCCCTATGTTCTAATGGAGAAGATTCAGCCACTGCATTTCCCGCAATATCTTTTGGTATGATTTTACCCGTGTCCATAGTTAAAATCATA
CGGCATAAAGTTAATATAGAGTTGGTTTCATCATCCTGATAATTATCTATTAATTCCTCTGACGAATCCATAATGGCTCTTCTCACATCAGAAAAT
GGAATATCAGGTAGTAATTCCTCTAAGTCATAATTCTTTTATTTTTCGTTTTGCTTGGTAAAGCATTATGGTTAAATCTGAATTT
AATTCCTTCTGAGGAATGTATCCTTGTTCATAAAGCTCTTGTAACCATTCTCCATAAATAAATTCTTGTTTGGGAGGATGATTCCACGGTACCATT
TCTTGCTGAATAATAATTGTTAATTCAATATCGTAAGTTGCTTTTATCTCCTATTTTTTTTTGAAATAGG

FIGURE 7 *(cont)*

TCTAATTTTTTGTATAAGTATTTCTTTACTTTGATCTGTCAATGGTTCAGATACGACGACTAAAAAGTCAAGATCACTATTTGGTTTTAGTCCACT
CTCAACTCCTGATCCAAACATGTAAGTACCAATAAGGTTATTTTTTAAATGTTTCCGAAGTATTTTTTTCACTTTATTAATTTGTTCGTATGTATT
CAAATATATCCTCCTCACTATTTTGATTAGTACCTATTTTATATCCATAGTTGTTAATTAAATAAACTTAATTTAGTTTATTTATAGATTTCATTG
GCTTCTAAATTTTTTATCTAGATAATAATTTTTAGTTTAATTTTATCTTCTAGATTTATATGATATGATCTTTCATTTCCATAAAACTAAAGTAAG
TGTAAACCTATTCATTGTTTTTAAAAATATCTCTTGCCAGTCACGTTACGTTATTAGTTATTATAACATGTATTCACGAACGAAAATCGCCC
ATTCGCCAGCTGCAGGTAAAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAAGCT
TACATAAGGAGGAACTACTATGCGTAAAGGCGAAGAGCTGTTCACTGGTGTCGTCCCTATTCTGGTGGAACTGGATGGTGATGTTCAACGGTCATAA
GTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCCGGTACCTTGGCC
GACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGA
AGGCTATGTGCAGGAACGCACGATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCG
CATTGAGCTGAAAGGCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACACAATTTTAACACAGCCACACAATGTTTACATCACCGC
CGATAAACAAAAAAATGGCATTAAAGCGAATTTTAAAAATTCGCCACACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACAC
TCCAATCGGTGATGGTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATAT
GGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAATGATGATAATAATCTAGACCAGGCATCAAATAAAA
CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGG
CCTTTCTCGCGTTTTATACGTTTCGGTGATGAAGATCTTCCCGATGATTAATTAATTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATGCAGCAAT
GGCAAGAACGTTGCTCGAGGGTAAATGTGAGCACTCACAATTCATTTTGCAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTGTGTAAT
TGTGAGCGGATAACAATTAAGCTTAGTCGACAGCTAGCACATAAGGAGGAACTACTATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACA
TCGAACTGGCTGCTATCCCGTTCAACACTCTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTTGAGCATGAGTCTTACGAGA
TGGGTGAAGCACGCTTCCGCAAGATGTTTGAGCGTCAACTTAAAGCTGGTGAGGTTGCGGATAACGCTGCCGCCAAGCCTCTCATCACTACCCTAC
TCCCTAAGATGATTGCACGCATCAACGACTGGTTTGAGGAAGTGAAAGCTAAGCGCGCCCGACACAGCCTTCCAGTTCCTGCAAGAAATCA
AGCCGGAAGCCGTAGCGTACATCACCATTAAGACCACACTCTGGCTTGCCTAACCAGTGCTGACAATACAACCGTTCAGGCTGTAGCAAGCGCAATCG
GTCGGGCCATTGAGGACGAGGCTCGCTTCGGTCGTATCCGTGACCTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACAACTCAACAAGCGCG
TAGGGCACGTCTACAAGAAAGCATTTATGCAAGTTGTCGAGGCTGACATGCTCTCTACTCGGTGGCGAGGCGTGGTCTTCGTGGCATA
AGGAAGACTCTATTCATGTAGGAGTACGCTGCATCGAGATGCTCATTGAGTCAACCGGAATGGTTAGCTTACACCGCCAAAATGCTGGCGTAGTAG
GTCAAGACTCTGAGACTATCGAACTCGCACCTGAATACGCTGAGGCTATCGGCAACCCGTGCAGGTGCGCTGGCATCTCTCCGATGTTCCAAC
CTTGCGTAGTTCCTCCTAAGCCGTGGACTGGCATTACTGGTGGTGGCTAATTGGGCTAACGGTCGTCGTCCTCTGGCGCTGGTGCGTACTCACAGTA
AGAAAGCACTGATGCGCTACGAAGACGTTTACATGCCTGAGGTGTACAAAGCGATTAACATTGCGCAAAACACCGCATGGAAAATCAACAAGAAAAG
TCCTAGCGGTCGCCAACGTAATCACCAAGTGGAAGCATTGTCCGGTCGAGGACATCCCTGCGATTGAGCGTGAAGAACTCCCGATGAAACCGGAAG
ACATCGACATGAATCCTGAGGCTCTCACCGCGTGGAAACGTGCTGCCGCTGCTGTGTACCGCAA

FIGURE 7 *(cont)*

GGACAAGGCTCGCAAGTCTCGCCGTATCAGCCTTGAGTTCATGCTTGAGCAAGCCAATAAGTTTGCTAACCATAAGGCCATCTGGTTCCCTTACAA
CATGGACTGGCCGCGGTCGTGTTTACGCCTGTGTCAATGTTCAACCCGCAAGGTAACGATATGACCAAAGGACTGCTTACGCCTGGCCGAAAGGTAAACC
AATCGGTAAGGAAGGTTACTACTGGCTGAAAATCCACGGTGCAAACTGTGCGGGTGTCGATAAGGTTCCGTTCCCTGAGCGCATCAAGTTCATTGA
GGAAAACCACGAGAACATCATGGCTTGCGCTAAGTCTCCACTGGAGAACACTTGGTGGGCTGAGCAAGATTCTCCGTTCTGCTTCCTTGCGTTCTG
CTTTGAGTACGCCTGGGGTACAGCACCACGGCCTGAGCTATAACTGCTCCCTTCCGCTGGCGTTTGACGGGTCTTGCTCTGGCATCCAGCACTTCTC
CGCGATGCTCCGAGATGAGGTAGGTGTCGCGCGGTTAACTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGGGATTGTTGCTAAGAAAGTCAA
CGAGATTCTACAAGCAGACGCAATCAATGGGACCGATAACGAAGTAGTTACCGTGACCGATGAGAACACTGGTGAAATCTCTGAGAAAGTCAAGCT
GGGCACTAAGGCACTGGCTGGTCAATGGCTGGCTTACTCGCAGTGTGACTAAGCGTTCAGTCATGACGCTGGCTTACGGGTCCAAAGA
GTTCGGCTTCCGTCAACAAGTGCTGGAAGATACCATTCAGCCAGCTATTGATTCCGGCAAGGGTCTGATGTTCACTCAGCCGAATCAGGCTGCTGG
ATACATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGACGGTGGTAGCTGCGGTTGAAGCAATGAACTGGCTTAAGTCTGCTGCTAAGCTGCTGGC
TGCTGAGGTCAAAGATAAGAAGACTGGAGAGATTCTTCGCAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGGTTTCCCTGTGTGGCAGGAATA
CAAGAAGCCTATTCAGACGCGCTTGAACCTGATGTTCCTCGGTCAGTTCCGCTTACAGCCTACCATTAACACCAACAAAGATAGCGAGATTGATGC
ACACAAACAGGAGTCTGGTATCGCTCCTAACTTTGTACACAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAGTGTGGGCACACGAGAAGTACGG
AATCGAATCTTTTGCACTGATTCACGACTCCTTCGGTACCATTCCGGCTGACGCTGCGCGAACCTGTTCAAAGCAGTGCGCGAAACTATGGTTGACAC
ATATGAGTCTTGTGATGTACTGGCTGATTTCTACGACCAGTTCGCTGACCAGAGTCTCAATTGGACAAAATGCCAGCACTTCCGGCTAA
AGGTAACTTGAACCTCCGTGACATCTTAGAGTCGGACTTCGCGTTCGCGTAAGCATGCAAGCTAATTCGGTGGAAACGAGGTCATCATTTCCTTCC
GAAAAAACGGTTGCATTTAAATCTTACATATGTAATACTTTCAAAGACTACATTTGTAAGATTTGATGTTTGAGTCGGCTGAAAGATCGTACGTAC
CAATTATTGTTTCGTGATTGTTCAAGCCATAACACTGTAGGGATAGTGGAAGAGTGCTTCATCTGGTTACGATCAATCAAATATTCAAACGGAGG
GAGACGATTTTGATGAAACCAGTAACGTTATACGATGTCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGC
CACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAAACAG
TCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCC
AGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCGAAGCCTGTAAAACGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATT
AACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATC
AACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCA
TTAAGTTCTGTCTCGGCGCGTCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGG
AGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGC
GCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCG
CCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAAT
CAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAG
CTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAG

FIGURE 7 *(cont)*

GATCCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGGTGTCAAAAATAATAATAACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAA
ATCCATTATGTACTATTTAATTCTGCGTGACATCCCATCGATCAGACCAGTTTTTAATTTGTGTGTTTCCATGTCGTCCAGTTTGGAATACTCTTAA
CCTCATTGGAAATCGCGGCATAATCACTGGTGGTATGATTGATCGACCGCGTCAACAATGACCTTTATGCCATATTCTTCAGCGGCTGCACACATTT
CTTTAAATTCTTGTTCAGTACCTAAGTAACGGTTGCCAATTTGATACGATGTCGGCTGATACAGCCAGTACCAGTTCGACATGCTTTTATCTCCTT
GATTCCCTTCCTTTACTTGGTTAATCGGAGATGTCTGAATGGCTGTATATCCTGCATCATGAATATCCTTCATATTGTGTTTTAACGTATTGAACG
ACCAATTCCATGCATGAAGAATGGTTCCGCTTTTGATCGACGGTGCTGTAAGCTCATTCGATTTGTTCGCCGTTTCAGCACTCGCAGCCGCCGGTC
CTGCCAGAACCAAATGAAACAGCAATAAAAATCCAGCGAATAACGGCAGTAAAGAGGTTTTGAATCGTTTTGCAAACATTCTTGACACTCCTTATT
TGATTTTTTGAAGACTTACTTCGGAGTCAAAAAATCCCTCTTACTTCATTCTTCCGCTTCCTCCTTTCAAACCGATGTGAAGACTGGAGAATTTTGT
TAACGCTTACATTTAAAATTATCACAATCACTCTATCAAAAACAACTTGGCAGAGTGAATACAAATCAATGTTCCTATAAAAAAAGCTGCCCGAAAC
TGTGAACGCTTCCCTCGCTTTCCAAACAAAAAAGATGATTTCTTTTTATTTCTTCTTTACCGCTCTTCTGTAAGCCAGCTTCACAATCACATAC
CGAAGCAGAAACAGAACGCCCGCCGCAGACGATAAAAAGAAGAATCATTTTCAGCCAAGCGAAGAATGTATGGGGAATCCCCCCTGTCGTACATCGTA
AGCACCACTACCGCAAAGGCGACCGCAATTGATTCTATTCGCTGCTGCTTCACCCGTTTTTGCAGTTCTTCTTTGGTGTCTATTCTCGGCATGAGC
ATTCCGGATGCCGCAGCCCTGATGCAGTAATATAAGATAATGATGAACAAAAACACAAGGCTTGCAGCCCATTCTTTAAACGGAAATTCAAACATA
ATGCCGCGGATGATCACATCAAGCAGCGCAAATGCTCCCGCTATCATCCCAATCTCCGACCAGATTTTTTGTTCTTTTTCTTTACTCTCTTATTT
TTCCCATTCACGCCTGAAAGTCTATTACGCAGATTATTCATGACCTTCCTCCCGCACTTTTACGAATGGCTTATGGCTTACATGTTACAATAACAG
CCTGCGCTTCTTGCAATGAATAACTCAAAAGCCCGCCGTACAAGCCGGCAGGGCAGCAATCGTTAAAAACGGCCCCCAATTGATCATGACTCCGATCAC
GATAAAGACAAGCCCGATCAAGAACCAAATCAGAGCAAGCGGCACCATAAACCGCAGCCACTTGACATACGGAATACCGCTGGCGGCAAGCACCGC
CATGAGAACGCCGGATGTCGGGTTCACACAGTTGACGACCCCTTCTCCAAGCATAACCGCTTCAACCGCAACCTGTCTCGTGATTCCCATCAAATC
AGCGAGCGGCGCCAGGATTGGAATAAATACAACGGCTTCGCCAGAACCTGAAGAGATGAGAAAATGAAGCAGCGCACTGGCGATATACATGCCGAT
TGCCCCAGCAATCGGGCTGAATCCATCCAAAAGTGAAGCCAAAGCATTGACGACAGTATCGAGAAGCTTTCCATTTCAAGGATAACGGAAATGCT
TCGTGCCATCCCGACAATCAGCGCCCCGTATACAAGACTTTGGCAGCCCGTAATGAAGGTTTTGGCAATATCGTTCGCCGCAAGCCCGCCTATTAA
ACCGGCAAGGACAGAAATAAAAATAAAATGTCGCAGCCATTTGGGAATCTGACCAGCCAAGCTTCAAAGCGCCGTATAAAAATCCGACAAGTGAGAG
TCCCGCCACAGCCAAAATCAGCTTATGGCGAACGGTAAACGGCACTGACTGATCTTCTTCTTTACCGGCTTCGC

FIGURE 8 Complete Vector without sfGFP SEQ ID NO 7

TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGA
CGAAACAGCCTCTACAAATAATTTTGTTTAAAAGCTTACATAAGGAGGAACTACT**ATG...TAA**CCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAA
AGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATAC
GTTTCGGTGATGAAGATCTTCCCGATGATTAATTAATTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATGCAGCAATGGCAAGAACGTTGCTCG
AGGGTAAATGTGAGCACTCACAATTCATTTGCAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTGTGTAATTGTGACGGATAACAAT
TAAGCTTAGTCGACAGCTAGCACATAAGGAGGAACTACTATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAACTGGCTGCTATC
CCGTTCAACACTCTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTTGAGCATGAGTCTTACGAGATGGGTGAAGCACGCTTC
CGCAAGATGTTTGAGCGTCAACTTAAAGCTGGTGAGGTTGCGGATAACGCTGCCGCCAAGCCTCTCATCACTACCCTAACTCCCTAAGATGATTGCA
CGCATCAACGACTGGTTTGAGGAAGTGAAAGCTAAGCGCGGCAAGGCGCCCGACAGCCTTCCAGTTCCTGCAAGAAATCAAGCCGGAAGCCGTAGCG
TACATCACCATTAAGACCACTCTGGCTTGCCTAACCAGTGCTGACAATACAACCGTTCAGGCTGTAGCAAGCGCAATCGGTCGGGCCATTGAGGAC
GAGGCTCGCTTCGGTCGTATCCGTGACCTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACACAACTCAACACAAGCGCGTAGGCGCACGTCTACAAG
AAAGCATTTATGCAAGTTGTCGAGGCTGACATGCTCTCTAAGGGTCTACTCGGTGGCGAGGCGTGGTCTTCGTGGCATAAGGAAGACTCTATTCAT
GTAGGAGTACGCTGCATCGAGATGCTCATTGAGTCAACCGGAATGGTTAGCTTACACCGCCAAAATGCTGGCGTAGTAGGTCAAGACTCTGAGACT
ATCGAACTCGCACCTGAATACGCTGAGGCTATCGCAACCCGTGCAGGTGCGCTGGCTGGCATCTCTCCGATGTTCCAACCTTGCGTAGTTCCTCCT
AAGCCGTGGACTGGCATTACTGGTGGTGGCTATTGGGCTAACGGTCGTCCTCTGGCGCTGGTGCGTACTCACAGTAAGAAAGCACTGATGCGC
TACGAAGACGTTTACATGCCTGAGGTGTACAAAGCGATTAACATTGCGCAAAACACCGCATGGAAAATCAACAAGAAAGTCCTAGCGGTCGCCAAC
GTAATCACCAAGTGGAAGCATTGTCCGGTCGAGGACATCCCTGCGATTGAGCGTGAAGAACTCCCGATGAAACCGGAAGACATCGACATGAATCCT
GAGGCTCTCACCGCGTGGAAACGTGCTGCCGCTGTGTACCGCAAGGACAAGGCTCGCAAGTCTCGCCGTATCAGCCTTGAGTTCATGCTTGAG
CAAGCCAATAAGTTTGCTAACCATAAGGCCATCTGGTTCCCTTACAACATGGACTGGCGCGGTCGTGTTTACGCTGTGTCAATGTTCAACCCGCAA
GGTAACGATATGACCAAAGGACTGCTTACGCTGGCGAAAGGTAAACCAATCGGTAAGGAAGGTTACTACTGGCTGAAAATCCACGGTGCAAACTGT
GCGGGTGTCGATAAGGTTCCGTTCCCTGAGCGCATCAAGTTCATTGAGGAAAACCACGAGAACATCATGGCTTGCGCTAAGTCTCCACTGGAGAAC
ACTTGGTGGGCTGAGCAAGATTCTCCGTTCTTCCTTGCGTTCTTGAGTACGCTGGGGTACAGCACCACGGCCTGAGCTATAACTGCTCC
CTTCCGCTGGCGTTTGACGGGTCTTGCTCTGGCATCCAGCACTTCTCCGCGATGCTCCGAGATGAGGTGGTCGCGCGGTTAACTTGCTTCCT
AGTGAAACCGTTCAGGACATCTACGGGATTGTTGCTAAGAAAGTCAACGAGATTCTACAAGCAGACGCAATCAATGGGACCGATAACGAAGTAGTT
ACCGTGACCGATGAGAACACTGGTGAAATCTCTTGAGAAAGTCAAGCTGGGCACTAAGGCACTGGCTGGTCAATGGCTGGCTTACGGTGTTACTCGC
AGTGTGACTAAGCGTTCAGTCATGACGCTGGCTTACGGGTCCAAAGAGTTCGGCTTCCGTCAACACAAGTGCTGGAAGATACCATTCAGCCAGCTATT
GATTCCGGCAAGGGTCTGATGTTCACTCAGCCGAATCAGGCTGCTGGATACATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGACGGTGGTAGCT
GCGGTTGAAGCAATGAACTGGCTTAAGTCTGCTGGCTGCTGAGGTCAAAGATAAGAAGACTGGAGAG

FIGURE 8 (cont)

ATTCTTCGCAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGGTTTCCCTGTGTGGCAGGAATACAAGAAGCCTATTCAGACGCGCTTGAACCTG
ATGTTCCTCGGTCAGTTCCGCTTACAGCCTACCATTAACACCAACAAAGATAGCGAGATTGATGCACACACAACAGGAGTCTGGTATCGCTCCTAAC
TTTGTACACAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAGTGTGGGCACACGAGAAGTACGGAATCGAATCTTTTGCACTGATTCACGACTCC
TTCGGTACCATTCCGGCTGACGCTGCGAACCTGTTCAAAGCAGTGCGCGAAACTATGGTTGACACATATGAGTCTTGTGATGTACTGGCTGATTTC
TACGACCAGTTCGCTGACCAGTTGCACGAGTCTCAATTGGACAAAATGCCAGCACTTCCGGCTAAAGGTAACTTGAACCTCCGTGACATCTTAGAG
TCGGACTTCGCGTTCGCGTAAGCATGCAAGCTAATTCGGTGGAAACGAGGTCATCATTTCCTTCCGAAAAAACGGTTGCATTTAAATCTTACATAT
GTAATACTTTCAAAGACTACATTTGTAAGATTTGAGTCGGCTGAAAGATCGTACGTACCAATTATTGTTTCGTGATTGTTCAAGCCATA
ACACTGTAGGGATAGTGGAAAGAGTGCTTCATCTGGTTACGATCAATCAAATATTCAAACGGAGGGAGACGATTTTGATGAAACCAGTAACGTTAT
ACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGG
AAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTC
TGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCG
GCGTCGAAGCCTGTAAAACGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTG
CTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTA
CGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGG
CTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGC
AAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGC
GCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGATTTTCGCC
TGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAA
AAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCG
GGCAGTGA

FIGURE 9. Variant *LacI-T7-sfgfp* SEQ ID NO 8

TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGA
CGAAACAGCCTCTACAAATAATTTTGTTTAAAAGCTTACATAAGGAGGAACTACTATGAGAAAAGGAGAAGAATTATTTACAGGAGTTGTTCCAAT
TTTAGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTGGCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTT
CATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGACGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATAT
GAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGGAACGCACCATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGC
GGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAGGCATTGACTTTAAGAAGACGGCAATATCCTGGGCCATAAGCTGGA
ATACAATTTTAACAGCCACACAATGTTTACATCACCGCCGATAAACAAAAAATGGCATTAAAGCGAATTTCAAAATTCGCCACACAACGTGGAGGATGG
CAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATGGTCCCTGTTCTGCCCAGACAATCACTATCTGAGCACGCAAAGCGT
TCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGTTCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAA
ATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCAC
ACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATACGTTTCGGTGATGAAGATCTTCCCGATGATTAATTCAGAACGCTCGGTTGCCGCC
GGGCGTTTTTTATGCAGCAATGGCAAGAACGTTGCTCGAGGGTAAATGTGAGCACTCACAATTCATTTGCAAAAGTTGTTGACTTTATCTACAAG
GTGTGGCATAATGTGTGTAATTGTGAGCGGATAACAATTAAGCTTAGTCGACAGCTAGCACATAAGGAGGAACTACTATGAACACGATTAACATCG
CTAAGAACGACTTCTCTGACATCGAACTGGCTGCTATCCCGTTCAACACTCTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCC
TTGAGCATGAGTCTTACGAGATGGGTGAAGCACGCTTCCGCAAGATGTTTGAGCGTCAACTTAAAGCTGGTGAGGTTGCGGATAACGCTGCCGCCA
AGCCTCTTCATCACTACCCTACTCCCTAAGATGATTGCACGCATCAACGACTGGTTTGAGGAAGTGAAAGCTAAGCGCGGCAAGCGCCCGACAGCCT
TCCAGTTCCTGCAAGAAATCAAGCCGGAAGCCGTAGCGTACATCACCATTAAGACCACTCTGGCTTGCCTAACCAGTGCTGACAATACAACCGTTC
AGGCTGTAGCAAGCGCAATCGGTCGGGCCATTGAGGACGAGGCTCGCTTCGGTCGTATCCGTGACCTTGAAGCTAAGCACTTCAAGAAAAACGTTG
AGGAACAACTCAACAAGCGCGTAGGGCACGTCTACAAGAAAAGCATTTATGCAAGTTGTCGAGGCTGACATGCTCTCTTAAGGGTCTACTCGGTGGCG
AGGCGTGGTCTTCGTGGCATAAGGAAGACTCTATTCATGTAGGAGTACGCTGCATCGAGATGCTCATTGAGTCAACCGGAATGGTTAGCTTACACC
GCCAAAATGCTGGCGTAGTAGGTCAAGACTCTGAGACTATCGAACTCGCACCTGAATACGCTGAGGCTATCGCAACCCGTGCAGGTGCGCTGGCTG
GCATCTCTCCGATGTTCCAACCTTGCGTAGTTCCTCCTAAGCCGTGGACTGGCATTACTGGTGGTGGCTATTGGGCTAACGGTCGTCCTCTGG
CGCTGGTGCGTACTCACAGTAAGAAAGCACTGATGCGCTACGAAGACGTTTACATGCCTGAGGTGTACAAAGCGATTAACATTGCGCAAAACACCG
CATGGAAAATCAACAAGAAAGTCCTAGCGGTCGCCAACGTAATCACCAAGTGGAAGCATTGTCCGGTCGAGGACATCCCTGCGATTGAGCGTGAAG
AACTCCCGATGAAACCGGAAGACATCGACATGAATCCTGAGGCTCTCACCGCGTGGAAAACGTGCTGCCGCTGCTGTGTACCGCAAGGACAAGGCTC
GCAAGTCTCGCCGTATCAGCCTTGAGTTCATGCTTGAGCAAGCCAATAAGTTTGCTAACCATAAGGCCATCTGGTTCCCTTACACAACATGGACTGGC
GCGGTCGTGTTTACGCTGTGTCAATGTTCAACCCGCAAGGTAACGATATGACCAAAGGACTGCTTACGCTGGCGAAAGGTAAACCAATCGGTAAGG
AAGGTTACTACTGGCTGAAAAATCCACGGTGCAAACTGTGCGGGTGTCGATAAGGTTCCGTTCCCTGAGCGCATCAAGTTCATTGAGGAAAACCACG
AGAACATCATGGCTTGCGCTAAGTCTCCACTGGAGAACACTTGGTGGGCTGAGCAAGATTCTCCGTTCTGCTTCCTTGCGTTCTTTGAGTACG
CTGGGGTACAGCACCACGGCCTGAGCTATAACTGCTCCCTTCCGCTGGCGTTTGACGGGTCTTG

FIGURE 9. *(cont)*

CTCTGGCATCCAGCACTTCTCCGCGATGCTCCGAGATGAGGTAGGTGGTCGCGCGGTTAACTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGG
GATTGTTGCTAAGAAAGTCAACGAGATTCTACAAGCAGACGCAATCAATGGGACCGAATAACGAAGTAGTTACCGTGACCGATGAGAACACTGGTGA
AATCTCTGAGAAAGTCAAGCTGGGCACTAAGGCACTGGCTTACGGTGTTACTCGCAGTGTGACTAAGCGTTCAGTCATGAC
GCTGGCTTACGGGTCCAAAGAGTTCGGCTTCCGTCAACAAGTGCTGGAAGATACCATTCAGCCAGCTATTGATTCCGGCAAGGGTCTGATGTTCAC
TCAGCCGAATCAGGCTGCTGGATACATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGGTAGCTGCCGGTTGAAGCAATGAACTGGCTTAA
GTCTGCTGCTAAGCTGCTGGCTGCTGAGGTCAAAGATAAGAAGACTGGAGAGATTCTTCGCAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGG
TTTCCCTGTGTGGCAGGAATACAAGAAGCCTATTCAGACGCGCTTGAACCTGATGTTCCTCGGTCAGTTCCGCTTACAGCCTACCATTAACACCAA
CAAAGATAGCGAGATTGATGCACACAAACAGGAGTCTGGTATCGCTCCTAACTTTGTACACAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAGT
GTGGGCACACGAGAAGTACGGAATCGAATCTTTTGCACTGATTCACGACTCCTTCGGTACCATTCCGGCTGACGCTGCGAACCTGTTCAAAGCAGT
GCGCGAAACTATGGTTGACACATATGAGTCTTGTGATGTACTGGCTGATTTCTACGACCAGTTCGCTGACCAGTTGCACGAGTCTCAATTGGACAA
AATGCCAGCACTTCCCGGCTAAAGGTAACTTGAACCTCCGTGACATCTTAGAGTCGGACTTCGCGTTCGCGTAAGCATGCAAGCTAATTCGGTGGAA
ACGAGGTCATCATTTCCTTCCGAAAAAACGGTTGCATTTAAATCTTACATATGTAATACTTTCAAAGACTACATTTGTAAGATTTGATGTTTGAGT
CGGCTGAAAGATCGTACGTACCAATTATTGTTTCGTGATTGTTCAAGCCATAACACTGTAGGGATAGTGGAAAGAGTGCTTCATCTGGTTACGATC
AATCAAATATTCAAACGGAGAGGAGACGATTTTGATGAAACCAGTAACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCC
CGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCA
CAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCT
CGCGCCGATCAACTGGGTGCCAGCGTGGTGGTCGATGGTAGAACGAAGCGGCGTCGAAGCCTGTAAAACGGCGGTGCACAATCTTCTCGCGCAA
CGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGAT
GTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAA
ATCGCGCTGTTAGCGGCCCATTAAGTTCTGTCGCGCGTCTCTGGCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATA
GCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCC
AACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAA
GACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAG
GGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCG
TTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGA

FIGURE 10: Compete Vector with sfGFP Variant SEQ ID NO 9

GTTCAGCTCAGTGATACCTGCGATCCCTCCGCGATTCACGACAGCCGGCACACCGATGTACACGTCATCTGCACCGTATTGCCCGTCCAAATATGT
GCTGACAGTTAATATGCTGTTTTCATTATGAAGAATGGCTTTTGTAATGCGAGCAAGACTCATCGCAACCCCATAATAAGTCGCGCCTTTTTCTC
AATGATATGGTAAGCTGCGTTTTTCACATCTACAATTTGGTCCAGCTCCTCTTGTTTGTACGCATCGTTTTTCTCAACGAGTTCACTGACCGG
CACACCGCCGACATTCGCGTGGCTCCAAACAGGAAGCTCTGTGTCGCCGTGCTCTCCGATAATATGCGCGTGTACGTTTTGAGGCGCTGCGCCAAA
GTATTCGCTCAGCATGAAACGGAATCTCGCAGAATCAAGTGTTGTGCCGCTTCCAATCACCCGCTCTTTGGCAGGCCGCTGAATTTCCATGTTGC
GTAAGTCAGGATATCAACCGGATTTGTCGCGACTAAGAAAATGCCGTCGTCAAATCCGCTCGCCATGACTTCACTAACGATGCCTTTGAAAATCTTCAA
GTTCTTTTCTACTAATTCAAGGCGTGTCTCACCAGGTTTTTGGTTTGCTCCGGCGCAAATGCAGACAATATCAGCATCCTTGCAGTCTTCATATGT
TCCGTAAGATGTTTTGACCGGTTGTGGCGCAAACGCCTTTCCGTGGTTTAAATCCATCACATCGCCCATTGCTTTTCTTTATTTACATCAATGAC
CACAAGCTCATCTGTGATTCCTTGGTTAATTAACGCAAATGCATAACTGCTTCCAACAAAACCCGCTCCGATTAAAGCTACTTTATTTACATGTTT
GTTCATCATTAATCATCCTTCCAGGGTATGTCTTTTGTTTGTGAAGTATTTCACATTTATATTGTGCAACACTTCACACAAACT
TTTGCAAGAGAAAAGTTTTGTCTGATTTATGAACAAAAAGAAACCATCATTGATGGTTTCTTTCGGTAAGTCCCGTCTAGCCTTGCCCTCAATGG
GGAAGAGAACCGCTTAAGCCCGAGTCATTATATAAACCATTTAGCACGTAATCAAAGCCAGGCTGATTCTGACCGGGCACTTGGGCGCTGCCATTA
TTAAAAATCACTTTTGCGTTGGTTGTATCCGTGTCCGCAGGCAGCGTCAGCGTGTAAATTCCGTCTGCATTTTTAGTCATTGGTTTCCAGGCCAA
GATCCGGTCAATTCAATTACTCGGCTCCCATCATGTTTATAGATATAAGCATTTACCTGGCTCCAATGATTCGGATTTTGATAGCCGATGGTTTTG
GCCGACGCTGGATCTCTTTTAACAAAACTGTATTTCTCGGTCCTCGTTACACCATCACTGTTCGTTCCTTTTAACATGATGGTGTATGTTTTGCCA
AATGGATCTCCTTTTCCGATTGTGAATTGATCTCCATCCTTAAACGCCGTCTCTGGTCCATTATTGATTTGATAAACGGCTTTTGTTGTATTCGCA
TCTGCACGCAAGGTAATCGTCAGTTGATCATTGAAAGAATGTGTTACACCTGTTTTGTAATTCTCAAGGAAAACATGAGGCGCTTTTGCAATATCA
TCAGGATAAAGCACAGCTACAGACCTGGCATTGATCGTGCCTGTTCACTTGAAATGAACCCGCTCCAGCTTTATTGTCATAC
CTGCCATCAGGCAATTTTGTTGCCGTATTGATAGAGACAGAGGATGAACCTGCATTTGCCAGCACAACGCCATGTGAGCCGCGCTGATTCATAAAT
ATCTGGTTGTTTCCATTCGGGTTCGAGAGTTCCTCAGCCATCACATTGTGAAATCTATTGACCGCAGTGATAGCCTGATCTTCAAAT
AAAGCACTCCCGCGATCGCCTATTTGGCTTTTCCCCGGGAACCTCACACCATTTCCGCCTCCCTCAGGTCTGGAAAGAAAGAGGCGTACTGCCT
GAACGAGAAGCTATCACCGCCCAGCCTAAACGGATATCATCATCGCTCATCCATGTCGAGATCCCCCCTATGCAAGGGTTTATTGTTTCTAAAATC
TGATTACCAATTAGAATGAATATTTCCCAAATATTAAATAATAAACAAAAAAATTGAAAAAAGTGTTTCCACCATTTTTTCAATTTTTTTATAAT
TTTTTTAATCTGTTATTTAAATAGTTTATAGTTTACATTTTCATTAGTCCATTCAATATTCTCTCCAAGATAACTACGAACTGCTAACAAA
ATTCTCTCCCTATGTTCTAATGGAGAAGATTCAGCCACTGCATTTCCCGCAATATCTTTTGGTATGATTTTACCCGTGTCCATAGTTAAAATCATA
CGGCATAAAGTTAATATAGAGTTGGTTTCATCATCCTGATAATTATCTATTAATTCCTCTGACGAATCCATAATGGCTCTTCTCACATCAGAAAAT
GGAATATCAGGTAGTAATTCCTCTAAGTCATAATTCTTTTATTTTTTCGTTTTGCTTGGTTAAGCATTATGGTTAAATCTGAATTT
AATTCCTTCTGAGGAATGTATCCTTGTTCATAAAGCTCTTGTAACCATTCTCCATAAATAAATTCTTGTTTGGGAGGATGATTCCACGGTACCATT
TCTTGCTGAATAATAATTGTTAATTCAATATATCGTAAGTTGCTTTTATCTCCTATTTTTTTTTGAAATAGGTCTAATTTTTTGTATAAGTATTTCT
TTACTTTGATCTGTCAATGGTTCAGATACGACGACTAAAAAGTCAAGATCACTATTTGGTTTTA

FIGURE 10 ***(cont)***

GTCCACTCTCAACTCCTGATCCAAACATGTAAGTACCAATAAGGTTATTTTTTAAATGTTTCCGAAGTATTTTTTTCACTTTATTAATTTGTTCGT
ATGTATTCAAATATATCCTCCTCACTATTTTGATTAGTACCTATTTTATATCCATAGTTGTTAATTAAATAAACTTAATTTAGTTTATTTATAGAT
TTCATTGGCTTCTAAATTTTTTATCTAGATAATAATTATTTTAGTTAATTTTATTCTAGATTATATATGATATGATCTTTCATTTCCATAAAACTA
AAGTAAGTGTAAACCTATTCATTGTTTTAAAAATATCTCTTGCCAGTCACGTTACGTTATTAGTTATAGTTATTATAACATGTATTCACGAACGAA
AATCGCCATTCGCCAGCTGCAGGTAAAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTA
GAAGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCTCTACAAATAATTTTGTTTAAAAGCTTACATAAGGAGGAA
CTACTATGAGAAAAGGAGAAGAATTATTTACAGGAGTTGTTCCAATTTTAGTGGAACTGGATGGTGATGTCAACGGTCATAAGTTTTCCGTGCGTG
GCGAGGGTGAAGGTGACGCAACTAATGGTAAACTGACGCTGAAGTTCATCTGTACTACTGGTAAACTGCCGGTACCTTGGCCGACTCTGGTAACGA
CGCTGACTTATGGTGTTCAGTGCTTTGCTCGTTATCCGGACCATATGAAGCAGCATGACTTCTTCAAGTCCGCCATGCCGGAAGGCTATGTGCAGG
AACGCACGATTTCCTTTAAGGATGACGGCACGTACAAAACGCGTGCGGAAGTGAAATTTGAAGGCGATACCCTGGTAAACCGCATTGAGCTGAAAG
GCATTGACTTTAAAGAAGACGGCAATATCCTGGGCCATAAGCTGGAATACAATTTTAACAGCCACAATGTTTACATCACCGCCGATAAACAAAAAA
ATGGCATTAAAGCGAATTTTAAAATTCGCCACAACGTGGAGGATGGCAGCGTGCAGCTGGCTGATCACTACCAGCAAAACACTCCAATCGGTGATG
GTCCTGTTCTGCTGCCAGACAATCACTATCTGAGCACGCAAAGCGTTCTGTCTAAAGATCCGAACGAGAAACGCGATCATATGGTTCTGCTGGAGT
TCGTAACCGCAGCGGGCATCACGCATGGTATGGATGAACTGTACAAATAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTT
TCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTATACGTTTCGGTGATG
AAGATCTTCCCGATGATTAATTAATTCAGAACGCTCGGTTGCCGCCGGGCGTTTTTTATGCAGCAATGGCAAGAACGTTGCTCGAGGGTAAATGTG
AGCACTCACAATTCATTTTGCAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATGTGTGTAATTGTGAGCGGATAACAATTAAGCTTAGTCG
ACAGCTAGCACATAAGGAGGAACTACTATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAACTGGCTGCTATCCCGTTCAACACT
CTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTTGAGCATGAGTCTTACGAGATGGGTGAAGCACGCTTCCGCAAGATGTTT
GAGCGTCAACTTAAAGCTGGTGAGGTTGCGGATAACGCTGCCGCCAAGCCTCTCATCACTACCCTACTCCCTAAGATGATTGCACGCATCAACGAC
TGGTTTGAGGAAGTGAAAGCTAAGCGCGGCAAGCGCCCGACAGCCTTCCAGTTCCTGCAAGAAATCAAGCCGGAAGCCGTAGCGTACATCACCATT
AAGACCACTCTGGCTTGCCTAACCAGTGCTGACAATACAACCGTTCAGGCTGTAGCAAGCGCAATCGGTCGGGCCATTGAGGACGAGGCTCGCTTC
GGTCGTATCCGTGACCTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACAACTCAACAAGCGCGTAGGGCACGTCTACAAGAAAGCATTTATG
CAAGTTGTCGAGGCTGACATGCTCTCTAAGGGTCTACTCGGTGGCGAGGCGTGGTCTTCGTGGCATAAGGAAGACTCTATTCATGTAGGAGTACGC
TGCATCGAGATGCTCATTGAGTCAACCGGAATGGTTAGCTTACACCGCCAAAATGCTGGCGTAGTAGGTCAAGACTCTGAGACTATCGAACTCGCA
CCTGAATACGCTGAGGCTATCGCAACCCGTGCAGGTGCGCTGGCTGGCATCTCTCCGATGTTCCAACCTTGCGTAGTTCCTCCTAAGCCGTGGACT
GGCATTACTGGTGGTGGCTATTGGGCTAACGGTCGTCGTCCTCTGGCGCTGGTGCGTACTCACAGTAAGAAAGCACTGATGCGCTACGAAGACGTT
TACATGCCTGAGGTGTACAAAGCGATTAACATTGCGCAAAACACCGCATGGAAAATCAACAAGAAAGTCCTAGCGGTCGCCAACGTAATCACCAAG
TGGAAGCATTGTCCGGTCGAGGACATCCCTGCGATTGAGCGTGAAGAACTCCCGATGAAACCGGAAGACAT

FIGURE 10 *(cont)*

CGACATGAATCCTGAGGCTCTCACCGCGTGGAAACGTGCTGCCGCTGTGTACCGCAAGGACAAGGCTCGCAAGTCTCGCCGTATCAGCCTTGA
GTTCATGCTTGAGCAAGCCAATAAGTTTGCTAACCATAAGGCCATCTGGTTCCCTTACAACATGGACTGGCGCCGGTCGTGTTTACGCTGTGTCAAT
GTTCAACCCGCAAGGTAACGATATGACCAAAGGACTGCTTACGCTGGCGAAAGGTAAACCAATCGGTAAGGAAGGTTACTACTGGCTGAAAATCCA
CGGTGCAAACTGTGCGGGTGTCGATAAGGTTCCGTTCCCTGAGCGCATCAAGTTCATTGAGGAAAACCACGAGAACATCATGGCTTGCGCTAAGTC
TCCACTGGAGAACACTTGGTGGGCTGAGCAAGATTCTCCGTTCTTCCTTGCGTTCTTGCTTTGAGTACGCCTGGGGTACAGCACCACGGCCTGAG
CTATAACTGCTCCCTTCCGCTGGCGTTTGACGGGTCTTGCTCTGGCATCCAGCACTTCTCCGCGATGCTCCGAGATGAGGTAGGTGGTCGCCGCGGT
TAACTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGGGATTGTTGCTAAGAAAGTCAACGAGATTCTACAAGCAGACGCAATCAATGGGACCGA
TAACGAAGTAGTTACCGTGACCGATGAGAACACTGGTGAAATCTCTGAGAAAGTCAAGCTGGGCACTAAGGCCACTGGCTGGTCAATGGCTGGCTTA
CGGTGTTACTCGCAGTGTGACTAAGCGTTCAGTCATGACGCTGGCTTCACGGGTCCAAAGAGTTCCGGCTTCCGTCAACAAGTGCTGGAAGATACCAT
TCAGCCAGCTATTGATTCCGGCAAGGGTCTGATGTTCACTCAGCCGAATCAGGCTGCTGGATACATGGCTAAGCTGATTTGGGAATCTGTGAGCGT
GACGGTGGTAGCTGCCGGTTGAAGCAATGAACTGGCTTAAGTCTGCTGCTAAGCTGCTGGCTGCTGAGGTCAAAGATAAGAAGACTGGAGACTGGA
TCGCAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGGTTTCCCTGTGTGGCAGGAAGAATACAAGAAGGCCTATTCAGACGCGCTTGAACCTGATGTT
CCTCGGTCAGTTCCGCTTACAGCCTACCATTAACACCAACAAAGATAGCGAGATTGATGCACACACAAACAGGAGTCTGGTATCGCTCCTAACTTTGT
ACACAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAGTGTGGGCACACGAGAAGTACGGAATCGAATCTTTTGCACTGATTCACGACTCCTTCGG
TACCATTCCGGCTGACGCTGCGAACCTGTTCAAAGCAGTGCGCGAAACTATGGTTGACACATATGAGTCTTGTGATGTACTGGCTGATTTCTACGA
CCAGTTCGCTGACCAGTTGCACGAGTCTCAATTGGACAAAATGCCAGCACTTCCGGCTAAAGGTAACTTGAACCTCCGTGACATCTTAGAGTCGGA
CTTCGCGTTCGCGTAAGCATGCAAGCTAATTCGGTGGAAACGAGGTCATCATTTCCTTCCGAAAAAACGGTTGCATTTAAATCTTACATATGTAAT
ACTTTCAAAGACTACATTTGTAAGATTTGATGTTTGAGTCGGCTGAAAGATCGTACGTACCAATTATTGTTTCGTGATTGTTCAAGCCATAACACT
GTAGGGATAGTGGAAAGAGTGCTTCATCTGGTTACGATCAATCAAATATTCAAACGGAGGAGACGATTTTGATGAAACCAGTAACGTTATACGAT
GTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCG
GCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCC
CTGCACGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTC
GAAGCCTGTAAAACGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTG
GAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCGA
CTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGC
TGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATG
CTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTT
GGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGATTTTCGCCTGCTG
GGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACC
ACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGG

FIGURE 10 *(cont)*

CACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGGATCCTGAGCGCCGGTCGCTACCATTACCAGTTGGT
CTGGTGTCAAAAATAATAATAACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTATGTACTATTTAAATTCTGCGTGACATCCC
ATCGATCAGACCAGTTTTTAATTTGTGTGTTTCCAGTTTGGAATACTCTTAACCTCATTGGAAATCGCGGCATAATCACTGGTGGTAT
GATTGATGACCGCGTCAACAATGACCTTTATGCCATATTCTTCAGCGGCTGCACACATTTCTTTAAATTCTTGTTCAGTACCTAAGTAACGGTTGC
CAATTTGATACGATGTCGGCTGATACAGCCAGTACCAGTTCGACATGCTTTTATCTCCTTCCTTTACTTGGTTAATCGGAGATGTCT
GAATGGCTGTATATCCTGCATCATGAATATCCTTCATATTGTGTTTTAACGTATTGAACGACCAATTCCATGCATGAAGAATGGTTCCGCTTTTGA
TCGACGGTGCTGTAAGCTCATTCGATTTGTTCGCCGTTTCAGCACTCGCAGCCGCCGGTCCTGCCAGAACCAAATGAAACAGCAATAAAAATCCAG
CGAATAACGGCAGTAAAGAGGTTTTGAATCGTTTTGCAAACATTCTTGACACTCCTTATTTGATTTTTTGAAGACTTACTTCGGAGTCAAAAATCC
CTCTTACTTCATTCTTTCCGCTTCCTCCTTTCAAACCGATGTGAAGACTGGAGAATTTTGTTAACGCTTACATTTAAAATTATCACAATCACTCTAT
CAAAACAACTTGGCAGAGTGAATACAAATCAATGTTCCTATAAAAAAAGCTGCCCGAAACTGTGAACGCTTCCCTCGCTTTCCAAACAAAAAAGAT
GATTTCTTTTTTATTTCTTCTTTACCGCTCTTCTGTAAGCCAGCTTCACAATCACATACCGAAGCAGAAACACAGAACGCCCGCCGCAGCACGATAAAA
AGAAGAATCATTTCAGCCAAGCGAAGAATGTATGGGGAATCCCCCCTGTCGTACATCGTAAGCACCACTACCGCAAAGGCGACCGCAATTGATTCT
ATTCGCTGCTGCTTCACCCGTTTTTGCAGTTCTTCTTTGGTGTCTATTCTCGGCATGAGCATTCCGGATGCCGCAGCCCTGATGCAGTAATATAAG
ATAATGATGAACAAAAAACACACAAGGCTTGCAGCCCATTCTTTAAACGGAAATTCAAACATAATGCCGCGGATGATCACATCAAGCAGCGCAAATGCT
CCCGCTATCATCCCAATCTCCGACCAGATTTTTTGTTCTTTTTCTCTTATTTTTCCCATTCACGCCTGAAAGTCTATTACGCAGATTA
TTCATGACCTTCCTCCCGCACTTTTACGAATGGCTTATGGCTTACATGTTACAATAACAGCCTGCGCTTCTGCAATGAATAACTCAAAAGCCCGCC
GTACAAGCCGGCGGGCAGCAATCGTTAAAACGGCCCCCAATTGATCATGACTCCGATCACGATAAAGACAAGCCCGATCAAGAACCAAATCAGAGC
AAGCGGCACCATAAACCGCAGCCACTTGACATACGGAATACCGCTGGCGGCAAGCACCGCCATGAGAACGCCGGATGTCGGGTTCACACAGTTGAC
GACCCCTTCTCCAAGCATAACCGCTTCAACCGCAACCTGTCTCGTGATTCCCATCAAATCAGCGAGCGGCGCCAGGATTGGAAATAAATACAACGGC
TTCGCCAGAACCTGAAGAGATGAGCAGCGCACTGGCGATATACATGCCGATTGCCCCAGCAATCGGGCTGAATCCATCCAAAAGTGA
AGCCAAAGCATTGACGACAGTATCGAGAAGCTTTCCATTTTCAAGGATAACGGAAATGCTTCGTGCCATCCCGACAATCAGCGCCCCGTATACAAG
ACTTTGGCAGCCCGTAATGAAGGTTTTGGCAATATCGTTCGCCGCAAGCCCGCCTATTAAACCGGCAAGGACAGAAATAAAAATAAAATGTCGCAGC
CATTTGGGAATCTGACCAGCCAAGCTTCAAAGCGCCGTATAAAAATCCGACACAAGTGAGAGTCCCGCCACAGCCCAAAATCAGCTTATGGCGAACGGT
AAACGGCACTGACTGATCTTCTTCTTTACCGGCTTCGC

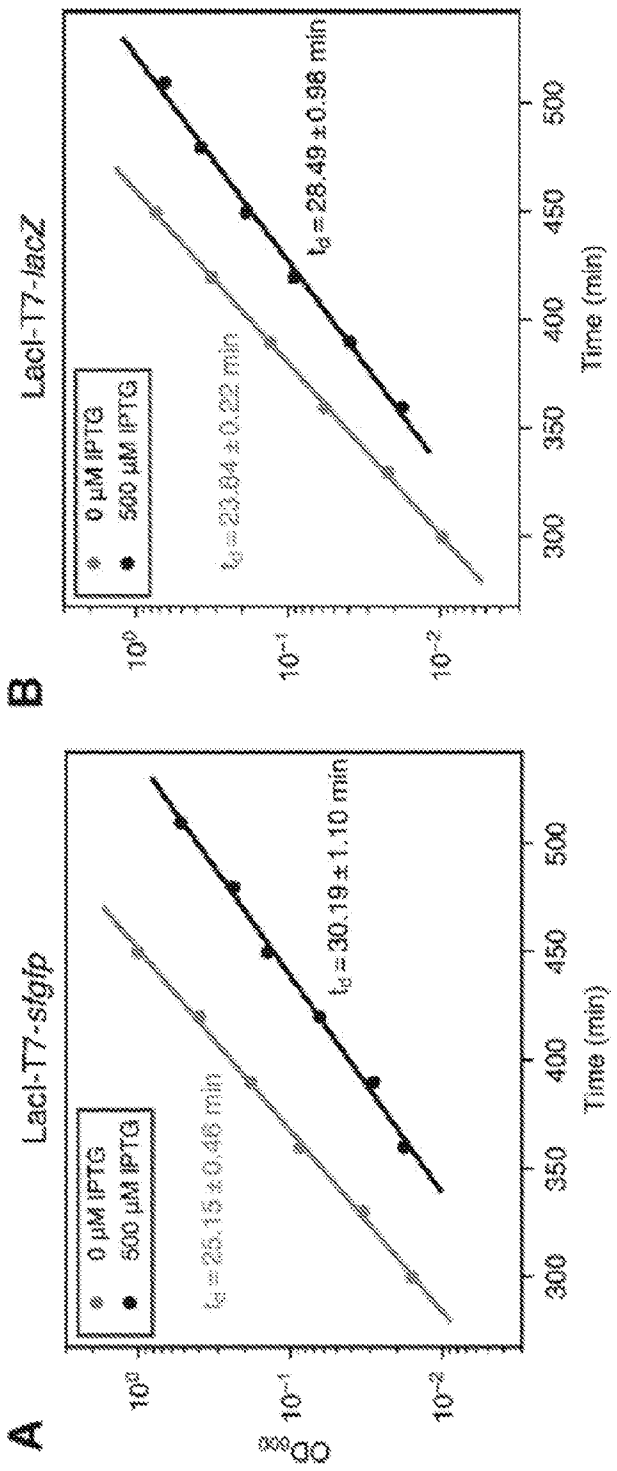

FIGURE 11 Growth curves of LacI-T7 strains

FIGURE 12 sfGFP expression from Phy-spank and LacI-T7

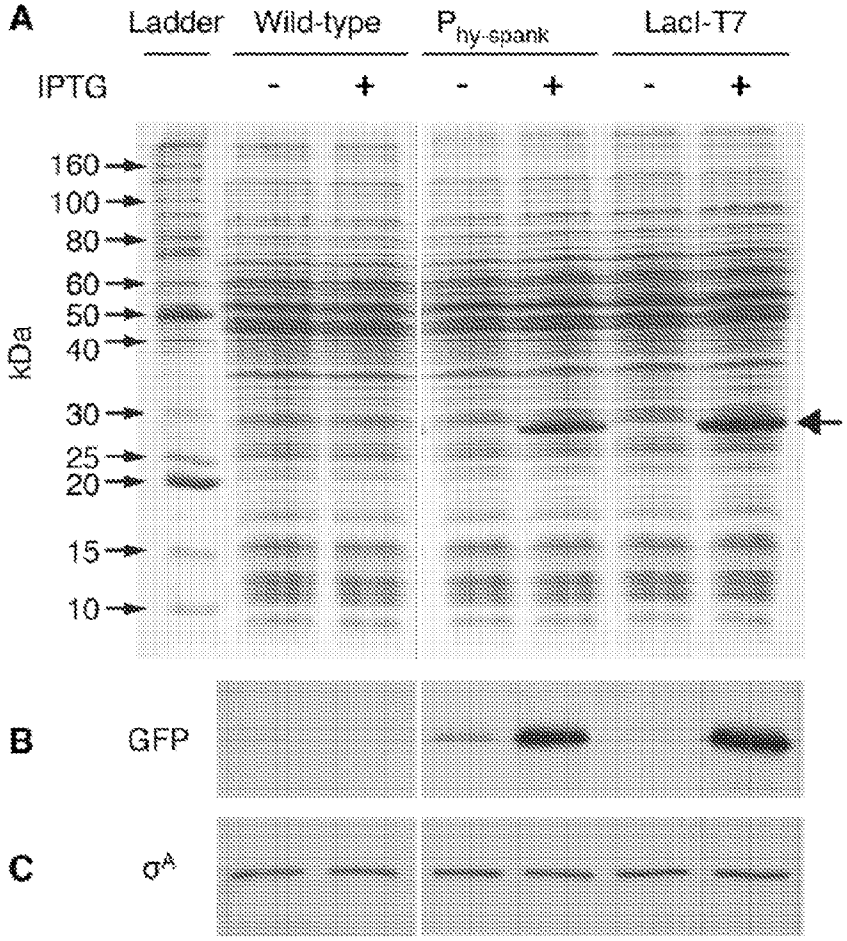

FIGURE 13 Performance of LacI-T7 measured via β-galactosidase