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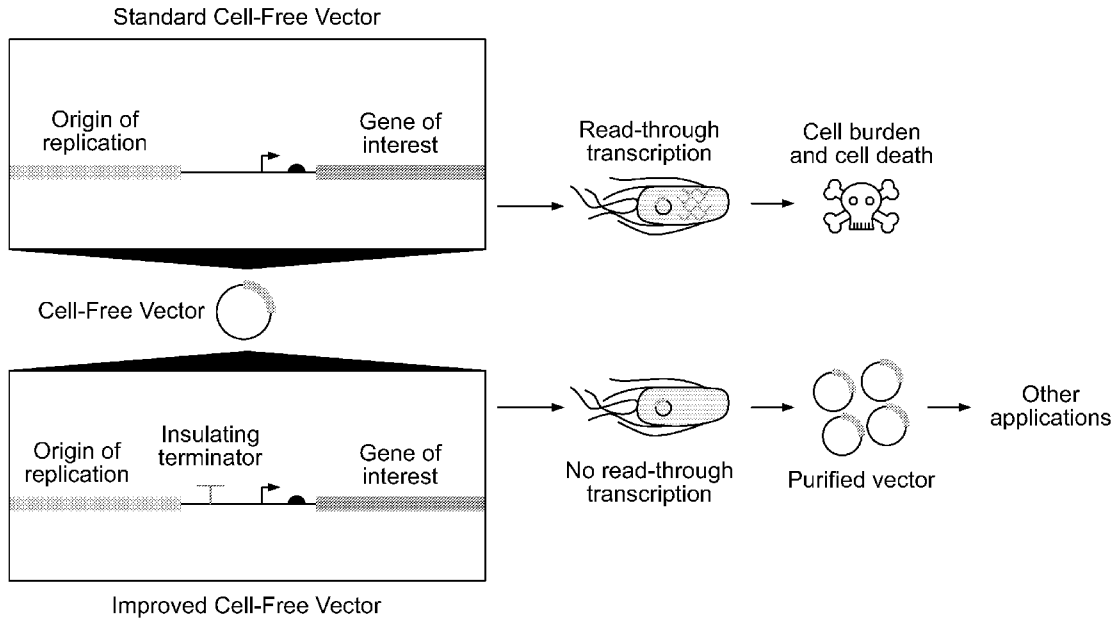

FIG. 1

(57) Abstract: The present disclosure relates to improved methods, compositions, and kits for cell-free expression of proteins *in vitro*.

[Continued on next page]

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CELL-FREE EXPRESSION VECTORS AND METHODS FOR IMPROVED PROTEIN PRODUCTION

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Application Serial No. 63/445,245, filed on February 13, 2023. The entire contents of the foregoing are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to cell-free systems, kits, and methods for producing proteins.

BACKGROUND

Cell-free protein-synthesis (CFPS) systems are emerging as an attractive alternative to conventional expression systems that rely on living cells (Katzen et al., "The Past, Present and Future of Cell-Free Protein Synthesis," Trends Biotechnol. 23: 150- 156 (2005)). This is because, over the past decade, cell-free protein synthesis reactions: (i) can be completed in less than a day; (ii) use cheaper reagents; (iii) fold complex proteins by routinely forming disulfide bonds; and (iv) can be scaled to 100 L. Two main approaches have been used for *in vitro* transcription/translation: one is based on cell-free extracts (CFEs), often derived from *Escherichia coli*, rabbit reticulocytes, or wheat germ, and the second is based on reconstituted protein synthesis from purified components (Shimizu et al., "Cell-Free Translation Reconstituted With Purified Components," Nat. Biotechnol. 19:75 1 -755 (2001)). Because of their ability to co-activate multiple biochemical networks in a single integrated platform (Jewett et al., "An Integrated Cell-Free Metabolic Platform for Protein Production and Synthetic Biology," Mol. Syst. Biol. 4:220 (2008)), CFPS systems are increasingly used in many important biotechnology and synthetic biology applications (Ryabova et al., "Functional Antibody Production Using Cell-Free Translation: Effects of Protein Disulfide Isomerase and Chaperones," Nat. Biotechnol. 15:79-84 (1997); Noireaux et al., "Principles of Cell-Free Genetic Circuit Assembly," Proc. Nat'l. Acad. Sci. U.S.A. 100: 12672- 12677 (2003); Yang et al., "Rapid Expression of Vaccine Proteins for B-Cell Lymphoma in a Cell-Free System," Biotechnol. Bioeng. 89:503-51 1 (2005)).

SUMMARY

The present disclosure relates to improved cell-free systems, methods, and kits for expressing proteins *in vitro*. In particular, the present disclosure relates to avoiding, reducing, or preventing read-through of toxic product proteins during generation of plasmids, while avoiding, decreasing, or preventing a reduction in protein synthesis of the product protein during CFPS at the commercial scale.

In one aspect, the disclosure provides methods of creating or modifying a cell-free expression vector, the methods including obtaining a cell-free expression vector including (i) an origin of replication (ori), (ii) a nucleic acid sequence encoding a protein or RNA, (iii) a promoter arranged to drive expression of the protein or RNA, and (iv) one or more selectable markers; and inserting into the cell-free expression vector an insulating terminator sequence at a location that is between 0 and 10,000 nucleotides in a 5' direction from the promoter.

In another aspect, the disclosure provides methods of performing protein or RNA synthesis *in vitro*, the methods including synthesizing protein or RNA *in vitro* using a cell-free expression vector, wherein the cell-free expression vector includes (i) an origin of replication (ori); (ii) a nucleic acid encoding protein or RNA to be synthesized; (iii) a promoter arranged to drive expression of the protein or RNA; (iv) an insulating terminator sequence located between 0 and 10,000 nucleotides in a 5' direction from the promoter; and (iv) one or more selectable markers.

These methods can avoid or reduce read-through of toxic product proteins during plasmid generation, while avoiding or decreasing a reduction in protein synthesis of the product protein during cell-free protein synthesis. For example, the methods can avoid or reduce production of toxic RNA products.

In general, the cell-free expression vectors with the insulating terminators enable synthesis of the protein or RNA at higher yields, with higher growth rates, and/or with fewer sequence mutations than synthesis of the protein or RNA using a cell-free expression vector without an insulating terminator. In some instances, the cell-free expression vector with the insulating terminator enables synthesis of the protein or RNA at higher levels compared to levels of synthesis of the protein or RNA using a cell-free expression vector without an insulating terminator.

In certain embodiments of the new methods, the cell-free expression vector contains a gene for a protein or RNA that inhibits or slows cellular replication in cells

containing the cell-free expression vector and/or a gene for a protein or RNA that reduces plasmid yield from the cells containing the cell-free expression vector.

In various embodiments, the insulating terminator sequence can be rnpB-T1 (SEQ ID NO: 820), rrnB T1 (SEQ ID NO: 14), L3S2P21 (SEQ ID NO: 318), or L3S2P56 (SEQ ID NO: 319). In various embodiments, the promoter can be a T7 phage promoter, a lac promoter, a trp promoter, a recA promoter, a ribosomal RNA promoter, a Sp6 promoter, an araBad promoter, a pTac promoter, or a J23119 promoter.

In some embodiments, the insulating terminator sequence is located 27 to 37 or 35 to 37, or 37 nucleotides in the 5' direction from the promoter. In some embodiments, the insulating terminator sequence is located about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500, about 8000, about 8500, about 9000, about 9500, or about 10,000 nucleotides in the 5' direction from the promoter that is arranged to drive expression of the protein or RNA.

In certain embodiments, the insulating terminator sequence is located 0 to 10,000 nucleotides in a 3' direction of the ori, e.g., the insulating terminator sequence is located 30 to 40 nucleotides, or 40 nucleotides, in the 3' direction of the ori. In some embodiments, the insulating terminator sequence is located about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500, about 8000, about 8500, about 9000, about 9500, or about 10,000 nucleotides in the 3' direction of the ori.

In some embodiments, the synthesizing step includes using a cell-free protein synthesis platform, for example, wherein the cell-free protein synthesis platform

includes a system for *in vitro* transcription of mRNA and/or translation of polypeptides.

In certain embodiments, the cell-free expression vector further includes a Ribosome-binding site (RBS) and/or an Open Reading Frame (ORF).

In another aspect, the disclosure provides kits for use in a method of modifying a cell-free expression vector, wherein the kits include a cell-free expression vector that includes (i) an origin of replication (ori), (ii) a nucleic acid sequence encoding a protein or RNA, (iii) a promoter arranged to drive expression of the protein or RNA, and (iv) one or more selectable markers; an insulating terminator sequence that is to be inserted at a location between 0 and 10,000 nucleotides in a 5' direction from the promoter in the cell-free expression vector; and cloning reagents.

In another aspect, the disclosure provides kits for performing protein or RNA synthesis *in vitro*, where the kits include reagents for cell-free protein or RNA synthesis; and a cell-free expression vector comprising (i) an origin of replication (ori); (ii) a nucleic acid encoding protein or RNA to be synthesized; (iii) a promoter arranged to drive expression of the protein or RNA; (iv) an insulating terminator sequence that is located between 0 and 10,000 nucleotides in a 5' direction from the promoter; and (iv) one or more selectable markers.

In these kits, the insulating terminator sequence can be rnpB-T1 (SEQ ID NO: 820), rrnB T1 (SEQ ID NO: 14), L3S2P21 (SEQ ID NO: 318), or L3S2P56 (SEQ ID NO: 319), and/or the promoter can be a T7 phage promoter, a lac promoter, a trp promoter, a recA promoter, a ribosomal RNA promoter, a Sp6 promoter, an araBad promoter, a pTac promoter, or a J23119 promoter.

In embodiments of these kits, the insulating terminator can be located between 0 and 10,000 nucleotides in a 5' direction from the promoter, e.g., 27 to 37, or 37, nucleotides in the 5' direction from the promoter. In some embodiments, the insulating terminator sequence is located about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500,

about 8000, about 8500, about 9000, about 9500, or about 10,000 nucleotides in the 5' direction from the promoter.

In some embodiments of the kits, the insulating terminator sequence is located 0 to 10,000 nucleotides, or 30 to 40 nucleotides, or 40 nucleotides, in a 3' direction of the ori. In some embodiments, the insulating terminator sequence is located about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500, about 8000, about 8500, about 9000, about 9500, or about 10,000 nucleotides in the 3' direction of the ori.

In certain embodiments of the kits, the cell-free expression vector further includes a Ribosome-binding site (RBS) and/or an Open Reading Frame (ORF).

Also provided herein are cell-free expression vectors including (i) an origin of replication (ori), (ii) a nucleic acid sequence encoding a protein or RNA, (iii) a promoter arranged to drive expression of the protein or RNA, (iv) one or more selectable markers, and (v) an insulating terminator sequence at a location that is between 0 and 10,000 nucleotides in a 5' direction from the promoter.

In these cell-free expression vectors, the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820), rrnB T1 (SEQ ID NO: 14), L3S2P21 (SEQ ID NO: 318), or L3S2P56 (SEQ ID NO: 319), and/or the promoter can be a T7 phage promoter, a lac promoter, a trp promoter, a recA promoter, a ribosomal RNA promoter, a Sp6 promoter, an araBad promoter, a pTac promoter, or a J23119 promoter.

In some embodiments of the cell-free expression vectors, the cell-free expression vectors includes a backbone. In some instances, the backbone is from one of the following vectors: pJL1, pY71, p70a, pBEST, pEXP5, or pT7CFE.

In certain embodiments of the cell-free expression vectors, at least a portion of the cell-free expression vector includes the following sequence: gggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttccggcttatcggtcagtttcacctg atttacgtaaaaacccgcttcggcgggtttttgcttttggaggggcagaaagatgaatgactgtccacgacgctatacccaaa agaaagctggccttttgctcacatgttcttatcccgcgaaattaatacgactcactatag (SEQ ID NO: 818).

In some aspects of the cell-free expression vectors, the vectors include, consist of, or consist essentially of, the features shown in FIG. 5.

In embodiments of these cell-free expression vectors, the insulating terminator can be located between 0 and 10,000 nucleotides in a 5' direction from the promoter, e.g., 27 to 37, or 37, nucleotides in the 5' direction from the promoter. In some embodiments, the insulating terminator sequence is located about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500, about 8000, about 8500, about 9000, about 9500, or about 10,000 nucleotides in the 5' direction from the promoter.

In some embodiments of the cell-free expression vectors, the insulating terminator sequence is located 0 to 10,000 nucleotides, or 30 to 40 nucleotides, or 40 nucleotides, in a 3' direction of the ori. In some embodiments, the insulating terminator sequence is located about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500, about 8000, about 8500, about 9000, about 9500, or about 10,000 nucleotides in the 3' direction of the ori.

In certain embodiments of the cell-free expression vectors, the cell-free expression vector further includes a Ribosome-binding site (RBS) and/or an Open Reading Frame (ORF).

Standard expression vectors for cell free protein synthesis (CFPS) exhibit some read-through expression of the protein of interest in common cloning strains of *E. coli*. This may not always be problematic for plasmid production, but can become so when the gene is toxic or confers any significant fitness defect. This expression results from transcriptional read-through from other parts of the plasmid. Accordingly, the methods provided herein reduce, avoid, or prevent read-through of

product proteins when preparing plasmids for use in CFPS, but decrease, avoid, or prevent a reduction of protein synthesis of the product protein during CFPS at the commercial scale. The present disclosure solves this problem, for example, by adding insulating terminator sequences that are in the 5' direction of the promoter and in the 3' direction of the origin of replication (ori).

Definitions

The terms "nucleic acid" and "oligonucleotide," as used herein, refer to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and to any other type of polynucleotide that is an N glycoside of a purine or pyrimidine base. There is no intended distinction in length between the terms "nucleic acid," "oligonucleotide," and "polynucleotide," and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. For use in the present invention, an oligonucleotide also can comprise nucleotide analogs in which the base, sugar or phosphate backbone is modified as well as non-purine or non-pyrimidine nucleotide analogs.

Oligonucleotides can be prepared by any suitable method, including direct chemical synthesis by a method such as the phosphotriester method of Narang et al., 1979, *Meth. Enzymol.* 68:90-99; the phosphodiester method of Brown et al., 1979, *Meth. Enzymol.* 68:109-151; the diethylphosphoramidite method of Beaucage et al., 1981, *Tetrahedron Letters* 22:1859-1862; and the solid support method of U.S. Pat. No. 4,458,066, each incorporated herein by reference. A review of synthesis methods of conjugates of oligonucleotides and modified nucleotides is provided in Goodchild, 1990, *Bioconjugate Chemistry* 1(3): 165-187, incorporated herein by reference.

The term "primer," as used herein, refers to an oligonucleotide capable of acting as a point of initiation of DNA synthesis under suitable conditions. Such conditions include those in which synthesis of a primer extension product complementary to a nucleic acid strand is induced in the presence of four different nucleoside triphosphates and an agent for extension (for example, a DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature.

A primer can be a single-stranded DNA. The appropriate length of a primer depends on the intended use of the primer but typically ranges from about 6 to about

225 nucleotides, including intermediate ranges, such as from 15 to 35 nucleotides, from 18 to 75 nucleotides and from 25 to 150 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template nucleic acid, but must be sufficiently complementary to hybridize with the template. The design of suitable primers for the amplification of a given target sequence is well known in the art and described in the literature cited herein.

Primers can incorporate additional features that allow for the detection or immobilization of the primer but do not alter the basic property of the primer, that of acting as a point of initiation of DNA synthesis. For example, primers may contain an additional nucleic acid sequence at the 5′ end which does not hybridize to the target nucleic acid, but which facilitates cloning or detection of the amplified product, or which enables transcription of RNA (for example, by inclusion of a promoter) or translation of protein (for example, by inclusion of a 5′-UTR, such as an Internal Ribosome Entry Site (IRES) or a 3′-UTR element, such as a poly$(A)_n$ sequence, where n is in the range from about 20 to about 200). The region of the primer that is sufficiently complementary to the template to hybridize is referred to herein as the hybridizing region.

The term "promoter" refers to a cis-acting DNA sequence that directs RNA polymerase and other trans-acting transcription factors to initiate RNA transcription from the DNA template that includes the cis-acting DNA sequence.

The terms "target, "target sequence," "target region," and "target nucleic acid," as used herein, are synonymous and refer to a region or sequence of a nucleic acid which is to be amplified, sequenced or detected.

The term "hybridization," as used herein, refers to the formation of a duplex structure by two single-stranded nucleic acids due to complementary base pairing. Hybridization can occur between fully complementary nucleic acid strands or between "substantially complementary" nucleic acid strands that contain minor regions of mismatch. Conditions under which hybridization of fully complementary nucleic acid strands is strongly preferred are referred to as "stringent hybridization conditions" or "sequence-specific hybridization conditions." Stable duplexes of substantially complementary sequences can be achieved under less stringent hybridization conditions; the degree of mismatch tolerated can be controlled by

suitable adjustment of the hybridization conditions. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length and base pair composition of the oligonucleotides, ionic strength, and incidence of mismatched base pairs, following the guidance provided by the art (see, e.g., Sambrook et al., 1989, Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Wetmur, 1991, Critical Review in Biochem. and Mol. Biol. 26(3/4):227-259; and Owczarzy et al., 2008, *Biochemistry*, 47: 5336-5353, which are incorporated herein by reference).

The term "amplification reaction" refers to any chemical reaction, including an enzymatic reaction, which results in increased copies of a template nucleic acid sequence or results in transcription of a template nucleic acid. Amplification reactions include reverse transcription, the polymerase chain reaction (PCR), including Real Time PCR (see U.S. Pat. Nos. 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications (Innis et al., eds, 1990)), and the ligase chain reaction (LCR) (see Barany et al., U.S. Pat. No. 5,494,810). Exemplary "amplification reactions conditions" or "amplification conditions" typically comprise either two or three step cycles. Two-step cycles have a high temperature denaturation step followed by a hybridization/elongation (or ligation) step. Three step cycles comprise a denaturation step followed by a hybridization step followed by a separate elongation step.

As used herein, a "polymerase" refers to an enzyme that catalyzes the polymerization of nucleotides. "DNA polymerase" catalyzes the polymerization of deoxyribonucleotides. Known DNA polymerases include, for example, *Pyrococcus furiosus* (Pfu) DNA polymerase, *E. coli* DNA polymerase I, T7 DNA polymerase and *Thermus aquaticus* (Taq) DNA polymerase, among others. "RNA polymerase" catalyzes the polymerization of ribonucleotides. The foregoing examples of DNA polymerases are also known as DNA-dependent DNA polymerases. RNA-dependent DNA polymerases also fall within the scope of DNA polymerases. Reverse transcriptase, which includes viral polymerases encoded by retroviruses, is an example of an RNA-dependent DNA polymerase. Known examples of RNA polymerase ("RNAP") include, for example, T3 RNA polymerase, T7 RNA polymerase, SP6 RNA polymerase and *E. coli* RNA polymerase, among others. The

foregoing examples of RNA polymerases are also known as DNA-dependent RNA polymerase. The polymerase activity of any of the above enzymes can be determined by means well known in the art.

As used herein, a primer is "specific," for a target sequence if, when used in an amplification reaction under sufficiently stringent conditions, the primer hybridizes primarily to the target nucleic acid. Typically, a primer is specific for a target sequence if the primer-target duplex stability is greater than the stability of a duplex formed between the primer and any other sequence found in the sample. One of skill in the art will recognize that various factors, such as salt conditions as well as base composition of the primer and the location of the mismatches, will affect the specificity of the primer, and that routine experimental confirmation of the primer specificity will be needed in many cases. Hybridization conditions can be chosen under which the primer can form stable duplexes only with a target sequence. Thus, the use of target-specific primers under suitably stringent amplification conditions enables the selective amplification of those target sequences that contain the target primer binding sites.

As used herein, "expression template" refers to a nucleic acid that serves as either a substrate for transcribing at least one RNA that can be translated into a polypeptide or protein or a substrate than can be translated into a polypeptide or protein. Expression templates include nucleic acids composed of DNA or RNA. Suitable sources of DNA for use a nucleic acid for an expression template include genomic DNA, cDNA and RNA that can be converted into cDNA. Genomic DNA, cDNA and RNA can be from any biological source, such as a tissue sample, a biopsy, a swab, sputum, a blood sample, a fecal sample, a urine sample, a scraping, among others. The genomic DNA, cDNA and RNA can be from host cell or virus origins and from any species, including extant and extinct organisms. As used herein, "expression template" and "transcription template" have the same meaning and are used interchangeably.

As used herein, "translation template" refers to an RNA product of transcription from an expression template that can be used by ribosomes to synthesize polypeptide or protein.

As used herein, the term "cap" (or "5′-cap") refers to a chemical modification of the 5′-terminus of a translation template. A cap for eukaryotic translation templates

can include a guanine nucleotide connected to the mRNA via a 5′ to 5′ triphosphate linkage ("5′,5′-GpppG" or "G(5′)ppp(5′)G"). The N-7 position guanine cap can methylated ("m^7GpppG" or "m^7G(5)ppp(5′)G"). Translation templates that include cap can be designated by 5′,5′-GpppG-, G(5′)ppp(5′)G-, m^7G(5′)ppp(5′)G- or m^7GpppG-translation templates.

As used herein, "cap-dependent," as the term modifies "translation" or "translation template," refers to the requirement of the translation template to include a 5′-cap for efficient protein synthesis from that translation template.

As used herein, "cap-independent," as the term modifies "translation" or "translation template," refers to the lack of a requirement that the translation template include a 5′-cap for efficient protein synthesis from that translation template.

The term "reaction mixture," as used herein, refers to a solution containing reagents necessary to carry out a given reaction.

To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes is at least 80% of the length of the reference sequence, and in some embodiments is at least 90% or 100%. The nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein nucleic acid "identity" is equivalent to nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. Percent identity between two polypeptides or nucleic acid sequences is determined in various ways that are within the skill in the art, for instance, using publicly available computer software such as Smith Waterman Alignment (Smith, T. F. and M. S. Waterman (1981) J Mol Biol 147:195-7); "BestFit" (Smith and Waterman, Advances in Applied Mathematics, 482-489 (1981)) as incorporated into GeneMatcher Plus™, Schwarz and Dayhof (1979) Atlas of Protein Sequence and Structure, Dayhof, M.O., Ed, pp 353-358; BLAST

program (Basic Local Alignment Search Tool; (Altschul, S. F., W. Gish, et al. (1990) J Mol Biol 215: 403-10), BLAST-2, BLAST-P, BLAST-N, BLAST-X, WU-BLAST-2, ALIGN, ALIGN-2, CLUSTAL, or Megalign™ (DNASTAR™) software. In addition, those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the length of the sequences being compared. In general, for proteins or nucleic acids, the length of comparison can be any length, up to and including full length (e.g., 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%).

Reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one element is present, unless the context clearly requires that there be one and only one element. The indefinite article "a" or "an" thus usually means "at least one."

The term "about" means within a statistically meaningful range of a value or values such as a stated concentration, length, molecular weight, pH, time frame, temperature, pressure or volume. Such a value or range can be within an order of magnitude, typically within 20%, more typically within 10%, and even more typically within 5% of a given value or range. The allowable variation encompassed by "about" will depend upon the particular system under study.

The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, and includes the endpoint boundaries defining the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their

entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1 shows a diagram of an example showing that standard cell-free vectors can result in cell growth issues when the gene of interest that is cloned into the plasmid is toxic or causes an increased cell burden (top) and that these issues can be mitigated when an insulating terminator is used as described herein (bottom).

FIG. 2 is a diagram of an example of an unmodified pJL1 expression vector.

FIG. 3 is a diagram of an example of a modified version of the pJL1 expression vector as described herein, where the insulating terminator sequence (rnpB-T1) is located immediately downstream of the origin of replication and 27 nucleotides upstream of the T7 promoter.

FIGs. 4A-4B are images of examples of Petri dishes that show that terminators in the 5` direction of the T7 promoter reduced read through expression.

FIG. 5 is a diagram of an example of a modified version of the pJL1 expression vector, where the insulating terminator sequence (rnpB-T1) is 40 nucleotides downstream of the origin of replication and 37 nucleotides upstream of the T7 promoter.

FIG. 6A are images of examples of Petri dishes that show that an alternate spacing of the insulating terminator resulted in a greater reduction of read through expression.

FIG. 6B is a bar graph that shows measured fluorescence (RFU) and culture optical density (OD) for three independent colonies chosen from each vector as described herein. Fluorescence is indicative of read through expression of the protein sfGFP from the vector. The measurement is normalized to cell culture density.

FIG. 7 is a bar graph showing that use of an insulating terminator resulted in comparable protein production as compared to experiments in which an insulating terminator was not used.

DETAILED DESCRIPTION

Standard expression vectors for cell free protein synthesis (CFPS) may exhibit read-through expression of the protein of interest in common cloning strains of *E. coli*. Normally, this is not problematic for plasmid production, but can become so when the gene is toxic or confers any significant fitness defect to the cells used to generate the plasmids. This read-through expression results from transcriptional read-through from other parts of the plasmid. The methods, compositions, and kits provided herein reduce read-through of product proteins when preparing large numbers of plasmids for use in CFPS, but decrease, avoid, or prevent a reduction of protein synthesis of the product protein during CFPS at the commercial scale.

I. Methods

As shown in FIG. 1, standard cell-free vectors can result in cell growth issues when the gene of interest that is cloned into the plasmid is toxic or causes increased cell burden (top of FIG. 1). The present disclosure solves this problem of read through expression by adding insulating terminator sequences that are in the 5' direction of the promoter and in the 3' direction of the origin of replication (ori) (bottom of FIG.1), for example.

The methods described herein can reduce read through expression by, for example, at least a 23-fold amount (that is, when compared to the parent plasmid).

Methods for Making a Modified Cell-Free Expression Vector

In one embodiment, this disclosure provides methods of creating or modifying a cell-free expression vector to avoid, inhibit, reduce, or prevent read-through of toxic product proteins during generation of plasmids, while avoiding, decreasing, or preventing a reduction in protein synthesis of the product protein during CFPS at the commercial scale. The methods include: (a) obtaining a cell-free expression vector including (i) a promoter, (ii) an ori, (ii) a nucleic acid sequence encoding a protein or RNA, (iii) a promoter arranged to drive expression of the protein or RNA, and (iv) one or more selectable markers and (b) inserting into the cell-free expression vector an insulating terminator sequence at a location that is between 0 and 10,000 nucleotides in a 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 10,000 nucleotides in the 5' direction from the

first nucleotide of the promoter sequence), and is also located 0 to 10,000 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 10,000 nucleotides in the 3' direction from the last nucleotide of the ori sequence).

For example, the insulating terminator sequence can be located between 0 and 500 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 500 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and can also be located 0 to 500 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 500 nucleotides in the 3' direction from the last nucleotide of the ori sequence).

(a) Obtaining a Cell-Free Expression Vector

As described below, cell-free expression vectors are known in the art and are explained below. Known cell-free expression vectors include pJL1, pY71, p70a, pBEST, pEXP5, pT7CFE.

(b) Inserting an Insulating Terminator Sequence

The preparation of cell-free expression vectors can be carried out using standard cloning procedures well known in the art, e.g., as described by Joseph Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL (Cold Springs Harbor 1989), including the November 18, 2014 updated version of Sambrook, and U.S. Patent No. 4,237,224 to Cohen and Boyer, which are hereby incorporated by reference in their entireties. For example, standard cloning procedures, well known to one skilled in the art, may be used to insert a terminator sequence according to the desired spacing as noted above (*i.e.*, an insulating terminator sequence that is located between 0 and 10,000 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 10,000 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and is also located 0 to 10,000 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 10,000 nucleotides in the 3' direction from the last nucleotide of the ori sequence).

For example, the insulating terminator sequence can be located between 0 and 500 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 500 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and can also be located 0 to 500 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 500 nucleotides in the 3' direction from the last nucleotide of the ori sequence). In some embodiments, the insulating terminator sequence can be located about 50 nucleotides in the 5' direction from the promoter (e.g., about 20, about 25, about 30, about 35, about 40, about 45, about 50 nucleotides in the 5' direction from the promoter). In some instances, the insulating terminator sequence can be located about 27 to 37 or 35 to 37, or 37 nucleotides in the 5' direction from the promoter. In certain embodiments, the insulating terminator sequence is located 0 to 50 nucleotides in a 3' direction of the ori, (e.g., about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50 nucleotides in the 3' direction of the ori). In some instances, the insulating terminator sequence is located 30 to 40 nucleotides, or 40 nucleotides, in the 3' direction of the ori.

Methods of Performing Protein Synthesis In Vitro

The disclosure also provides methods of performing protein synthesis *in vitro.* the method comprising synthesizing protein or RNA *in vitro* using a cell-free expression vector, wherein the cell-free expression vector includes: (i) an origin of replication (ori); (ii) a nucleic acid encoding protein or RNA to be synthesized; (iii) a promoter arranged to drive expression of the protein or RNA; (iv) an insulating terminator sequence located between 0 and 10,000 nucleotides in a 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 10,000 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and is also located 0 to 10,000 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 10,000 nucleotides in the 3' direction from the last nucleotide of the ori sequence); and (iv) one or more selectable markers.

For example, the insulating terminator sequence can be located between 0 and 500 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 500 nucleotides in the 5' direction from the first

nucleotide of the promoter sequence), and can also be located 0 to 500 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 500 nucleotides in the 3' direction from the last nucleotide of the ori sequence).

(a) Providing a Nucleic Acid That Encodes a Protein or RNA product

A nucleic acid molecule is any nucleic acid sequence that encodes a desired protein or RNA product.

(b) Preparing a Cell-Free Expression Vector from the Nucleic Acid

As provided herein, a desired source nucleic acid is cloned into the modified cell-free expression vector as described above. Generally, the use of cell-free expression vectors to produce and isolate a protein of interest involves inserting a source nucleic acid molecule (for protein or RNA) into a cell-free expression vector to which the molecule is heterologous (i.e., not normally present). One or more desired nucleic acid molecules encoding one or more proteins may be inserted into the vector. When multiple nucleic acid molecules are inserted, the multiple nucleic acid molecules may encode the same or different enzymes. The heterologous nucleic acid molecule is inserted into the expression system or vector in proper sense (5' → 3') orientation relative to the promoter and any other 5' regulatory molecules, and correct reading frame. The preparation of the cell-free expression vectors can be carried out using standard cloning procedures well known in the art, e.g., as described by Joseph Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL (Cold Springs Harbor 1989), including the November 18, 2014 updated version of Sambrook, and, e.g., in U.S. Patent No. 4,237,224 to Cohen and Boyer, which are hereby incorporated by reference in their entireties.

(c) Synthesizing the Product Protein in Vitro Using the Cell-Free Expression Vector

The modified cell-free expression vectors including the nucleic acid molecule is then introduced by means of transformation and replicated in a suitable host cell. The components, systems, and methods disclosed herein may be applied to, or adapted to cell-free protein synthesis methods as known in the art. See, for example,

U.S. Patent Nos. 5,478,730; 5,556,769; 5,665,563; 6,168,931; 6,548,276; 6,869,774; 6,994,986; 7,118,883; 7,186,525; 7,189,528; 7,235,382; 7,338,789; 7,387,884; 7,399,610; 7,776,535; 7,817,794; 8,703,471; 8,298,759; 8,715,958; 8,734,856; 8,999,668; and 9,005,920. See also U.S. Published Application Nos. 2018/0016614, 2018/0016612, 2016/0060301, 2015-0259757, 2014/0349353, 2014-0295492, 2014-0255987, 2014-0045267, 2012-0171720, 2008-0138857, 2007-0154983, 2005-0054044, and 2004-0209321. See also U.S Published Application Nos. 2005-0170452; 2006-0211085; 2006-0234345; 2006-0252672; 2006-0257399; 2006-0286637; 2007-0026485; 2007-0178551. See also Published PCT International Application Nos. 2003/056914; 2004/013151; 2004/035605; 2006/102652; 2006/119987; and 2007/120932. See also Jewett, M.C., Hong, S.H., Kwon, Y.C., Martin, R.W., and Des Soye, B.J.2014, "Methods for improved in vitro protein synthesis with proteins containing non-standard amino acids," U.S. Patent Application Serial No.: 62/044,221; Jewett, M.C., Hodgman, C.E., and Gan, R.2013, "Methods for yeast cell-free protein synthesis," U.S. Patent Application Serial No.: 61/792,290; Jewett, M.C., J.A. Schoborg, and C.E. Hodgman. 2014, "Substrate Replenishment and Byproduct Removal Improve Yeast Cell-Free Protein Synthesis," U.S. Patent Application Serial No.: 61/953,275; and Jewett, M.C., Anderson, M.J., Stark, J.C., Hodgman, C.E.2015, "Methods for activating natural energy metabolism for improved yeast cell-free protein synthesis," U.S. Patent Application Serial No.: 62/098,578. See also Guarino, C., & DeLisa, M. P. (2012). A prokaryote-based cell-free translation system that efficiently synthesizes glycoproteins. Glycobiology, 22(5), 596-601. The contents of all of these references are incorporated in the present application by reference in their entireties.

II. Cell-Free Expression Vectors

The disclosure further provides modified cell-free expression plasmids, methods for making them, and methods for using them. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double-stranded DNA loop into which additional DNA segments can be ligated. Such vectors are referred to herein as "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of

plasmids. In the present specification, "plasmid" and "vector" are used interchangeably.

While cell-free expression vectors are well known in the art, we briefly describe below the required and optional features of the cell-free expression vectors.

Necessary Features of Cell-Free Expression Vectors

Insulating Terminator Sequences

Provided herein are expression vectors that include an insulating terminator sequence. The term "insulating" means that the terminator sequence is located between 0 and 10,000 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 10,000 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and is also located 0 to 10,000 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 10,000 nucleotides in the 3' direction from the last nucleotide of the ori sequence).

For example, the insulating terminator sequence can be located between 0 and 500 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 500 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and can also be located 0 to 500 nucleotides in the 3' direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 500 nucleotides in the 3' direction from the last nucleotide of the ori sequence).

Examples of terminator sequences are described in Chen, YJ., Liu, P., Nielsen, A. *et al.* Characterization of 582 natural and synthetic terminators and quantification of their design constraints. *Nat Methods* 10, 659–664 (2013); Guillaume Cambray, Joao C. Guimaraes, Vivek K. Mutalik, Colin Lam, Quynh-Anh Mai, Tim Thimmaiah, James M. Carothers, Adam P. Arkin, Drew Endy, Measurement and modeling of intrinsic transcription terminators, *Nucleic Acids Research*, Volume 41, Issue 9, 1 May 2013, Pages 5139–5148; Diana G Calvopina-Chavez, Mikaela A Gardner, Joel S Griffitts, Engineering efficient termination of bacteriophage T7 RNA polymerase transcription, *G3 Genes|Genomes|Genetics*, Volume 12, Issue 6, June 2022, jkac070, each of which are hereby incorporated in their entireties by reference.

Numerous examples of specific terminator sequences (SEQ ID NOs:1 to 581 and SEQ ID NO:820) are listed in Appendix 1, which is appended to this application (parts.igem.org/Terminators/ Catalog). This list contains a useful, but non-exhaustive, list of terminators that may be used in certain embodiments. The iGEM parts registry name, descriptions (such as natural contexts or synthetic origins), and sequences are provided.

In some embodiments, the terminator sequence is rnpB-T1, rrnB-T1, L3S2P21, or L3S2P56 (or a terminator sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence similarity to one of these terminator sequences). In some embodiments, more than one terminator sequence is included in the cell-free expression vector. In some embodiments, only one terminator sequence is included in the cell-free expression vector.

Promoters

For the purposes of expressing a nucleic acid sequence encoding one or more desired proteins, different promoters can be used to produce genes at various levels and rates. Depending upon the host system utilized, any one of a number of suitable promoters may be used. Promoters are well known in the art to one of skill in the art. For instance, T7 phage promoter, lac promoter, trp promoter, recA promoter, ribosomal RNA promoter, Sp6 promoter, araBad promoter, pTac promoter, or J23119 promoter.

Numerous examples of additional promoter sequences (SEQ ID NOs:582 to 814 and SEQ ID NO:819) are also listed in Appendix 2, which is appended to this application. This list contains a useful, but non-exhaustive, list of promoters that may be used in certain embodiments. The iGEM parts registry name, descriptions (such as natural contexts or synthetic origins), and sequences are provided.

Origin of Replication (ori)

The ori is the place where DNA replication begins, enabling a plasmid to reproduce itself as it must to survive within cells. The replicons of plasmids are generally different from those used to replicate the host's chromosomal DNA, but they still rely on the host machinery to make additional copies. In some embodiments, more than one ori is included in the cell-free expression vector. In some embodiments, only one ori is included in the cell-free expression vector.

Selectable Marker(s)

Selectable marker(s) are well known in the art and can be categorized into those based on resistance genes that confer the ability to grow in the presence of toxic compounds such as antibiotics or herbicides that kill or otherwise compromise untransformed tissue (negative selection). Alternatively, a range of positive selection systems are available which provide transformed tissues with an enhanced ability to utilize, for example, an unusual carbohydrate or amino acid supply and thus enrich the culture for transformed tissue expressing the marker gene.

In some embodiments, the selectable marker is Kanamycin, Spectinomycin, Streptomycin, Ampicillin, Carbenicillin, Bleomycin, Erythromycin, Polymyxin B, Tetracycline, or Chloramphenicol.

Open Reading Frame (ORF)

As would be recognized by one skilled in the art, the cell-free expression vectors include an open reading frame.

Ribosomal Binding Site (RBS)

There are other specific initiation signals required for efficient gene transcription and translation in prokaryotic cells that can be included in the nucleic acid construct to maximize peptide production, e.g., the Shine -Dalgarno ribosome binding site. Depending on the vector system and host utilized, any number of suitable transcription and/or translation elements, including constitutive, inducible, and repressible promoters, as well as minimal 5' promoter elements, enhancers or leader sequences may be used. For a review on maximizing gene expression see Roberts and Lauer, "Maximizing Gene Expression on a Plasmid Using Recombination *In Vitro* " Methods in Enzymology 68:473-82 (1979), which is hereby incorporated by reference in its entirety.

Optional Features of the Plasmid

In some embodiments, the cell-free expression vector includes a multiple cloning site. Alternatively, the cell-free expression vector does not include a multiple cloning cite. When included, a multiple cloning site, as recognized by one of skill in

the art, is a short segment of DNA that contains many (up to ~20) restriction sites. For example, any of the following restriction sites: SgrAI, SrfI, XmaI, SpeI, BamHI, BglII, XholI, SacII, RsrII, PacI, NruI, NotI, NdeI, MscI, MluI, KpnI, Fse1, BssHII, BsrGI, BspEI, BclI, BbvC1, PmeI, BssHII, AscI, XbaI.

In some embodiments, the cell-free expression vector includes one or more transcriptional or translational regulation sites. For example, transcription factor operator sites.

In some embodiments, the cell-free expression vector includes one or more inducer elements that can be utilized for the induction of gene expression. For example, LacO (for use with Lactose or IPTG), P(BAD) (for use with arabinose), or Tet (for use with tetracycline).

III. Kits

Also provided herein in some embodiments are kits for use in a method of creating or modifying a cell-free expression vector or kits for performing protein or RNA synthesis *in vitro*.

In some embodiments, the kits for creating or modifying a cell-free expression vector include: (a) a cell-free expression vector comprising (i) an origin of replication (ori), (ii) a nucleic acid sequence encoding a protein or RNA, (iii) a promoter arranged to drive expression of the protein or RNA, and (iv) one or more selectable markers; and (b) an insulating terminator sequence that is to be inserted at a location between 0 and 10,000 nucleotides in a 5' direction from the promoter in the cell-free expression vector; and (c) cloning reagents. In certain instances, the kits for performing protein or RNA synthesis *in vitro*, the kit comprising: (a) reagents for cell-free protein or RNA synthesis; and (b) a cell-free expression vector comprising (i) an origin of replication (ori); (ii) a nucleic acid encoding protein or RNA to be synthesized; (iii) a promoter arranged to drive expression of the protein or RNA; (iv) an insulating terminator sequence that is located between 0 and 10,000 nucleotides in a 5' direction from the promoter; and (iv) one or more selectable markers.

For example, the insulating terminator sequence can be located between 0 and 500 nucleotides in the 5' direction from the promoter (that is, the last nucleotide of the terminator sequence is between 0 and 500 nucleotides in the 5' direction from the first nucleotide of the promoter sequence), and can also be located 0 to 500 nucleotides in

the 3` direction of the ori (that is, the first nucleotide sequence of the terminator sequence is 0 to 500 nucleotides in the 3` direction from the last nucleotide of the ori sequence).

The kit can include an unmodified cell-free expression vector (e.g., pJL1, pY71, p70a, pBEST, pEXP5, pT7CFE), a terminator sequence (e.g., rnpB-T1, rrnB-T1, L3S2P21, L3S2P56), and the necessary cloning reagents to insert the terminator sequence. The preparation of the cell-free expression vectors can be carried out using standard cloning reagents (e.g., dNTPs, DNA polymerase, buffers, DNA ligase, DNA restriction endonuclease, DNA exonuclease) and procedures that are well known in the art, e.g., as described by Joseph Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL (Cold Springs Harbor 1989), including the November 18, 2014 updated version of Sambrook, and U.S. Patent No. 4,237,224 to Cohen and Boyer, which are hereby incorporated by reference in their entireties.

In some embodiments, provided herein is a kit that includes the modified plasmids as described above and the reagents for cell-free expression, e.g., a "CFPS reaction mixture." In some embodiments, a "CFPS reaction mixture" typically contains a crude or partially-purified cell extract (e.g., a yeast or bacterial extract), an RNA translation template, and a suitable reaction buffer for promoting cell-free protein synthesis from the RNA translation template.

As used herein, the term "crude" may mean components obtained by disrupting and lysing cells and, at best, minimally purifying the crude components from the disrupted and lysed cells, for example by centrifuging the disrupted and lysed cells and collecting the crude components from the supernatant and/or pellet after centrifugation. The term "isolated or purified" refers to components that are removed from their natural environment, and are, for example at least 60% free, at least 75% free, at least 90% free, or at least 95% free from other components with which they are naturally associated.

In some embodiments, the cells used to derive the crude or partially purified extract are selected based on the presence or absence of specific endogenous biochemical pathways and/or engineered biochemical pathways. For example, cells that direct carbon flux, prevent or minimize side product formation, and prevent or minimize promiscuous background activity can be advantageous as compared to other cells. In some embodiments, the cell is a prokaryotic cell (e.g., bacterial cell) or a

eukaryotic cell (e.g., a yeast cell). In some embodiments, the cell is a prokaryotic cell and comprises and *E. coli* cell. In some embodiments, the *E. coli* cell comprises a modified *E. coli* cell, such as BL21, JST07, MB263, MP263sucD, and JC01. In some embodiments, the *E. coli* cell comprises JST07.

As used herein, "translation template" for a polypeptide refers to an RNA product of transcription from an expression template that can be used by ribosomes to synthesize polypeptides or proteins. For example, a CFPS reaction mixture may include an expression template, a translation template, or both an expression template and a translation template. The expression template serves as a substrate for transcribing at least one RNA that can be translated into a sequence-defined biopolymer (e.g., a polypeptide or protein). The translation template is an RNA product that can be used by ribosomes to synthesize the sequence-defined biopolymer. In certain embodiments, the platform comprises both the expression template and the translation template.

The CFPS reaction mixture may comprise one or more polymerases capable of generating a translation template from an expression template. The polymerase may be supplied exogenously or may be supplied from the organism used to prepare the extract. In certain specific embodiments, the polymerase is expressed from a plasmid present in the organism used to prepare the extract and/or an integration site in the genome of the organism used to prepare the extract.

The reaction mixture may include any organic anion suitable for CFPS. In certain aspects, the organic anions can be glutamate, acetate, among others. In certain aspects, the concentration for the organic anions is independently in the general range from about 0 mM to about 200 mM, including intermediate specific values within this general range, such as about 0 mM, about 10 mM, about 20 mM, about 30 mM, about 40 mM, about 50 mM, about 60 mM, about 70 mM, about 80 mM, about 90 mM, about 100 mM, about 110 mM, about 120 mM, about 130 mM, about 140 mM, about 150 mM, about 160 mM, about 170 mM, about 180 mM, about 190 mM and about 200 mM, among others. The reaction mixture may include any halide anion suitable for CFPS. In certain aspects the halide anion can be chloride, bromide, iodide, among others. A preferred halide anion is chloride. Generally, the concentration of halide anions, if present in the reaction, is within the general range from about 0 mM to

about 200 mM, including intermediate specific values within this general range, such as those disclosed for organic anions generally herein.

The reaction mixture may include any organic cation suitable for CFPS. In certain aspects, the organic cation can be a polyamine, such as spermidine or putrescine, among others. In some embodiments, polyamines are present in the CFPS reaction. In certain aspects, the concentration of organic cations in the reaction can be in the general about 0 mM to about 3 mM, about 0.5 mM to about 2.5 mM, about 1 mM to about 2 mM. In certain aspects, more than one organic cation can be present.

The reaction mixture may include any inorganic cation suitable for CFPS. For example, suitable inorganic cations can include monovalent cations, such as sodium, potassium, lithium, among others; and divalent cations, such as magnesium, calcium, manganese, among others. In certain aspects, the inorganic cation is magnesium. In such aspects, the magnesium concentration can be within the general range from about 1 mM to about 50 mM, including intermediate specific values within this general range, such as about 1 mM, about 2 mM, about 3 mM, about 5 mM, about 6 mM, about 7 mM, about 8 mM, about 9 mM, about 10 mM, among others. In some implementations, the concentration of inorganic cations can be within the specific range from about 4 mM to about 9 mM. In some implementations, the concentration of inorganic cations can be within the range from about 5 mM to about 7 mM.

The reaction mixture may include endogenous NTPs (i.e., NTPs that are present in the cell extract) and or exogenous NTPs (i.e., NTPs that are added to the reaction mixture). In certain aspects, the reaction use ATP, GTP, CTP, and UTP. In certain aspects, the concentration of individual NTPs is within the range from about 0.1 mM to about 2 mM.

The reaction mixture may include any alcohol suitable for CFPS. In certain aspects, the alcohol may be a polyol, and more specifically glycerol. In certain aspects the alcohol is between the general range from about 0% (v/v) to about 25% (v/v), including specific intermediate values of about 5% (v/v), about 10% (v/v) and about 15% (v/v), and about 20% (v/v), among others.

As recognized by one skill in the art, components for a reaction mixture may be stored separately in separate containers, each containing one or more of the total components. Components may be packaged separately for commercialization and

useful commercial kits may contain one or more of the reaction components for a reaction mixture.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Insulating Terminators Reduced Read-Through Expression

We first assessed if inclusion of a terminator sequence could reduce read through expression. To do this, we split the pJL1 vector into two pieces of synthetic DNA (backbone and insert). We then designed different inserts with different combinations of the T7 promoter and different terminators that were in the 5' direction of the T7 promoter (see, Table 1 below showing the various terminators tested (note Sample #6 was discarded)).

Table 1

Sample #	Promoter	Terminator	Condition
1	T7	None	Positive Control
2	None	None	Negative Control
3	T7	rnpB	Endogenous Terminator
4	T7	rrnB	Endogenous Terminator
5	T7	T7	Phage Terminator
7	T7	M13 & rrnD	Phage Terminator
8	T7	L3S2P21	Synthetic Endogenous Terminator
9	T7	L3S2P56	Synthetic Endogenous Terminator

The terminator design is shown below. A portion of the sequence of the pJL1 vector is shown below (bold text is the end of the pMB1 origin of replication and italicized text is the T7 RNA polymerase promoter):

ctgtcgggtttcgccacctctgacttgagcgtcgattttgtgatgc
tcgtcaggg**gggcggagcctatggaaa**aacgccagcaacgcgatccc
gcgaaat*taatacgactcactatag* (SEQ ID NO: 815).

FIG. 2 shows a full map of the unmodified pJL1 expression vector.

In these initial experiments, the terminators were placed immediately downstream of the origin of replication and 27 nucleotides upstream of the T7 promoter, as shown below (bold text is the end of the pMB1 origin of replication and italicized text is the T7 RNA polymerase promoter; and the bold and italicized text is the terminator sequence):

ctgtcgggtttcgccacctctgacttgagcgtcgattttgtgatgc
tcgtcaggggggcggagcctatggaa*accggcttatcggtcagtttc*
acctgatttacgtaaaaacccgcttcggcgggtttttgcttttggag
gggcagaaagatgaatgactgtccacgacgctatacccaaaagaaaa
acgccagcaacgcgatcccgcgaaat*taatacgactcactatag*
(SEQ ID NO: 816).

FIG. 3 shows a full map of a modified pJL1 expression vector, where the insulating terminator rnpB-T1 sequence was inserted into the pJL1 vector immediately 3'of the origin of replication and 27 nucleotides 5'of the T7 promoter.

In general, the T7 promoter regulates the expression of GFP, so if read-through is observed it can be visualized by fluorescent readout. Gibson assembly reactions were performed and NEB5a *E.coli* cells were transformed with the vectors and grown overnight at 37°C. The next day, the plates were imaged using a fluorescent imager (to visualize GFP expression) and with a cell phone (to visualize cell grown and roughly gauge transformation efficiency).

FIG. 4A shows the GFP expression in the cells cultured in Petri dishes. Condition 2 (which lacks a T7 promoter) showed appreciable GFP fluorescence, indicating that read through expression is due to run-on transcription from somewhere upstream of the gene of interest (GOI). It also indicated that read through expression was not due to endogenous RNA polymerases recognizing the T7 promoter. In Samples 3, 4, 8, and 9, endogenous terminators appeared to be better at inhibiting read through expression than phage terminators (Samples 5 and 7), because Sample 5 showed considerable GFP expression, and Sample 7 had less, but still significant GFP expression.

FIG. 4B shows an overall cell growth for the plates shown in Fig 4A. The large number of colonies in each condition indicated that the lack of fluorescence in

Samples # 3, 4, 8, and 9 are not due to a lack of cell growth, but rather the specific decrease in fluorescence due to the presence of an insulating terminator.

Example 2: Modification of Terminator Location to Improve Plasmid Production Yields

Initial placement of terminators in Example 1 reduced plasmid yields. NEB5a cells harboring the plasmids of interest were cultured and then a large scale plasmid purification was performed. Yields were determined using absorbance (A280). For the plasmids with terminators inserted, the purified plasmid yields were consistently only about 25% of the yields of the unmodified plasmid. As a result, we altered the spacing of the terminator in the insert design to see if this alteration could improve the plasmid yields (see, Table 2 below). Specifically, in this set of experiments, the terminators were placed 40 nucleotides 3'to the origin of replication and 37 nucleotides 5' to the T7 promoter, as shown below (bold text is the end of the pMB1 origin of replication and italicized text is the T7 RNA polymerase promoter; and the bold and italicized text is the terminator sequence):

ctgtcgggtttcgccacctctgacttgagcgtcgattttgtgatgc
tcgtcaggg**gggcggagcctatggaaa**aacgccagcaacgcggcctt
tttacggttcctggccttttt***ccggcttatcggtcagtttcacctgat***
ttacgtaaaaacccgcttcggcgggtttttgctttggaggggcaga
aagatgaatgactgtccacgacgctatacccaaaagaaagctggcct
tttgctcacatgttcttatcccgcgaaat*taatacgactcactatag*
(SEQ ID NO: 817).

FIG. 5 shows a full map of the modified pJL1 expression vector, where the insulating terminator is 40 nucleotides 3' of the origin of replication and 37 nucleotides 5' of the T7 promoter.

Table 2:

Sample #	Promoter	Terminator	Condition
10	T7	None	Positive Control
11	None	None	Negative Control
12	T7	rnpB	Altered terminator location
13	T7	rrnB	Altered terminator location

14	T7	L3S2P21	Altered terminator location
15	T7	L3S2P56	Altered terminator location

As in Example 1, read through expression was greatly reduced with the endogenous terminators (see, e.g., Samples 12-15 in FIG. 6A). Similar results to those seen in the solid culture were observed in liquid culture as well. That is, we observed that endogenous terminators nearly eliminated read through expression of GFP in liquid culture tests using standard techniques. As shown in the bar graph of FIG. 6B, the wildtype (WT) and no promoter showed significant GFP expression as evidenced by fluorescence (high RFU/OD), whereas the insertion of the terminators rnpB, rrnB, L3S2P21, and L3S2P56 all significantly reduced GFP expression. The results were consistent across three clones, A, B, and C.

We also cultured NEB5a cells harboring the plasmids of interest and performed large-scale plasmid purification. Yields were again determined using absorbance (A280). As compared to Example 1, for the vectors with terminators inserted in the new location, the purified plasmid yields were comparable to the unmodified plasmid. Overall, this example shows that terminators need to be placed in the correct location to enable comparable plasmid yields, but still achieve the goal of decreasing leaky or read-through expression of the downstream open reading frame.

Example 3: Modified Plasmids Exhibit GFP Production in CFPS Comparable to GFP Production in the Original Plasmid

Purified plasmids were evaluated in cell-free protein synthesis (CFPS) for their ability to produce GFP. Two independent plasmid isolates (A and B) were tested for each design. In FIG. 7, the bars indicate a measurement of GFP expression in the strain used for plasmid production. We observed a similar optical density (OD) for all the different vectors, but variable expression of GFP. When a terminator was not present, a significant fluorescence was observed due to the expression of GFP. When the terminator was absent, little GFP expression was observed. Data was normalized to the concentration of cells in each well, so that samples are cross-comparable.

The circles in FIG. 7 represent the GFP production from the plasmid in a cell free protein synthesis system. All conditions yield GFP expression. As shown in FIG. 7, the modified plasmids from Example 2 showed comparable protein production

(ug/mL sfGFP) to the original vector (uninsulated). Overall, this example shows that plasmids modified with insulating terminators provide similar yields when used in cell-free protein synthesis, making them fully compatible with this expression systems while making it easier to prepare these plasmids by decreasing background expression of downstream open reading frames.

APPENDICES

APPENDIX 1: Terminator Sequences

Name	Description	Sequence	SEQ ID NO:
ECK120029600	spy	TTCAGCCAAAAAACTTAAGACCGCCGGTCTTGTCCACTACCTTGCAGTAATGCGGTGGACAGGATCGGCGGTTTTCTTTTCTCTTCTCAA	1
ECK120033737	thrLABC	ggaaacacagAAAAAAGCCCGCACCTGACAGTGCGGGCTTTTTTTTTcgaccaaagg	2
pheA-1	pheA	gacgaacaaTAAGGCCTCCCAAATCGGGGGGCCTTTTTTATTgaTaacaaaa	3
ECK120034435	secG-leuU	CTCGGTACCAAATTCCAGAAAAGAGACGCTGAAAAGCGTCTTTTTTCGTTTTGGTCC	4
ECK120033736	hisLGDCBHAFI	aacgcatgagAAAGCCCCCGGAAGATCACCTTCCGGGGGCTTTtttattgcgc	5
ECK120010818	garPLRK-rnpB	GTCAGTTTCACCTGTTTTACGTAAAAACCCGCTTCGGCGGGTTTTTACTTTTGG	6
ECK125109870	metZWV	ccaattattgAACACCCTAACGGGTGTTTTTTTGTTTCtggtctccc	7
ECK120015440	lpdpdhR-aceEF-lpd	tccggcaattAAAAAAGCGGCTAACCACGCCGCTTTTTTTacgtctgca	8
BBa_B0062-R	rrnC	cagataaaaaaaatccttagctttcgctaaggatgatttct	9
ECK120010799	csrC	gttatgagtcAGGAAAAAAGGCGACAGAGTAATCTGTCGCCTTTTTTTCTTtgcttgcttt	10
ECK120010876	creABCDcreD	taaggttgaaAAATAAAAACGGCGCTAAAAAGCGCCGTTTTTTTTgacggtggta	11
ECK120015170	rplM-rpsI	ACAATTTTCGAAAAAACCCGCTTCGGCGGGTTTTTTTATAGCTAAAA	12
ECK120010869	rplJL-rpoBCrplKAJL-rpoBCrpoBC	taacgtaaaaACCCGCTTCGGCGGGttttttttatg	13
BBa_B0010	rrnB T1	ccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctc	14

ECK120017009	ihfApheMST-ihfA	GATCTAACTAAAAAGGCCGCTCTGCGGCCTTTTTTCTTTTCACT	15
ECK120051401	xapR	CGCAGATAGCAAAAAAGCGCCTTTAGGGCGCTTTTTTACATTGGTGG	16
ECK120010855	osmE	GTAACAACGGAAACCGGCCATTGCGCCGGTTTTTTTTGGCCT	17
ECK120010850	clpPX	agttaaccaaAAAGGGGGGATTTTATCTCCCCTTTaatttttcct	18
thr	thr	AAAAAAGCCCGCACCTGACAGTGCGGGCTTTTTTTTT	19
ECK120035137	istR-1istR-2	AGGCGACTGACGAAACCTCGCTCCGGCGGGGTTTTTTGTTATCTGCA	20
ECK120051382	rrlB-rrfBrrsB-gltT-rrlB-rrfB	caggcatcaaATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATctgttgttg	21
ECK120035133	rphrph-pyrE	ACTGATTTTTAAGGCGACTGATGAGTCGCCTTTTTTTTGTCT	22
tonB_P14	tonB/P14	CCTGTTGAGTAATAGTCAAAAGCCTCCGGTCGGAGGCTTTTGACTTTCTGCTTAC	23
ECK120023928	proS	GCTGATGCCAGAAAGGGTCCTGAATTTCAGGGCCCTTTTTTTACATGGATTG	24
BBa_J61053	fmn T1	gcttgctgaggatcctaaagccccgaatttttataaattcggggcttttt	25
ECK120016882	rpsT	TGTGAAAAAGCCCGCGCAAGCGGGTTTTTTTATG	26
pyrBI	pyrBI	AGCCCCTCAATCGAGGGGCTTTTTTTTGC	27
ECK120026481	arcA	TACCACCGTCAAAAAAAACGGCGCTTTTTAGCGCCGTTTTTATTTTTCAACCTT	28
ECK120015444	seqA-pgm	acatttaataAAAAAAGGGCGGTCGCAAGATCGCCCTTTTTTacgtatgaca	29
ECK120010782	fhuE	ACCTGTAAAAAAGGCAGCCATCTGGCTGCCTTAGTCTCCCCA	30
ilvBN	ilvBN ilvGEDA	AAGACCCCCGCACCGAAAGGTCCGGGGGTTTTTTTTT	31
ECK120016586	infA	AAGAACGAGTAAAAGGTCGGTTTAACCGGCCTTTTTATTTTGTGA	32
ECK120010797	crrptsHI-crr	cagtgaaaaaTGGCGCCCATCGGCGCCAttttttatg	33
ECK120014970	valS	TGAAAACGAAGGCCGGAGCATGCTCCGGCCTTTTTTATCTCTTACA	34
ECK120015452	nagE	caacaatgacAAGCGGTGGAGATCTTCTCTGCCGCTTttttttcat	35
ECK120010863	atpBEFHAGDCatpIBEFHAGDC	AAGCACAAAAGCCAGTCTGGAAACAGGCTGGCTTTTTTTTGCG	36

ECK120010815	gadBC	GTTATAAGACAAGGGAGCGATAATTCATCGCTCCCTTTTTCGTGCTT	37
ECK120010867	xapAB	AAAAGGATTCGCGGCTCTGCTCTTCAGAGCTGCTTTTATGATA	38
ECK120051383	rrlB-rrfBrrsB-gltT-rrlB-rrfB	caaattaagcAGAAGGCCATCCTGACGGATGGCCTTTTtgcgtttcta	39
ECK120035134	yicC	ccgccttcacAAATGCCGCCACTCAAACAGAGCGGCATTTttcttccccg	40
ECK120010822	ilvAilvEDAilvLXG_1G_2MEDA	tgcctcaattAGCGGCTCATGTAGCCGCTttttctgcgc	41
ECK120010856	dcuB-fumBfumB	GGCAGCATGCTGCCAGGTGATCCCCCTGGCCACCTCTTTT	42
ECK120010793	rnpB	TACGTAAAAACCCGCTTCGGCGGGTTTTTACTTT	43
ECK120015459	talB	taatcattctTAGCGTGACCGGGAAGTCGGTCACGCTAcctcttctga	44
ECK120051385	glmY	CTTATTCCATAACAAAGCCGGGTAATTCCCGGCTTTGTTGTATCTGAAC	45
ECK120010864	glmUS	AATAAATGGATGCCCTGCGTAAGCGGGGCATTTTTCTTCCT	46
ECK120010781	ompC	ATCGAACAAAGGGCCTGCGGGCCCTTTTTTCATTG	47
BBa_K088008	glnRS	agaaacagcaaacaatccaaaacgccgcgttcagcggcgttttttctgcttttct	48
ECK120010846	uspA	cgtgttcctgAACGCCCGCATATGCGGGCGTTttgctttttg	49
ECK120010840	cutC	TGCTCGTACCAGGCCCCTGCAATTTCAACAGGGGCCTTTTTTTATCC	50
ECK120010868	ampC	AATTCCATCGGGTCCGAATTTTCGGACCTTTTCTCCGC	51
ECK120010853	htpG	ttcctgatgtAATGCCGGATGACCTTCGTGTCATCCGGCATTtttcttttca	52
pheA_2	pheA	ATGGGAGGCGTTTCGTCGTGTGAAACAGAATGCGAAGACGAACAATAAAGGCCTCCCAAATCGGGGGGCCTTTTTT	53
ECK125108723	mgrR	TAGCGTAAAAGCAAAACACAAATCTATCCATGCAAGCATTCACCGCCGGTTTACTGGCGGTTTTTTTTCGCCGTCATA	54
ECK120034950	sspAB	GTGAAGTAATACAAAACAGGCCCAGGCGGCCTGTTTTGTCTTTTTAATG	55
ECK120010825	rnb	ATCTCCTTTCACGGCCCATTCCTCATGGATGGGCCGTTTATTTCCCC	56
ECK120030221	relBE-hokD	CCCGCACTTAACCCGCTTCGGCGGGTTTTTGTTTTT	57
ECK120015454	glnS	caaacaatccAAAACGCCGCGTTCAGCGGCGTTTTttctgctttt	58

ECK120010865	rpoDrpsU-dnaG-rpoD	ctctgcacaaACGCCACCTTTTCGGTGGCGtttttatcg	59
ECK120010806	glnA	GATGGCTCCGATGGATAACCAGCGCCGCTTAAGTCAGGAA	60
ECK120020522	accBC	agcgtcaaaaGGCCGGATTTTCCGGCCttttttatta	61
ECK120010833	fliAZYfliY	GCATAATGACAAAAAAGGGCGCTTTCACTAGCGCCTTTTTTATTTACGCGT	62
ECK120029341	aroH	tctgaatgcgTGCCCATTCCTGACGGAATGGGCAtttctgcgca	63
BBa_B0052	rrnC	agaaatcatccttagcgaaagctaaggatttttttatctg	64
BBa_B0021	LuxICDABEG reversed	aaataataaaaaagccggattaataatctggctttttatattctct	65
ECK120011170	fldA-uof-furfuruof-fur	AACGAGAAAAGCCAACCTGCGGGTTGGCTTTTTTATGCA	66
ECK120051408	fadD-sroD	GTCAGTCGTCAGACGCCGGTTAATCCGGCGTTTTTTTTGACGCCCAC	67
ECK120034551	gpt	ctaatctttCAACGCCTGGCACTGCCGGGCGTTGTTCTTTTTaacttcaggc	68
ECK120035136	tisB	aagtcgcaccAAAGGGGAGCGGGAAACCGCTCCCCTTTtatatttagc	69
BBa_B0057	BBa_B0057	cagaaatcatccttagcgaaagctaaggatttttttatctg	70
ECK120029529	dusB-fis	tgcttgattaAAAAGGCGCTACTCGGCATGGGGAAGCGCCTTTTtataggtgt	71
ECK120051400	ivbL-ilvBN	TGAACAACATCGCGCTTATCGTTAAGGTAAGCGCGTATTTTTTTTACCCGCCAG	72
ECK120020525	metY	TTATATAAAGCCCCGATTTATCGGGGTTTTTTGTTA	73
ECK120010851	rlmE-ftsH	ATTTGTACCGAAAACCCCGGGGCGTGCTCCGGGGTTTTTTCTTATCAA	74
ECK120034436	argP	atcaaataatGCCTGATAGCACATATCAGGCgttgtcctca	75
ECK120010852	hslVU	CATTGTTTGATGGGGCTGAAAGGCCCCATTTTTATTGG	76
ECK120048898	fadR	tttaaagagcAAACCCCTCAAACGAGGGGTTTtttgttgttt	77
ECK120051404	mtlADR	cctctacctgCTTCGGCCGATAAAGCCGACGataatactcc	78
ECK120021270	infC-rpmI-rplTrplTrpmI-rplT	GCCAGTTGAAAGAGGGAGCTAGTCTCCCTCTTTTCGTTTC	79
ECK120030802	ldrD	GCCCGGACCAGGCCGCAGGGGGGAAACTCTGCGGCCTTTTTCGTTCTTACT	80
ECK120010857	focA-pflBpflB	TGTAATTAGATTTGACTGAAATCGTACAGTAAAAAGCGTACAATAAAGGCTCCACGAAAGTGGGGCCTTTTTTAGCGCGA	81

ECK125095454	tff-rpsB-tsf	atcaaaaaggAGCCGCCTGAGGGCGGCTtcttttt gtg	82
ECK120020622	rpsO	CGAGTTTCAGAAAAGGGGGCCTGAGTG GCCCCTTTTTTCAAGCTGAC	83
ECK120010874	rpsJ-rplCDWB-rpsS-rplV-rpsC-rplP-rpmC-rpsQ	TACGAATAAACGGCTCAGAAATGAGCC GTTTATTTTTTC	84
ECK120010783	mdoGH	acgagccaatAAAAATACCGGCGTTATGCCG GTATTTTTttacgaaaga	85
ECK120030671	ileS-lspA-fkpB-ispHlspA-fkpB-ispHribF-ileS-lspA-fkpB-ispH	aagttatgcgAAAATGCCGGTCTTGTTACCG GCATTTTttatggagaa	86
ECK120010796	csrB	GAAACGAACCGGGAGCGCTGTGAATAC AGTGCTCCCTTTTTTTATT	87
ECK125095211	micM	ctctttgacgGGCCAATAGCGATATTGGCCAT TTTTTTagcgcaacat	88
ECK120010832	lolB-ispE-prsprs	GGCTCAAAGACCCGCTGCGGCGGGTTT TTTTGTCT	89
ECK120030218	rseX	tatttttttggGCCGGCATGATGCCGGCttttttttat	90
rpsO_pnp	rpsO-pnp	AAAAGGGGCCTGAGTGGCCCCTTTTTTC A	91
ECK120029531	tyrB	atgcaggaaaGCAGGCTGGAGCTACCCAGCC TGCagtgaaatta	92
ECK120020528	glyQS	GTTATTAAATAGCCTGCCATCTGGCAG GCTTTTTTTATCG	93
BBa_B0061	yciA/tonA	aagtcaaaagcctccggtcggaggcttttgacttt	94
ECK120030798	tatABCD	agaataaattCAACCGCCCGTCAGGGCGGTT Gtcatatggag	95
ECK120015460	pheP	caaccatccgAAACCGCTCTCATCCATTCGA TGAGAGCGGTTTttttaattac	96
frd_ampC	frd-ampC	CGGCCCGCCTATGGCGGGCCGTTTTGTA T	97
ECK120051403	mtlADR	gcctttcaaaAGTAAGCAACGTCTGCTTACTg cccctctac	98
ECK120010790	cpxP	tccctgtcttCCCCCACATGCTGTGGGGGttttttt at	99
ECK120033262	glgS	GCACTGATATAACGGGCCTGATGGCCC GTTTTAGTGTTTG	100
ECK120010831	aldB	GGTATTCATTGCCTGATGCGACGCTTAC GCGTCTTATCATGCCTACGGGAACCTG A	101

ECK120033265	cspA	cacagaatctAAGATCCCTGCCATTTGGCGGGGATTTTtttatttgtt	102
ECK120010826	glnK-amtB	caagcactgcAAAAAACAGCCGGACGGTTTTCACCTCCGGCTATTTTTTtaattgtgat	103
nusA_infB	nusA-infB	CCCCGATTTATCGGGGTTTTTTGTTATCTGACTACAGAATAACTGGGCTTTAGGCCCTTTTTTT	104
ECK120035132	pstSCA	CTCACCCTAACCCTCTCCCCAGAGGGGCGAGGGGACCGACCGA	105
ECK120010834	rpsU	tgtagttgtaAGGCCGTGCTTCCGAAAGGAATGCGCGGCTTattttcgttt	106
ECK120010812	gadAXgadX	GGGAAGAGGATAGTCTGCCGTCTCCAGACTAATAAACCGTT	107
ECK120030672	dapD	AGTATGCACACGGGCAGCACGACGCTGCCCGATTTTTTTGCA	108
ECK120029599	aroG	tagcaacaaaAAAGCCGACTCACTTGCAGTCGGCTTTctcattttaa	109
trp	trp	AGCCCGCCTAATGAGCGGGCTTTTTTTT	110
ECK120010873	metY-rimP-nusA-infB-rbfA-truB-rpsO-pnppnprpsO-pnp	GTTGCCATTTGCCCTCCGCTGCGGCGGGGGGCTTTTAACCGGG	111
ECK120010841	rpoH	AAGCAGAGAACCCTGGATGAGAGTCCGGGGTTTTTGTTTTT	112
BBa_B0051	yciA/tonA	aaagtcaaaagcctccgaccggaggcttttgactt	113
ECK120010805	gadY	ctatcctcttCCCGGTCCCCTATGCCGGGtttttttat	114
ECK125109867	arcZ	gtattcgcgcACCCCGGTCTAGCCGGGGTCATTTTTTagtggctttt	115
ECK120030673	dapB	tattgattatAAAGGGCTTTAATTTTTGGCCCTTTtatttttggt	116
ECK120034956	rhoL-rho	cgtctgcgtaTGGAACGTGGTAACGGTTCTActgaagattt	117
ECK120010780	glyA	TGATCATCAAGGCTTCCTTCGGGAAGCCTTTCTACGTTA	118
ECK120029530	guaBA	ATGAATGAACAAAACCCTCTGTTACTACAGAGGGTTTTTTATCTTCAA	119
ECK120010784	bamCdapA-bamC	GTAAAAATACAGGGCTGGAATCATCCGGCCCTTTTTTCTGAT	120
ECK120010798	bglGFB	CTAAGCGGGCAAAACCTGAAAAAAATTGCTTGATTCACGTCAGGCCGTTTTTTTCAGGTTTTTTTTTGGAGT	121
ECK120010830	tgt-yajC	gctgttcagaTCACTGGTGCGCGCGGAGTCGCCGCCAGTGAgcaaacgctg	122

ECK125095455	greA	GTGAATTGTAGCTGACCTGGGACTTGTACCCGGGTCGGTATTTTTTTGCTTCTGGTCCCGG	123
ECK120010800	gntKUgntRKU	AGTTTGTTCGCCCGGTAGTTGTGACGCTACCGGGTTCTTTTCGA	124
ECK120048901	cstA	ttagtgcccaGGGTTCCCTCTCACCCTAACCCTCTCCCCGGTGGGGCGAGGGGACTgaccgagcgc	125
ECK120034434	feaB	gctcataagtAAAAAACGGCACCTGGTGCCGTTTTTTTgtctgaaac	126
ECK120010809	malZ	ccgtttaacaCGTTCTGGATGAAATCCATATCGcgatagcgca	127
ECK120048902	cysK	gcgtaaaaaaGCACCTTTTTAGGTGCttttttgtgg	128
ECK120035138	cyaR	gaaccacctcCTTAGCCTGTGTAATCTCCCTTACACGGGCTTATTTTTTacgcgtaata	129
ECK120010821	hupB	gaagttcaagGGCGCATCTACTGATGTGCCttttttattt	130
ECK120030219	gcvPgcvTHP	ACTATTTTCTAAAGGCGCTTCGGCGCCTTTTTAGTCAGAT	131
ECK120026395	acrEF	gataaatcagAAACATAAAGGCGCTTTCGGGTGCCTTTATTATTTccagtgaaac	132
ECK125109871	metZWV	ccataaaaaaGCGCCATTCAGCGCCTTTTTAtcatcccctt	133
ECK120010827	adiA	TTCCGCTGAAGGCGTAATTGTTTAAATAACATTACGCCGCCTGGCCTT	134
ECK120010849	hypABCDEhypBCDE	atgacctttGCACCGCTTTGCGGTGCTttcctggaag	135
ECK120029975	trpR	taagacgtggCGCATCAGGCATCGTGCACCGAATGCCGGATGCGgcgtgaacgc	136
ECK120010791	malEFG	ACTGTTATTCGGCGCTCCACGGAGCGCCTTTTTTTCT	137
ECK120010819	hupA	agttttaacgAAGGGGTGGTTTCACCCCTTttgtctttct	138
ECK120048897	malI	CCGGCTCATTGCAGCGAAATAATCCTCTCTTTATCTGCTATACCTGGT	139
ECK120027937	bolA	gattttatgaAAAACGGCCTGCGGGCCGTTTTgttttgtctg	140
ECK120010802	gntP	AAGGACACCAGAGCCTGCCAATGGCAGGCTCAGACTGATGA	141
tonB	tonB	AGTCAAAAGCCTCCGACCGGAGGCTTTTGACTATTACT	142
ECK120010803	edaedd-eda	AAAGTCAAAATGCCCGATCGAGGATCGGGCATTTTTGTAGC	143
ECK120010836	ryjAsoxR	aagatgaacaAAACTAAAGCGCCACAAGGGCGCTTTAGTTTgttttccggt	144
ECK120029528	aroP	ATCTCTCTACGCCCTCACCCGTACAGGGTGAGGGCAATAATCTTT	145

pheST	pheST	AGCCTCCCAGTGGAGGCTTTTTTTGT	146
his	his	AAAGCCCCCGGAAGATCACCTTCCGGGGGCTTT	147
ECK120010813	exbBDexbD	CACAATGATGCCCGGTTGCTTTTCACAACCGGGCATTTTTTTAAC	148
ECK120015957	treR	CCTGTCCTGATCGTTTCCTGAACGATAAATTGTGA	149
ECK120010824	ilvY	GCGCGGGTAGGCCTGATAAGCGAAGCGCTATCAGGCATTTTTCCCTA	150
BBa_B0053	his	tccggcaaaaaaacgggcaaggtgtcaccaccctgccctttttcttttaaaaccgaaaagattacttcgcgtt	151
ECK120033127	sdaA	tacttcttacTCGCCCATCTGCAACGGATGGGCGAatttataccc	152
ECK120015448	amyA	gaggtgatttAAATTCATCCCCGGCGGCAAGCCGGGGAGATTTcattacggca	153
ECK120010792	tonB	gtaagcagaaAGTCAAAAGCCTCCGACCGGAGGCTTTTGACTattactcaac	154
ECK120015446	gcd	CAGATTGCTGACAACGTGCGCGTTGTTCATGCCGGA	155
ECK120010843	tsx	CTGATTATGAAAATGCCGGGATTTATTCCCGGCATTTCTGATTGTTA	156
ECK120010848	malS	cagccctaatCAGCGTTGCAGGATAAAGCACCGCTCactcttcaac	157
ECK120010816	glpEGRglpGRglpR	ACGGCTTCCCACGTCAGACCAAAACGCGCCAGGTATTTGCGTAGCCGATCCGCGTCATTGACGCTGGCTTTGCCCTGGCGCGAAACGTCAAAAAGCTG	158
ECK120015439	nudB-yebC-ruvCruvCyebC-ruvC	ACTGCGTTAAGTTATACCGCCTCGGTCAGTTCCGGCTGAGGCGTTTCCACTCCCTCCGCA	159
ECK120010845	cvpA-purF-ubiXubiX	AACTCCGCTGTTGCCCTGTTTCAGGGCAATTTTGCAACC	160
ECK120010787	tyrR	agtaagcgcgAATATGCCTGATGGTGCAACACCATCAGGCATATTaaattatgct	161
ECK120051405	mtlADR	aatcgcgttaCGGGGAGGAAGTTTTTTCAGATACTCCCggaacgcctg	162
ECK120048899	ansB	TAATCGCCTCGCCCCGGTATCGTGCCGGGGCTTTTTCACTT	163
ECK120030220	cynTS	ttacccgcaaAGTGCGCTGCTGCTTAGCAGCGCACTgcttggtggg	164
BBa_B0060	pBR322	aaaatcaaaggatcttcttgagatccttttttt	165
ECK120010807	bglGFB	TTAAAGCACCTTAATTATCGTCGCATTCAGAACAGTCTGGATGCGATGCGTTAATTCTTTCTTT	166
ECK120026315	dnaTC-yjjAyjjB-dnaTC-yjjA	CTGCCCTTTTCCCTGCTCCTGGACGGTTTTACCCCT	167

ECK120033264	ivbL-ilvBN	CCCAATGACTACTTCCATGCTCAACGCAAAACTACTACCAACTGCGCCATCCGCCGCAGTGGTCGTCGTGCGTGTGGTGGTGGTCGTCGGCAATGCGCCGTAGGGACTGGAACAACACACGATTCCAAAACCCCGCCGGCGCAAACCGGGCGGGGTTTTTCGTTTAAG	168
ECK120051406	mtlADR	ccgtattaccCCGCGCCGGGAATGCGCGGccgccaattt	169
ECK125122040	yciEyciGFE	TACCATGTCCTTATTGACCCCGTATATTACGGGGTCGTTTTTGTGCGGAAT	170
ECK120010844	nuoABCEFGHIJKLMNnuoMN	AGTCAGAAAGCCGCCGACATGCTCGGCGGTTTTTCTGAA	171
ECK120010779	aroF-tyrApheLA	ctgatgaaaaGGTGCCGGATGATGTGAATCATCCGGCACtggattatta	172
ECK120030670	mreB	TAATCGGATGCAGGCAGGGGAAGTGTCTGTTTACCCTGCCTGGTCTGATACG	173
ECK120015455	malT	gtgtaagtttAGCCGGATAACGCGCCAGATCCGGCTtacatctctg	174
ECK120015450	bglX	ATGCTTTAGTAAGGGCGCGACGTTTGCGCCCTTTGTAGGCCGG	175
ECK120015456	epd-pgkpgk	AAAAAATCACAGGGCAGGGAAACCTGCCCTTGTTTCAGCG	176
ECK120010862	putP	gctccgccgtCACGGTTGCAGGAAAGCTAAGGGACTTAGCCTGCGGCGGTTTTGTttggcttcag	177
ECK125108943	selD-topBydjA-selD-topB	CTGAAATATCCAGCGGATCAAGAAAATTCGTTGGATATTTTTT	178
ECK120035135	dacD	CTTTCTTTTGCAGCAGACTGGCAGGAGTGCGAGTCTGCTCGCATAATCA	179
ECK120015449	glpFKX	CGATTGAGCCTTCCAGTCCTTCGGGACTGGAATTTTTTTGTT	180
ECK120010842	hyaABCDEF	ccgacgtaaaAAGACGGTAAGTATCGCTTTCAGTCTTatgaatatcg	181
ECK125095210	pldA	gtttctcgcgCAGGCGCTGAAAATAGCGCCTGtttttatttc	182
ECK120034954	rhoL-rho	ggctggaaaaCCTGGCTCGTATGCGTAAGCAGGacattatttt	183
ECK120010788	cynTSXlacYAlacZYA	gaacttgtagGCCTGATAAGCGCAGCGTATCAGGCaatttttata	184
ECK120010860	araBAD	TCAAACGAAACCAGGCTATACTCAAGCCTGGTTTTTTGATGG	185
ECK120010801	asr	cagtaatgctGGCGCGCCCCCTCGCGCCtgaaaattac	186
ECK120015458	aspS	CTGAGTGAACTCCCATGAGCATAGATAACTATGTGAATGGGATGAGCGAAGG	187

ECK120034948	ecnB	aataagcaatAACGGTACGACAGCTGTGTCG TGCCGTttgtttttc	188
ECK120015453	katE	aaacacgtagGCCTGATAAGCGAAGCGCATC AGGCagttttgcgt	189
ECK125109037	otsAotsBA	GGCAATAACTCTTTTCGCCGAGCAGGA TGCTCGCGAAAAGAAACTGTGATT	190
ECK125109868	gadWgadXW	AGCAGGAAAGAGTAAGGCTGAACCTTC ATGTTCAACCTTACTCTCATTTAC	191
ECK120010828	cysB	tgcgttatttTCGGCACCTTTTATGTAGCGAA GGTGCCGGaatatattct	192
ECK120010814	aceEF	tgatgtaagtAAAAGAGCCGGCCCAACGGCC GGCTTTTTTctggtaatct	193
ECK120034949	yjaZ	caaaataacaAAACCCACCTTAAGGTGGGTT Tcgccagagaa	194
ECK125108944	selD-topB	ATATTCTGAAATATCCAGCGGATCAAG AAAATTCGTTGGATAT	195
ECK120010820	hupB	ctaagcgttgTCCCCAGTGGGGAtgtgacgaag	196
ECK120010829	phoH	gttatcggtgCAGAGCCCGGGCGAACCGGGC TTTGttttgggtgt	197
ECK120010837	fliDST	cgaataatccGATTACGGCTACGCTTCTAAT GTTCCCCTTGAATGGAGTCGAAGAATG CGTAATCccacgctgtt	198
BBa_B0011	luxICDABEG	agagaatataaaaagccagattattaatccggctttttattattt	199
ECK120015443	rpiA	AATGATCTGACGGGGGAACCTCCCCCG TTAAAAAAAT	200
ECK120020526	metY	CAGAATAACTGGGCTTTAGGCCCTTTTT TTATG	201
trp_t	trp t	GCCGCCAGTTCCGCTGGCGGCATTTT	202
ECK120010839	endA	ttaacccctaCCCCACGCGTACAACCGCGTG GGGagacgacgcg	203
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ECK120026312	dnaTCyjjB-dnaTC	TTCTGTGCTGTGCCATTGCCGCCAGCGC ATTAATTTCCA	206
ECK120010866	fldA-uof-furfuruof-fur	TAAGAAAAGCGAGAGTTACAGCTCTCA CTTATTTGTT	207
tetA	tetA	CCCTCTTGATAACCCAAGAGGGCATTTT TTA	208
rplKAJL_rpoBC	rplKAJL-rpoBC	GGCGTGAGATTGGAATACAATTTCGCG CCTTTTGTT	209
ECK120015526	ruvAB	CGAACCGTAGGTCGGATAAGGCGCTCG CGCCGCATCCGACAAATGTGTTC	210
ECK120030801	trmA	AGTAAAACCCATGCCGGATGCGCCAGC ATCCGGCATAATACCGATTAC	211

ECK120015445	agp	ataacagaaaACTCCCCCGCGAGAAGCGGGGGAGTcgctggttaa	212
ECK120030800	psrD	aattattggcAAAAGGCAACCACAGGCTGCCTTTTTtctttgactc	213
ECK120010810	malZ	cagccactgcTCTGACCACAAGTAATTGTTCAGAttgataaaac	214
ECK120011168	gntRgntRKU	CCGGCTGGACAATGTTACCGATAACAGTTACCCGTAACATTTTTAATTCTT	215
ECK120015451	hycABCDEFGHI	ATGCTAAATTGCCCGATGCGCTGCGCTTATCGGGCCTTCATGGTT	216
ECK120048900	cdh	aggaaagtaaGTGCCGGATATGAAATCCGGCACctgtcagact	217
ECK120033738	rpsP-rimM-trmD-rplS	TTTAATATGACACCGGACTCCGTTCCTCGATGGGGTCCGGTTGTTTTATTCAC	218
ECK120010847	malS	aacagtaactTTTCCGGCTTCCCGTTCGTCAGTACCTCGGGAAGCCGCCAAccaggataaa	219
orfL	orfL	CCCTCTTGGGTTATCAAGAGGGTCATTATATTT	220
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ECK120010817	cmr	gggtaaaaaaATGCCTGACTGCTTTGTGCGATCAGGCATtctcgaatta	222
ECK120020527	yhdT-panF-prmA	ccgattatttACGCAAATTTGCGTgccaaaattt	223
ECK120035131	pstS	AAAACTCCAGGCCGGGTACGGTGTTTTACGCCGCATCCGGCATTACAAAAT	224
ECK120010838	endA	acgcgtacaaCCGCGTGGGGAGACGACGCGGatttttaact	225
ECK120027917	yacC-speED	GTTTAACGGCTCTGGCGGAGCTCCCAGGCTCCGCCAGATTTATTTACT	226
ECK120010785	rpiB	agaaattgagATTCATCCACTACTTGCATGGATGAGTaatgattaat	227
ECK120029978	yjbE	taaagtatgtATCCCCAAAATAATTCGAGTCATTGCATCTGTGGCTAGAAGTATGAAGGGAttaaccataa	228
ECK120010858-R	aspA	acaagaaaaaAGGCACGTCATCTGACGTGCCttttttattt	229
ECK120026481-R	arcA	aaggttgaaaAATAAAAACGGCGCTAAAAAGCGCCGTTTTTTTTgacggtggta	230
ECK120010835-R	cysDNC	cgcaaataacCAGGAGATAAAACCGACCACGGCACCAGGCAGTGACCATGTGGTTTCTTCAtcctcagtaa	231
ECK120015457-R	ruvAB	ctctggtagtCCTGGTAAGACGCGAACAGCGTCGCATCAGGcatattgcca	232
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ECK120026314-R	valS	tgtaagagatAAAAAAGGCCGGAGCATGCTCCGGCCttcgtttca	234
ECK120010812-R	gadAXgadX	aacggtttatTAGTCTGGAGACGGCAGACTAtcctcttccc	235
ECK120010832-R	lolB-ispE-prsprs	agacaaaaaaACCCGCCGCAGCGGGtctttgagcc	236
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ECK120026315-R	dnaTC-yjjAyjjB-dnaTC-yjjA	aggggtaaaaCCGTCCAGGAGCAGGGaaaagggcag	239
ECK120020622-R	rpsO	gtcagcttgaAAAAAGGGGCCACTCAGGCCCCCTTTTctgaaactcg	240
ECK120015439-R	nudB-yebC-ruvCruvCyebC-ruvC	tgcggagggaGTGGAAACGCCTCAGCCGGAACTGACCGAGGCGGTATAACttaacgcagt	241
ECK120010825-R	rnb	ggggaaataaACGGCCCATCCATGAGGAATGGGCCGTgaaaggagat	242
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ECK120015447-R	glk	tttaaaagatTATCGGGAGAGTTACCTCCCGATAtaaaaggaag	253
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ECK120010867-R	xapAB	tatcataaaaGCAGCTCTGAAGAGCAGAGCCGCgaatcctttt	256

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ECK120010794-R	rnpB	aaagcaaaaaCCCGCCGAAGCGGGtttttacgta	258
ECK120034435-R	secG-leuU	ggaccaaaacGAAAAAAGACGCTTTTCAGCGTCTCTTTTCTGGAATTtggtaccgag	259
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ECK120010803-R	edaedd-eda	gctacaaaaaTGCCCGATCCTCGATCGGGCAttttgacttt	261
ECK120010864-R	glmUS	aggaagaaaaATGCCCCGCTTACGCAGGGCAtccatttatt	262
ECK120015526-R	ruvAB	gaacacatttGTCGGATGCGGCGCGAGCGCCTTATCCGACctacggttcg	263
ECK120010802-R	gntP	tcatcagtctGAGCCTGCCATTGGCAGGCTCtggtgtcctt	264
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ECK120010851-R	rlmE-ftsH	ttgataagaaAAAACCCCGGAGCACGCCCCGGGGTTTTcggtacaaat	268
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ECK120027917-R	yacC-speED	agtaaataaaTCTGGCGGAGCCTGGGAGCTCCGCCAGAgccgttaaac	275
ECK120030221-R	relBE-hokD	aaaaacaaaaACCCGCCGAAGCGGGTtaagtgcgg	276
ECK120015449-R	glpFKX	aacaaaaaaaTTCCAGTCCCGAAGGACTGGAAggctcaatcg	277
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ECK120021269-R	pheMpheMST-ihfArplT-pheM	tacaaaaaaaGCCTCCACTGGGAGGCtttcaggcgc	279
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ECK120023928-R	proS	caatccatgtAAAAAAAGGGCCCTGAAATTCAGGACCCTTTCtggcatcagc	282
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ECK120010868-R	ampC	gcggagaaaaGGTCCGAAAATTCGGACCcgatggaatt	304
ECK120015443-R	rpiA	atttttttaaCGGGGGAGGTTCCCCCGtcagatcatt	305
ECK120011169-R	gntKU	catgactaaaAACAGCAGCAGTAAAACAGACCCTACTGCTGTTaaaacaagcg	306
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ECK120010866-R	fldA-uof-furfuruof-fur	aacaaataagTGAGAGCTGTAACTCTCgcttttctta	313
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L3S1P11	synthetic	GACGAACAATAAGGCCTCCCTTCGGGGGGGCCTTTTTTATTGATAACAAAA	357

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L2U1H09	synthetic	ACGGCCCTAGATAGGGCCGTTTTTTTTT T	387
L3S1P15	synthetic	GACGAACAATAAGGCCTCCCTTTAGGG GGGGCCTTTTTTATTGATAACAAAA	388
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L1U1H11	synthetic	CCCGCTTCGGCGGGTTTTTTTTT	390
L1U1H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTT TTTTTTT	391
L3S1P23	synthetic	GACGAACAATAAGACGCTAAATCAGCG TCTTTTTTATTGATAACAAAA	392
L3S1P25	synthetic	GACGAACAATAAGCGGCAAATCGCCGC TTTTTTATTGATAACAAAA	393
L3S2P32	synthetic	CTCGGTACCAAATTCCAGAAAAGAGAC GCTGAAAAGCGTCTTTTTATTTTTCGGT CC	394
L3S3P46	synthetic	GACGAACAATAACACCCTAACGGGTGT TTTTTATTGATTGGTCTCCC	395
L2U1H07	synthetic	AGGCCTCCCAGATGGGGGGCCTTTTTTT TTT	396

L1U4H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTTTCTCTG	397
L1U4H07	synthetic	GATCCAGCTTCGGCTGGATCTTTTCTCTG	398
L1U4H11	synthetic	CCCGCTTCGGCGGGTTTTCTCTG	399
L1U4H08	synthetic	CCCGCATGTTCGCATGCGGGTTTTCTCTG	400
L1U4H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTTTCTCTG	401
L1U4H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTTTCTCTG	402
L2U1H08	synthetic	TGCCGGGAAGATTCCCGGCATTTTTTTTT	403
L2U1H12	synthetic	CGCCCGCAGATGCGGGCGTTTTTTTTT	404
L1U4H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTTTCTCTG	405
L1U4H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTTTCTCTG	406
L1U4H09	synthetic	CGACGATGTTCGCATCGTCGTTTTCTCTG	407
L3S1P24	synthetic	GACGAACAATAACACCCAAATCGGGTGTTTTTTATTGATAACAAAA	408
L1U4H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTTTCTCTG	409
L2U6H06	synthetic	CTAAAGCGCCTAACCACGGCGCTTTAGTTTTTTGTA	410
L2U1H06	synthetic	CTAAAGCGCCAGATGGCGCTTTAGTTTTTTTTT	411
L2U2H09	synthetic	ACGGCCCTCGCAAGGGCCGTTTTTTTGTA	412
L2U6H09	synthetic	ACGGCCCTTAACCACAGGGCCGTTTTTTTGTA	413
L2U2H06	synthetic	CTAAAGCGCCCGCAGGCGCTTTAGTTTTTTGTA	414
L1U1H07	synthetic	GATCCAGCTTCGGCTGGATCTTTTTTTTT	415
L2U6H07	synthetic	AGGCCTCCCTAACCACGGGGGGCCTTTTTTTGTA	416
L1U4H12	synthetic	CGACGTTCGCGTCGTTTTCTCTG	417
L1U1H08	synthetic	CCCGCATGTTCGCATGCGGGTTTTTTTTT	418
L2U7H07	synthetic	AGGCCTCCCGAACAGGGGGGCCTTTTTGCAAC	419
L1U4H10	synthetic	CGACGATATTCGTATCGTCGTTTTCTCTG	420
L2U1H11	synthetic	TAGCGTGCAGATGCACGCTATTTTTTTTT	421

L1U2H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTTTTTGGG	422
L1U5H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTTATTGTC	423
L2U4H09	synthetic	ACGGCCCTCGCAAGGGCCGTTTTTGCAAC	424
L2U1H04	synthetic	GCCGGAGCGGAGATCTGCTCCGGCTTTTTTTTT	425
L1U3H07	synthetic	GATCCAGCTTCGGCTGGATCTTTTGTTGG	426
L2U6H12	synthetic	CGCCCGCTAACCACGCGGGCGTTTTTTGTA	427
L2U7H06	synthetic	CTAAAGCGCCGAACAGGCGCTTTAGTTTTGCAAC	428
L1U5H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTTATTGTC	429
L1U2H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTTTTTGGG	430
L1U5H07	synthetic	GATCCAGCTTCGGCTGGATCTTTATTGTC	431
L1U2H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTTTTTGGG	432
L2U7H09	synthetic	ACGGCCCTGAACAAGGGCCGTTTTTGCAAC	433
L1U5H09	synthetic	CGACGATGTTCGCATCGTCGTTTATTGTC	434
L2U2H12	synthetic	CGCCCGCCGCAGCGGGCGTTTTTTGTA	435
L1U1H09	synthetic	CGACGATGTTCGCATCGTCGTTTTTTTTT	436
L1U5H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTTATTGTC	437
L2U2H11	synthetic	TAGCGTGCCGCAGCACGCTATTTTTTGTA	438
L2U4H12	synthetic	CGCCCGCCGCAGCGGGCGTTTTGCAAC	439
L1U2H07	synthetic	GATCCAGCTTCGGCTGGATCTTTTTTGGG	440
L2U4H10	synthetic	GGGCGGTCCGCAGATCGCCCTTTTGCAAC	441
L2U3H09	synthetic	ACGGCCCTCGCAAGGGCCGTTTTGTTGAG	442
L1U5H08	synthetic	CCCGCATGTTCGCATGCGGGTTTATTGTC	443
L1U2H11	synthetic	CCCGCTTCGGCGGGTTTTTTGGG	444
L1U5H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTTATTGTC	445
L1U2H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTTTTTGGG	446

L1U5H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTTATTGTC	447
L1U1H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTTTTTTTT	448
L1U5H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTTATTGTC	449
L1U8H12	synthetic	CGACGTTCGCGTCGTTAAAATAA	450
L2U6H04	synthetic	GCCGGAGCGGTAACCACCTGCTCCGGCTTTTTTGTA	451
L2U6H11	synthetic	TAGCGTGCTAACCACGCACGCTATTTTTTGTA	452
L2U2H10	synthetic	GGGCGGTCCGCAGATCGCCCTTTTTTGTA	452
L2U6H10	synthetic	GGGCGGTCTAACCACGATCGCCCTTTTTTGTA	453
L2U2H07	synthetic	AGGCCTCCCCGCAGGGGGGCCTTTTTTTGTA	454
L2U4H06	synthetic	CTAAAGCGCCCGCAGGCGCTTTAGTTTTGCAAC	455
L2U1H05	synthetic	GCCGGATCGGAGATCTGATCCGGCTTTTTTTTT	456
L1U1H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTTTTTTT	457
L1U5H10	synthetic	CGACGATATTCGTATCGTCGTTTATTGTC	458
L1U2H08	synthetic	CCCGCATGTTCGCATGCGGGTTTTTTGGG	459
L1U2H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTTTTTGGG	460
L2U2H08	synthetic	TGCCGGGACGCATCCCGGCATTTTTTGTA	461
L2U3H07	synthetic	AGGCCTCCCCGCAGGGGGGCCTTTTGTTGAG	462
L2U6H08	synthetic	TGCCGGGATAACCACTCCCGGCATTTTTTGTA	463
L2U6H05	synthetic	GCCGGATCGGTAACCACCTGATCCGGCTTTTTTGTA	464
L1U3H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTTTGTTGG	465
L2U1H03	synthetic	TAGCGTGACCGGAGATTCGGTCACGCTATTTTTTTTT	466
L1U2H09	synthetic	CGACGATGTTCGCATCGTCGTTTTTTGGG	467
L1U5H12	synthetic	CGACGTTCGCGTCGTTTATTGTC	468
L1U1H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTTTTTTTT	469
L1U3H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTTTGTTGG	470

L1U2H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTTTTTGGG	471
L2U7H05	synthetic	GCCGGATCGGGAACACTGATCCGGCTTTTGCAAC	472
L1U1H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTTTTTTTT	473
L1U2H10	synthetic	CGACGATATTCGTATCGTCGTTTTTTGGG	474
L2U7H12	synthetic	CGCCCGCGAACAGCGGGCGTTTTGCAAC	475
L1U1H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTTTTTTTT	476
L1U3H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTTTGTTGG	477
L1U3H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTTTGTTGG	478
L2U3H12	synthetic	CGCCCGCCGCAGCGGGCGTTTGTTGAG	479
L2U2H04	synthetic	GCCGGAGCGGCGCACTGCTCCGGCTTTTTTGTA	480
L1U3H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTTTGTTGG	481
L1U3H11	synthetic	CCCGCTTCGGCGGGTTTTGTTGG	482
L1U3H08	synthetic	CCCGCATGTTCGCATGCGGGTTTTGTTGG	483
L1U1H10	synthetic	CGACGATATTCGTATCGTCGTTTTTTTTT	484
L2U4H07	synthetic	AGGCCTCCCCGCAGGGGGGCCTTTTTGCAAC	485
L2U7H08	synthetic	TGCCGGGAGAACATCCCGGCATTTTGCAAC	486
L2U4H11	synthetic	TAGCGTGCCGCAGCACGCTATTTTGCAAC	487
L2U3H06	synthetic	CTAAAGCGCCCGCAGGCGCTTTAGTTTGTTGAG	488
L2U7H10	synthetic	GGGCGGTCGAACAGATCGCCCTTTTGCAAC	489
L2U8H07	synthetic	AGGCCTCCCTAACCACGGGGGGCCTTTGTTGTAT	490
L1U2H12	synthetic	CGACGTTCGCGTCGTTTTTTGGG	491
L2U2H02	synthetic	CCTGGTAAGACGCCGCAGCGTCTTATCAGGTTTTTTGTA	492
L1U8H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTAAAATAA	493
L2U4H04	synthetic	GCCGGAGCGGCGCACTGCTCCGGCTTTTGCAAC	494
L2U3H11	synthetic	TAGCGTGCCGCAGCACGCTATTTGTTGAG	495

L2U7H02	synthetic	CCTGGTAAGACGCGAACAGCGTCTTATCAGGTTTTGCAAC	496
L1U7H08	synthetic	CCCGCATGTTCGCATGCGGGTTATCAAAA	497
L1U3H09	synthetic	CGACGATGTTCGCATCGTCGTTTTGTTGG	498
L2U2H05	synthetic	GCCGGATCGGCGCACTGATCCGGCTTTTTTGTA	499
L2U4H08	synthetic	TGCCGGGACGCATCCCGGCATTTTGCAAC	500
L2U5H06	synthetic	CTAAAGCGCCGAACAGGCGCTTTAGTTGTTGTAT	501
L2U6H03	synthetic	TAGCGTGACCGGTAACCACTCGGTCACGCTATTTTTTGTA	502
L1U3H12	synthetic	CGACGTTCGCGTCGTTTTGTTGG	503
L2U8H10	synthetic	GGGCGGTCTAACCACGATCGCCCTTGTTGTAT	504
L1U3H10	synthetic	CGACGATATTCGTATCGTCGTTTTGTTGG	505
L2U2H01	synthetic	CCTGGTAAGACGCCGCAGCGTCGCATCAGGTTTTTTGTA	506
L2U8H12	synthetic	CGCCCGCTAACCACGCGGGCGTTGTTGTAT	507
L2U1H02	synthetic	CCTGGTAAGACGCAGATGCGTCTTATCAGGTTTTTTTTT	508
L2U2H03	synthetic	TAGCGTGACCGGCGCATCGGTCACGCTATTTTTTGTA	509
L2U3H04	synthetic	GCCGGAGCGGCGCACTGCTCCGGCTTTGTTGAG	510
L2U3H08	synthetic	TGCCGGGACGCATCCCGGCATTTGTTGAG	511
L2U8H08	synthetic	TGCCGGGATAACCACTCCCGGCATTGTTGTAT	512
L2U4H01	synthetic	CCTGGTAAGACGCCGCAGCGTCGCATCAGGTTTTGCAAC	513
L2U3H03	synthetic	TAGCGTGACCGGCGCATCGGTCACGCTATTTGTTGAG	514
L2U7H11	synthetic	TAGCGTGCGAACAGCACGCTATTTTGCAAC	515
L2U4H03	synthetic	TAGCGTGACCGGCGCATCGGTCACGCTATTTTGCAAC	516
L2U7H04	synthetic	GCCGGAGCGGGAACACTGCTCCGGCTTTTGCAAC	517
L1U8H09	synthetic	CGACGATGTTCGCATCGTCGTTAAAATAA	518
L2U5H05	synthetic	GCCGGATCGGGAACACTGATCCGGCTTGTTGTAT	519
L2U5H10	synthetic	GGGCGGTCGAACAGATCGCCCTTGTTGTAT	520

L2U5H12	synthetic	CGCCCGCGAACAGCGGGCGTTGTTGTAT	521
L1U8H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTAAAATAA	522
L1U6H08	synthetic	CCCGCATGTTCGCATGCGGGTTTCAACAA	523
L1U7H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTATCAAAA	524
L2U4H02	synthetic	CCTGGTAAGACGCCGCAGCGTCTTATCAGGTTTTGCAAC	525
L2U3H10	synthetic	GGGCGGTCCGCAGATCGCCCTTTGTTGAG	526
L2U6H01	synthetic	CCTGGTAAGACGCTAACCACGCGTCGCATCAGGTTTTTTGTA	527
L1U7H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTATCAAAA	528
L2U3H01	synthetic	CCTGGTAAGACGCCGCAGCGTCGCATCAGGTTTGTTGAG	529
L2U8H02	synthetic	CCTGGTAAGACGCTAACCACGCGTCTTATCAGGTTGTTGTAT	530
L1U7H12	synthetic	CGACGTTCGCGTCGTTATCAAAA	531
L1U8H11	synthetic	CCCGCTTCGGCGGGTTAAAATAA	532
L2U7H03	synthetic	TAGCGTGACCGGGAACATCGGTCACGCTATTTTGCAAC	533
L1U8H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTAAAATAA	534
L2U5H02	synthetic	CCTGGTAAGACGCGAACAGCGTCTTATCAGGTTGTTGTAT	535
L1U6H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTTCAACAA	536
L2U1H01	synthetic	CCTGGTAAGACGCAGATGCGTCGCATCAGGTTTTTTTTT	537
L2U5H04	synthetic	GCCGGAGCGGGAACACTGCTCCGGCTTGTTGTAT	538
L2U3H05	synthetic	GCCGGATCGGCGCACTGATCCGGCTTTGTTGAG	539
L1U7H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTATCAAAA	540
L1U6H04	synthetic	CGACGATGCCATTCGTGGCATCGTCGTTTCAACAA	541
L2U5H08	synthetic	TGCCGGGAGAACATCCCGGCATTGTTGTAT	542
L2U4H05	synthetic	GCCGGATCGGCGCACTGATCCGGCTTTTGCAAC	543
L1U6H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTTCAACAA	544
L1U7H07	synthetic	GATCCAGCTTCGGCTGGATCTTATCAAAA	545

L1U8H07	synthetic	GATCCAGCTTCGGCTGGATCTTAAAATAA	546
L1U6H07	synthetic	GATCCAGCTTCGGCTGGATCTTTCAACAA	547
L1U7H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTATCAAAA	548
L1U1H12	synthetic	CGACGTTCGCGTCGTTTTTTTTT	549
L2U3H02	synthetic	CCTGGTAAGACGCCGCAGCGTCTTATCAGGTTTGTTGAG	550
L2U5H09	synthetic	ACGGCCCTGAACAAGGGCCGTTTGTTGTAT	551
L1U8H08	synthetic	CCCGCATGTTCGCATGCGGGTTAAAATAA	552
L1U8H10	synthetic	CGACGATATTCGTATCGTCGTTAAAATAA	553
L1U7H09	synthetic	CGACGATGTTCGCATCGTCGTTATCAAAA	554
L1U6H12	synthetic	CGACGTTCGCGTCGTTTCAACAA	555
L2U5H07	synthetic	AGGCCTCCCGAACAGGGGGGCCTTTGTTGTAT	556
L1U7H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTATCAAAA	557
L1U8H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTAAAATAA	558
L2U6H02	synthetic	CCTGGTAAGACGCTAACCACGCGTCTTATCAGGTTTTTTGTA	559
L1U8H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTAAAATAA	560
L2U8H05	synthetic	GCCGGATCGGTAACCACCTGATCCGGCTTGTTGTAT	561
L1U7H02	synthetic	CCCGCATGCCATTCGTGGCATGCGGGTTATCAAAA	562
L1U8H01	synthetic	TCGGTTACCGCTTCGGCGGTAACCGATTAAAATAA	563
L1U7H11	synthetic	CCCGCTTCGGCGGGTTATCAAAA	564
L1U6H11	synthetic	CCCGCTTCGGCGGGTTTCAACAA	565
L1U6H06	synthetic	TCGGTTACATGTTCGCATGTAACCGATTTCAACAA	566
L1U7H10	synthetic	CGACGATATTCGTATCGTCGTTATCAAAA	567
L2U8H09	synthetic	ACGGCCCTTAACCACAGGGCCGTTTGTTGTAT	568
L2U7H01	synthetic	CCTGGTAAGACGCGAACAGCGTCGCATCAGGTTTTGCAAC	569
L2U8H01	synthetic	CCTGGTAAGACGCTAACCACGCGTCGCATCAGGTTGTTGTAT	570
L1U6H09	synthetic	CGACGATGTTCGCATCGTCGTTTCAACAA	571

L1U6H05	synthetic	CGACGATACCATTCGTGGTATCGTCGTTTCAACAA	572
L1U6H03	synthetic	GATCCAGCCCATTCGTGGGCTGGATCTTTCAACAA	573
L2U5H03	synthetic	TAGCGTGACCGGGAACATCGGTCACGCTATTGTTGTAT	574
L2U8H04	synthetic	GCCGGAGCGGTAACCACCTGCTCCGGCTTGTTGTAT	575
L2U5H01	synthetic	CCTGGTAAGACGCGAACAGCGTCGCATCAGGTTGTTGTAT	576
L2U8H03	synthetic	TAGCGTGACCGGTAACCACTCGGTCACGCTATTGTTGTAT	577
L1U6H10	synthetic	CGACGATATTCGTATCGTCGTTTCAACAA	578
L2U8H11	synthetic	TAGCGTGCTAACCACGCACGCTATTGTTGTAT	579
L2U8H06	synthetic	CTAAAGCGCCTAACCACGGCGCTTTAGTTGTTGTAT	580
L2U5H11	synthetic	TAGCGTGCGAACAGCACGCTATTGTTGTAT	581
BBa_J61048	rnpB-T1	ccggcttatcggtcagtttcacctgatttacgtaaaaacccgcttcggcgggtttttgcttttggaggggcagaaagatgaatgactgtccacgacgctatacccaaaagaaa	820

APPENDIX 2: Promotor Sequences

Name	Description	Promoter Sequence	SEQ ID NO:
BBa_J23100	constitutive promoter family member	. . . ggctagctcagtcctaggtacagtgctagc	582
BBa_J23101	constitutive promoter family member	. . . agctagctcagtcctaggtattatgctagc	583
BBa_J23102	constitutive promoter family member	. . . agctagctcagtcctaggtactgtgctagc	584
BBa_J23103	constitutive promoter family member	. . . agctagctcagtcctagggattatgctagc	585
BBa_J23104	constitutive promoter family member	. . . agctagctcagtcctaggtattgtgctagc	586
BBa_J23105	constitutive promoter family member	. . . ggctagctcagtcctaggtactatgctagc	587
BBa_J23106	constitutive promoter family member	. . . ggctagctcagtcctaggtatagtgctagc	588
BBa_J23108	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc	589
BBa_J23109	constitutive promoter family member	. . . agctagctcagtcctagggactgtgctagc	590
BBa_J23110	constitutive promoter family member	. . . ggctagctcagtcctaggtacaatgctagc	592

BBa_J23111	constitutive promoter family member	. . . ggctagctcagtcctaggtatagtgctagc	593
BBa_J23112	constitutive promoter family member	. . . agctagctcagtcctagggattatgctagc	594
BBa_J23113	constitutive promoter family member	. . . ggctagctcagtcctagggattatgctagc	595
BBa_J23114	constitutive promoter family member	. . . ggctagctcagtcctaggtacaatgctagc	596
BBa_J23115	constitutive promoter family member	. . . agctagctcagcccttggtacaatgctagc	597
BBa_J23116	constitutive promoter family member	. . . agctagctcagtcctagggactatgctagc	598
BBa_J23117	constitutive promoter family member	. . . agctagctcagtcctagggattgtgctagc	599
BBa_J23118	constitutive promoter family member	. . . ggctagctcagtcctaggtattgtgctagc	600
BBa_J44002	pBAD reverse	. . . aaagtgtgacgccgtgcaaataatcaatgt	601
BBa_K256002	J23101:GFP	. . . caccttcgggtgggcctttctgcgtttata	602
BBa_K823004	Anderson promoter J23100	. . . ggctagctcagtcctaggtacagtgctagc	603
BBa_K823005	Anderson promoter J23101	. . . agctagctcagtcctaggtattatgctagc	604
BBa_K823006	Anderson promoter J23102	. . . agctagctcagtcctaggtactgtgctagc	605
BBa_K823007	Anderson promoter J23103	. . . agctagctcagtcctagggattatgctagc	606
BBa_K823008	Anderson promoter J23106	. . . ggctagctcagtcctaggtatagtgctagc	607
BBa_K823010	Anderson promoter J23113	. . . ggctagctcagtcctagggattatgctagc	608
BBa_K823011	Anderson promoter J23114	. . . ggctagctcagtcctaggtacaatgctagc	609
BBa_K823013	Anderson promoter J23117	. . . agctagctcagtcctagggattgtgctagc	610
BBa_K823014	Anderson promoter J23118	. . . ggctagctcagtcctaggtattgtgctagc	611
BBa_I14018	P(Bla)	. . . gtttatacataggcgagtactctgttatgg	612
BBa_I14033	P(Cat)	. . . agaggttccaactttcaccataatgaaaca	613
BBa_I14034	P(Kat)	. . . taaacaactaacggacaattctacctaaca	614
BBa_J23119	constitutive promoter family member	. . . agctagctcagtcctaggtataatgctagc	615
BBa_J23150	1bp mutant from J23107	. . . ggctagctcagtcctaggtattatgctagc	616
BBa_K1330002	Constitutive promoter (J23105)	. . . ggctagctcagtcctaggtactatgctagc	617
BBa_I742126	Reverse lambda cI-regulated promoter	. . . gaggtaaaatagtcaacacgcacggtgtta	618
BBa_J01006	Key Promoter absorbs 3	. . . caggccggaataactccctataatgcgcca	619
BBa_J23151	1bp mutant from J23114	. . . ggctagctcagtcctaggtacaatgctagc	620

BBa_J48104	NikR promoter, a protein of the ribbon helix-helix family of trancription factors that repress expre	. . . gacgaatacttaaaatcgtcatacttattt	623
BBa_J54200	lacq_Promoter	. . . aaacctttcgcggtatggcatgatagcgcc	624
BBa_J56015	lacIQ - promoter sequence	. . . tgatagcgcccggaagagagtcaattcagg	625
BBa_J64951	E. Coli CreABCD phosphate sensing operon promoter	. . . ttatttaccgtgacgaactaattgctcgtg	626
BBa_K119000	Constitutive weak promoter of lacZ	. . . ttatgcttccggctcgtatgttgtgtggac	627
BBa_K119001	Mutated LacZ promoter	. . . ttatgcttccggctcgtatggtgtgtggac	628
BBa_K137085	optimized (TA) repeat constitutive promoter with 13 bp between -10 and -35 elements	. . . tgacaatatatatatatataatgctagc	629
BBa_K137086	optimized (TA) repeat constitutive promoter with 15 bp between -10 and -35 elements	. . . acaatatatatatatatataatgctagc	630
BBa_K137087	optimized (TA) repeat constitutive promoter with 17 bp between -10 and -35 elements	. . . aatatatatatatatatataatgctagc	631
BBa_K137088	optimized (TA) repeat constitutive promoter with 19 bp between -10 and -35 elements	. . . tatatatatatatatatataatgctagc	632
BBa_K137089	optimized (TA) repeat constitutive promoter with 21 bp between -10 and -35 elements	. . . tatatatatatatatatataatgctagc	633
BBa_K137090	optimized (A) repeat constitutive promoter with 17 bp between -10 and -35 elements	. . . aaaaaaaaaaaaaaaaaatataatgctagc	634
BBa_K137091	optimized (A) repeat constitutive promoter with 18 bp between -10 and -35 elements	. . . aaaaaaaaaaaaaaaaaatataatgctagc	635
BBa_K1585100	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	636
BBa_K1585101	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	637
BBa_K1585102	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	638
BBa_K1585103	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	639

BBa_K1585104	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	640
BBa_K1585105	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	641
BBa_K1585106	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	642
BBa_K1585110	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	643
BBa_K1585113	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	644
BBa_K1585115	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	645
BBa_K1585116	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	646
BBa_K1585117	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	647
BBa_K1585118	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	648
BBa_K1585119	Anderson Promoter with lacI binding site	. . . ggaattgtgagcggataacaatttcacaca	649
BBa_K2486171	A reverse complement version of BBa_J23114	. . . cattgtacctaggactgagctagccataaa	650
BBa_K418000	IPTG inducible Lac promoter cassette	. . . ttgtgagcggataacaagatactgagcaca	651
BBa_M13101	M13K07 gene I promoter	. . . cctgtttttatgttattctctctgtaaagg	652
BBa_M13102	M13K07 gene II promoter	. . . aaatatttgcttatacaatcttcctgtttt	653
BBa_M13103	M13K07 gene III promoter	. . . gctgataaaccgatacaattaaaggctcct	654
BBa_M13104	M13K07 gene IV promoter	. . . ctcttctcagcgtcttaatctaagctatcg	655
BBa_M13105	M13K07 gene V promoter	. . . atgagccagttcttaaaatcgcataaggta	656
BBa_M13106	M13K07 gene VI promoter	. . . ctattgattgtgacaaaataaacttattcc	657
BBa_M13108	M13K07 gene VIII promoter	. . . gtttcgcgcttggtataatcgctgggggtc	658
BBa_M13110	M13110	. . . ctttgcttctgactataatagtcagggtaa	659
BBa_M31519	Modified promoter sequence of g3.	. . . aaaccgatacaattaaaggctcctgctagc	660
BBa_R1074	Constitutive Promoter I	. . . caccacactgatagtgctagtgtagatcac	661
BBa_R1075	Constitutive Promoter II	. . . gccggaataactccctataatgcgccacca	662
BBa_S03331	--Specify Parts List--	ttgacaagcttttcctcagctccgtaaact	663
BBa_J23107	constitutive promoter family member	. . . ggctagctcagccctaggtattatgctagc	664
BBa_K088007	GlnRS promoter	. . . catacgccgttatacgttgtttacgctttg	665
BBa_K137029	constitutive promoter with (TA)10 between -10 and -35 elements	. . . atatatatatatataatggaagcgtttt	666

BBa_K137030	constitutive promoter with (TA)9 between -10 and -35 elements	. . . atatatatatatataatggaagcgtttt	667
BBa_K137031	constitutive promoter with (C)10 between -10 and -35 elements	. . . ccccgaaagcttaagaatataattgtaagc	668
BBa_K137032	constitutive promoter with (C)12 between -10 and -35 elements	. . . ccccgaaagcttaagaatataattgtaagc	669
BBa_K1824896	J23100 + RBS	. . . gattaaagaggagaaatactagagtactag	670
BBa_K256018	J23119:IFP	. . . caccttcgggtgggcctttctgcgtttata	671
BBa_K256020	J23119:HO1	. . . caccttcgggtgggcctttctgcgtttata	672
BBa_K256033	Infrared signal reporter (J23119:IFP:J23119:HO1)	. . . caccttcgggtgggcctttctgcgtttata	673
BBa_K292000	Double terminator + constitutive promoter	. . . ggctagctcagtcctaggtacagtgctagc	674
BBa_K292001	Double terminator + Constitutive promoter + Strong RBS	. . . tgctagctactagagattaaagaggagaaa	675
BBa_K418002	IPTG inducible Lac promoter cassette	. . . ttgtgagcggataacaagatactgagcaca	676
BBa_K418003	IPTG inducible Lac promoter cassette	. . . ttgtgagcggataacaagatactgagcaca	677
BBa_I0500	Inducible pBad/araC promoter	. . . gtttctccatacccgtttttttgggctagc	678
BBa_I1051	Lux cassette right promoter	. . . tgttatagtcgaatacctctggcggtgata	679
BBa_I12006	Modified lamdba Prm promoter (repressed by 434 cI)	. . . attacaaactttcttgtatagatttaacgt	680
BBa_I12007	Modified lambda Prm promoter (OR-3 obliterated)	. . . atttataaatagtggtgatagatttaacgt	681
BBa_I12036	Modified lamdba Prm promoter (cooperative repression by 434 cI)	. . . tttcttgtatagatttacaatgtatcttgt	682
BBa_I12040	Modified lambda P(RM) promoter: -10 region from P(L) and cooperatively repressed by 434 cI	. . . tttcttgtagatacttacaatgtatcttgt	683
BBa_I12210	plac Or2-62 (positive)	. . . ctttatgcttccggctcgtatgttgtgtgg	684
BBa_I13406	Pbad/AraC with extra REN sites	. . . ttttttgggctagcaagctttaccatggat	685
BBa_I13453	Pbad promoter	. . . tgtttctccataccgtttttttgggctagc	686
BBa_I14015	P(Las) TetO	. . . ttttggtacactccctatcagtgatagaga	687
BBa_I14016	P(Las) CIO	. . . ctttttggtacactacctctggcggtgata	688
BBa_I14017	P(Rhl)	. . . tacgcaagaaaatggtttgttatagtcgaa	689
BBa_I721001	Lead Promoter	. . . gaaaaccttgtcaatgaagagcgatctatg	690

BBa_I723020	Pu	. . . ctcaaagcgggccagccgtagccgttacgc	691
BBa_I731004	FecA promoter	. . . ttctcgttcgactcatagctgaacacaaca	692
BBa_I739104	Double Promoter (LuxR/HSL, positive / P22 cII, negative)	. . . gttctttaattatttaagtgttctttaatt	693
BBa_I739105	Double Promoter (LuxR/HSL, positive / cI, negative)	. . . cgtgcgtgttgataacaccgtgcgtgttga	694
BBa_I741018	Right facing promoter (for xylF) controlled by xylR and CRP-cAMP	. . . gttacgtttatcgcggtgattgttacttat	695
BBa_I741019	Right facing promoter (for xylA) controlled by xylR and CRP-cAMP	. . . gcaaaataaaatggaatgatgaaactgggt	696
BBa_I741020	promoter to xylF without CRP and several binding sites for xylR	. . . gttacgtttatcgcggtgattgttacttat	697
BBa_I741021	promoter to xylA without CRP and several binding sites for xylR	. . . atttcacactgctattgagataattcacaa	698
BBa_I746104	P2 promoter in agr operon from S. aureus	. . . agattgtactaaatcgtataatgacagtga	699
BBa_I746360	PF promoter from P2 phage	. . . gacatctccggcgcaactgaaaataccact	700
BBa_I746361	PO promoter from P2 phage	. . . gaggatgcgcatcgtcgggaaactgatgcc	701
BBa_I746362	PP promoter from P2 phage	. . . catccgggactgatggcggaggatgcgcat	702
BBa_I746363	PV promoter from P2 phage	. . . aacttttatatattgtgcaatctcacatgc	703
BBa_I746364	Psid promoter from P4 phage	. . . tgttgtccggtgtacgtcacaattttctta	704
BBa_I746365	PLL promoter from P4 phage	. . . gtctgctgaaaatattcacaaaataaagcg	705
BBa_I751501	plux-cI hybrid promoter	. . . gtgttgatgcttttatcaccgccagtggta	706
BBa_I751502	plux-lac hybrid promoter	. . . agtgtgtggaattgtgagcggataacaatt	707
BBa_I760005	Cu-sensitive promoter	atgacaaaattgtcat	708
BBa_I761011	CinR, CinL and glucose controlled promotor	. . . acatcttaaaagttttagtatcatattcgt	709
BBa_I765001	UV promoter	. . . ctgaaagcgcataccgctatggaggggtt	710
BBa_I765007	Fe and UV promoters	. . . ctgaaagcgcataccgctatggaggggtt	711
BBa_J01005	pspoIIE promoter (spo0A J01004, positive)	. . . aacgaatataacaggtgggagatgagagga	712
BBa_J03007	Maltose specific promotor	. . . aatatttcctcattttccacagtgaagtga	713
BBa_J06403	RhlR promoter repressible by CI	. . . tacgcaagaaaatggtttgttatagtcgaa	714
BBa_J07007	ctx promoter	. . . atttaattgttttgatcaattatttttctg	715

BBa_J102001	Reverse Lux Promoter	. . . tcttgcgtaaacctgtacgatcctacaggt	716
BBa_J13210	pOmpR dependent POPS producer	. . . attattctgcatttttggggagaatggact	717
BBa_J15502	copA promoter	. . . ccttgctggaaggtttaacctttatcacag	718
BBa_J16101	BanAp - Banana-induced Promoter	atgatgtgtccatggatta	719
BBa_J16105	HelPp - "Help" Dependant promoter	atgatagacgatgtgcggacaacgtg	720
BBa_J45503	hybB Cold Shock Promoter	. . . cattagccgccaccatggggttaagtagca	721
BBa_J58100	AND-type promoter synergistically activated by cI and CRP	. . . atttataaatagtggtgatagatttaacgt	722
BBa_J61051	[Psal1]	. . . ataaagccatcacgagtaccatagaggatc	723
BBa_J61054	[HIP-1] Promoter	. . . tttgtcttttcttgcttaataatgttgtca	724
BBa_J61055	[HIP-1fnr] Promoter	. . . tttgtcttttcttgcttaataatgttgtca	725
BBa_J64000	rhlI promoter	. . . atcctcctttagtcttccccctcatgtgtg	726
BBa_J64010	lasI promoter	. . . taaaattatgaaatttgcataaattcttca	727
BBa_J64712	LasR/LasI Inducible & RHLR/RHLI repressible Promoter	. . . gaaatctggcagtttttggtacacgaaagc	728
BBa_J64800	RHLR/RHLI Inducible & LasR/LasI repressible Promoter	. . . tgccagttctggcaggtctaaaaagtgttc	729
BBa_J64804	The promoter region (inclusive of regulator binding sites) of the B. subtilis RocDEF operon	. . . cacagaacttgcatttatataaagggaaag	730
BBa_K091107	pLux/cI Hybrid Promoter	. . . acaccgtgcgtgttgatatagtcgaataaa	731
BBa_K091117	pLas promoter	. . . aaaattatgaaatttgtataaattcttcag	732
BBa_K091143	pLas/cI Hybrid Promoter	. . . ggttctttttggtacctctggcggtgataa	733
BBa_K091146	pLas/Lux Hybrid Promoter	. . . tgtaggatcgtacaggtataaattcttcag	734
BBa_K091156	pLux	. . . caagaaaatggtttgttatagtcgaataaa	735
BBa_K091157	pLux/Las Hybrid Promoter	. . . ctatctcatttgctagtatagtcgaataaa	736
BBa_K100000	Natural Xylose Regulated Bi-Directional Operator	. . . gttacgtttatcgcggtgattgttacttat	737
BBa_K100001	Edited Xylose Regulated Bi-Directional Operator 1	. . . gttacgtttatcgcggtgattgttacttat	738
BBa_K100002	Edited Xylose Regulated Bi-Directional Operator 2	. . . gttacgtttatcgcggtgattgttacttat	739
BBa_K112118	rrnB P1 promoter	. . . ataaatgcttgactctgtagcgggaaggcg	740
BBa_K112320	{< ftsAZ promoter >} in BBb format	. . . aaaactggtagtaggactggagattggtac	741
BBa_K112322	{Pdps} in BBb format	. . . gggacacaaacatcaagaggatatgagatt	742

BBa_K112402	promoter for FabA gene - Membrane Damage and Ultrasound Senstitive	. . . gtcaaaatgaccgaaacgggtggtaacttc	743
BBa_K112405	Promoter for CadA and CadB genes	. . . agtaatcttatcgccagtttggtctggtca	744
BBa_K112406	cadC promoter	. . . agtaatcttatcgccagtttggtctggtca	745
BBa_K112701	hns promoter	. . . aattctgaacaacatccgtactcttcgtgc	746
BBa_K112900	Pbad	. . . tcgataagattaccgatcttacctgaagct	747
BBa_K116001	nhaA promoter, that can be regulated by pH and nhaR protein.	. . . cgatctattcacctgaaagagaaataaaaa	748
BBa_K116401	external phosphate sensing promoter	. . . attaatgatcgcaacctatttattacaaca	749
BBa_K116500	OmpF promoter that is activated or repressesed by OmpR according to osmolarity.	. . . aaacgttagtttgaatggaaagatgcctgc	750
BBa_K116603	pRE promoter from λ phage	. . . tttgcacgaaccatatgtaagtatttcctt	751
BBa_K117002	LsrA promoter (indirectly activated by AI-2)	. . . taacacttatttaattaaaaagaggagaaa	752
BBa_K118011	PcstA (glucose-repressible promoter)	. . . tagaaacaaaatgtaacatctctatggaca	753
BBa_K121011	promoter (lacI regulated)	. . . acaggaaacagctatgaccatgattacgcc	754
BBa_K135000	pCpxR (CpxR responsive promoter)	. . . agcgacgtctgatgacgtaatttctgcctc	755
BBa_K136010	fliA promoter	. . . gttcactctataccgctgaaggtgtaatgg	756
BBa_K145150	Hybrid promoter: HSL-LuxR activated, P22 C2 repressed	. . . tagtttataatttaagtgttctttaatttc	757
BBa_K1520010	Prlux-rbs-rfp-Ter	. . . caccttcgggtgggcctttctgcgtttata	758
BBa_K1520515	MC7-rbs-luxI-Ter-Pcons2-rbs-luxR-Ter-Prlux-rbs-OMPA-golB-rbs-luxI-Ter.	. . . caccttcgggtgggcctttctgcgtttata	759
BBa_K1520516	MC31-rbs-luxI-Ter-Pcons2-rbs-luxR-Ter-Prlux-rbs-OMPA-golB-rbs-luxI-Ter	. . . caccttcgggtgggcctttctgcgtttata	760
BBa_K1660005	RFP controlled by the PompR promoter	. . . caccttcgggtgggcctttctgcgtttata	761
BBa_K180000	Hybrid promoter (trp & lac regulated -- tac pR)	. . . cgagcacttcaccaacaaggaccatagcat	762
BBa_K180002	tac pR testing plasmid (GFP)	. . . caccttcgggtgggcctttctgcgtttata	763
BBa_K180003	PTAC testing plasmid (GFP) - basic	. . . catggcatggatgaactatacaaataataa	764

BBa_K180004	Game of Life - Primary plasmid	. . . caccttcgggtgggcctttctgcgtttata	765
BBa_K180005	GoL - Primary plasmid (part 1)/RPS - Paper primary plasmid (part 1) [LuxR generator]	. . . caccttcgggtgggcctttctgcgtttata	766
BBa_K180006	Game of Life - Primary plasmid (part 2) [lux pR, GFP and LacI generator]	. . . caccttcgggtgggcctttctgcgtttata	767
BBa_K180007	Game of Life - Secondary plasmid [tac pR, LuxI generator]	. . . caccttcgggtgggcctttctgcgtttata	768
BBa_K180010	Rock-paper-scissors - Rock primary plasmid	. . . caccttcgggtgggcctttctgcgtttata	769
BBa_K180011	Rock - Primary plasmid (part 1) [RhlR generator]	. . . caccttcgggtgggcctttctgcgtttata	770
BBa_K180012	Rock - Primary plasmid (part 2) [tac pR, mCherry and LasI generator]	. . . caccttcgggtgggcctttctgcgtttata	771
BBa_K180013	Rock-paper-scissors - Rock secondary plasmid [rhl pR, LacI generator]	. . . caccttcgggtgggcctttctgcgtttata	772
BBa_K180014	Rock-paper-scissors - Paper primary plasmid	. . . caccttcgggtgggcctttctgcgtttata	773
BBa_K180015	Paper - Primary plasmid (part 2) [tac pR, GFP and RhlI generator]	. . . caccttcgggtgggcctttctgcgtttata	774
BBa_K180016	Rock-paper-scissors - Paper secondary plasmid [lux pR, LacI generator]	. . . caccttcgggtgggcctttctgcgtttata	775
BBa_K180017	Rock-paper-scissors - Scissors primary plasmid	. . . caccttcgggtgggcctttctgcgtttata	776
BBa_K180018	Scissors - Primary plasmid (part 1) [LasR generator]	. . . caccttcgggtgggcctttctgcgtttata	777
BBa_K180019	Scissors - Primary plasmid (part 2) [tac pR, mBanana and LuxI generator]	. . . caccttcgggtgggcctttctgcgtttata	778
BBa_K180020	Rock-paper-scissors - Scissors secondary plasmid [las pR, LacI generator]	. . . caccttcgggtgggcctttctgcgtttata	779
BBa_K206000	pBAD strong	. . . tgtttctccataccgtttttttgggctagc	780
BBa_K206001	pBAD weak	. . . tgtttctccataccgtttttttgggctagc	781
BBa_K2558001	lux pR-HS	. . . caagaaaatggtttgttactttcgaataaa	782
BBa_K259005	AraC Rheostat Promoter	. . . ttttatcgcaactctctactgtttctccat	783
BBa_K259007	AraC Promoter fused with RBS	. . . gtttctccattactagagaaagaggggaca	784

BBa_K266000	PAI+LasR -> LuxI (AI)	. . . caccttcgggtgggcctttctgcgtttata	785
BBa_K266005	PAI+LasR -> LasI & AI+LuxR --| LasI	. . . aataactctgatagtgctagtgtagatctc	786
BBa_K266006	PAI+LasR -> LasI+GFP & AI+LuxR --| LasI+GFP	. . . caccttcgggtgggcctttctgcgtttata	787
BBa_K266007	Complex QS -> LuxI & LasI circuit	. . . caccttcgggtgggcctttctgcgtttata	788
BBa_K3205003	luxPR_3A	. . . caagaaaatggtttgttatagtcgaataaa	789
BBa_K3205004	luxPR_3G	. . . caagaaaatggtttgttatagtcgaataaa	790
BBa_K3205005	luxPR_4G12T	. . . caagaaaatggtttgttatagtcgaataaa	791
BBa_K3254014	Optimized Ptac promoter	. . . taatgtgtggaattgtgagcgctcacaatt	792
BBa_K3254015	Mutant Ptac promoter No.1	. . . tactgtgtggaattgtgagcgctcacaatt	793
BBa_K3254016	Mutant Ptac promoter No.2	. . . taatgtgtggaattgtgagcgctcacaatt	794
BBa_K3254017	Mutant Ptac promoter No.3	. . . tactgtgtggaattgtgagcgctcacaatt	795
BBa_K338029	+OmpR, +(CinR-HSL) Double Promoter	. . . tgctttccacgaacttgaaaacgctggagg	796
BBa_K427003	Pm promoter of Mu bacteriophage	. . . tcctcaatatcctgtgatgaataaccgtac	797
BBa_K427004	Pmom promoter of Mu bacteriophage	. . . tttttaagatagtggcgaattgatgcaaag	798
BBa_K658006	position 3 mutated promoter lux pR-3 (luxR & HSL regulated)	. . . caagaaaatggtttgttatagtcgaataaa	799
BBa_K658007	position 5 mutated promoter lux pR-5 (luxR & HSL regulated)	. . . caagaaaatggtttgttatagtcgaataaa	800
BBa_K658008	position 3&5 mutated promoter lux pR-3/5 (luxR & HSL regulated)	. . . caagaaaatggtttgttatagtcgaataaa	801
BBa_K731201	Arabinose inducible araC-pBAD promoter	. . . ctctactgtttctccatacccgttttttg	802
BBa_K808000	araC-Pbad - Arabinose inducible regulatory promoter/repressor unit	. . . tgtttctccatacccgtttttgggctaac	803
BBa_K864400	Ptac, trp & lac regulated promoter	. . . aatgtgtggaattgtgagcggataacaatt	804
BBa_R0062	Promoter (luxR & HSL regulated -- lux pR)	. . . caagaaaatggtttgttatagtcgaataaa	805
BBa_R0065	Promoter (lambda cI and luxR regulated -- hybrid)	. . . gtgttgactattttacctctggcggtgata	806
BBa_R0071	Promoter (RhlR & C4-HSL regulated)	. . . gttagctttcgaattggctaaaaagtgttc	807
BBa_R0078	Promoter (cinR and HSL regulated)	. . . ccattctgctttccacgaacttgaaaacgc	808

BBa_R0079	Promoter (LasR & PAI regulated)	. . . ggccgcgggttctttttggtacacgaaagc	809
BBa_R0080	Promoter (AraC regulated)	. . . ttttatcgcaactctctactgtttctccat	810
BBa_R0082	Promoter (OmpR, positive)	. . . attattctgcatttttggggagaatggact	811
BBa_R0083	Promoter (OmpR, positive)	. . . attattctgcatttttggggagaatggact	812
BBa_R0084	Promoter (OmpR, positive)	. . . aacgttagtttgaatggaaagatgcctgca	813
BBa_R1062	Promoter, Standard (luxR and HSL regulated -- lux pR)	. . . aagaaaatggtttgttgatactcgaataaa	814
BBa_K1614000	T7 bacteriophage Promoter	TAATACGACTCACTATAG	819

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of creating or modifying a cell-free expression vector, the method comprising:

(a) obtaining a cell-free expression vector comprising

(i) an origin of replication (ori),

(ii) a nucleic acid sequence encoding a protein or RNA,

(iii) a promoter arranged to drive expression of the protein or RNA, and

(iv) one or more selectable markers; and

(b) inserting into the cell-free expression vector an insulating terminator sequence at a location that is between 0 and 10,000 nucleotides in a 5' direction from the promoter.

2. A method of performing protein or RNA synthesis *in vitro*, the method comprising synthesizing protein or RNA *in vitro* using a cell-free expression vector, wherein the cell-free expression vector comprises:

(i) an origin of replication (ori);

(ii) a nucleic acid encoding protein or RNA to be synthesized;

(iii) a promoter arranged to drive expression of the protein or RNA;

(iv) an insulating terminator sequence located between 0 and 10,000 nucleotides in a 5' direction from the promoter; and

(iv) one or more selectable markers.

3. The method of claim 1 or claim 2, wherein the method avoids or reduces read-through of toxic product proteins during plasmid generation, while avoiding or decreasing a reduction in protein synthesis of the protein during cell-free protein synthesis.

4. The method of any one of claims 1-3, wherein the method avoids or reduces production of toxic RNA products.

5. The method of any one of claims 1-4, wherein the cell-free expression vector with the insulating terminator enables synthesis of the protein or RNA at a higher yield, with a higher growth rate, and/or with fewer sequence mutations than synthesis of the protein or RNA using a cell-free expression vector without an insulating terminator.

6. The method of any one of claims 1-4, wherein the cell-free expression vector with the insulating terminator enables synthesis of the protein or RNA at a higher level compared to a level of synthesis of the protein or RNA using a cell-free expression vector without an insulating terminator.

7. The method of any one of claims 1-6, wherein the cell-free expression vector contains a gene for a protein or RNA that inhibits or slows cellular replication in cells containing the cell-free expression vector.

8. The method of any one of claims 1-2 wherein the cell-free expression vector contains a gene for a protein or RNA that reduces plasmid yield from the cells containing the cell-free expression vector.

9. The method of any one of claims 1-8, wherein the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820), rrnB T1 (SEQ ID NO: 14), L3S2P21 (SEQ ID NO: 318), or L3S2P56 (SEQ ID NO: 319).

10. The method of claim 9, wherein the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820).

11. The method of any one of claims 1-10, wherein the promoter is a T7 phage promoter, a lac promoter, a trp promoter, a recA promoter, a ribosomal RNA promoter, a Sp6 promoter, a araBad promoter, a pTac promoter, or a J23119 promoter.

12. The method of claim 11, wherein the promoter is a T7 phage promoter.

13. The method of any one of claims 1-12, wherein the insulating terminator sequence is located 27 to 37 nucleotides in the 5' direction from the promoter.

14. The method of any one of claims 1-13, wherein the insulating terminator sequence is located 35 to 37 nucleotides in the 5' direction from the promoter.

15. The method of any one of claims 1-14, wherein the insulating terminator sequence is located 37 nucleotides in the 5' direction from the promoter.

16. The method any one of claims 1-15, wherein the insulating terminator sequence is 0 to 10,000 nucleotides in a 3' direction of the ori.

17. The method of claim 16, wherein the insulating terminator sequence is located 30 to 40 nucleotides in the 3' direction of the ori.

18. The method of claim 17, wherein the insulating terminator sequence is located 40 nucleotides in the 3' direction of the ori.

19. The method of claim 2, wherein the synthesizing step comprises using a cell-free protein synthesis platform.

20. The method of claim 19, wherein the cell-free protein synthesis platform comprises a system for *in vitro* transcription of mRNA and/or translation of polypeptides.

21. The method of any one of claims 1-20, wherein the cell-free expression vector further comprises a Ribosome-binding site (RBS).

22. The method of any one of claims 1-21, wherein the cell-free expression vector further comprises an Open Reading Frame (ORF).

23. A kit for use in a method of creating or modifying a cell-free expression vector, the kit comprising:

(a) a cell-free expression vector comprising

(i) an origin of replication (ori),

(ii) a nucleic acid sequence encoding a protein or RNA,

(iii) a promoter arranged to drive expression of the protein or RNA, and

(iv) one or more selectable markers;

(b) an insulating terminator sequence that is to be inserted at a location between 0 and 10,000 nucleotides in a 5' direction from the promoter in the cell-free expression vector; and

(c) one or more cloning reagents.

24. A kit for performing protein or RNA synthesis *in vitro*, the kit comprising:

(a) reagents for cell-free protein or RNA synthesis; and

(b) a cell-free expression vector comprising

(i) an origin of replication (ori);

(ii) a nucleic acid encoding protein or RNA to be synthesized;

(iii) a promoter arranged to drive expression of the protein or RNA;

(iv) an insulating terminator sequence that is located between 0 and 10,000 nucleotides in a 5' direction from the promoter; and

(iv) one or more selectable markers.

25. The kit of any one of claims 23-24, wherein the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820), rrnB T1 (SEQ ID NO: 14), L3S2P21 (SEQ ID NO: 318), or L3S2P56 (SEQ ID NO: 319).

26. The kit of claim 25, wherein the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820).

27. The kit of any one of claims 23-26, wherein the promoter is a T7 phage promoter, a lac promoter, a trp promoter, a recA promoter, a ribosomal RNA promoter, a Sp6 promoter, a araBad promoter, a pTac promoter, or a J23119 promoter.

28. The kit of claim 27, wherein the promoter is a T7 phage promoter.

29. The kit of any one of claims 23-28, wherein the insulating terminator is located between 0 and 10,000 nucleotides in a 5' direction from the promoter

30. The kit of any one of claims 23-29, wherein the insulating terminator sequence is located 27 to 37 nucleotides in the 5' direction from the promoter.

31. The kit of any one of claims 23-30, wherein the insulating terminator sequence is located 35 to 37 nucleotides in the 5' direction from the promoter.

32. The kit of any one of claims 23-31, wherein the insulating terminator sequence is located 37 nucleotides in the 5' direction from the promoter.

33. The kit of any one of claims 23-32, wherein the insulating terminator sequence is 0 to 10,000 nucleotides in a 3' direction of the ori.

34. The kit of any one of claims 23-33, wherein the insulating terminator sequence is located 30 to 40 nucleotides in the 3' direction of the ori.

35. The kit of any one of claims 23-34, wherein the insulating terminator sequence is located 40 nucleotides in the 3' direction of the ori.

36. The kit of any one of claims 23-35, wherein the cell-free expression vector further comprises a Ribosome-binding site (RBS).

37. The kit of any one of claims 23-36, wherein the cell-free expression vector further comprises an Open Reading Frame (ORF).

38. A cell-free expression vector comprising:

(i) an origin of replication (ori),

(ii) a nucleic acid sequence encoding a protein or RNA,

(iii) a promoter arranged to drive expression of the protein or RNA,

(iv) one or more selectable markers, and

(v) an insulating terminator sequence at a location that is between 0 and 10,000 nucleotides in a 5' direction from the promoter.

39. The cell-free expression vector of claim 38, wherein the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820), rrnB T1 (SEQ ID NO: 14), L3S2P21 (SEQ ID NO: 318), or L3S2P56 (SEQ ID NO: 319).

40. The cell-free expression vector of claim 39, wherein the insulating terminator sequence is rnpB-T1 (SEQ ID NO: 820).

41. The cell-free expression vector of any one of claims 38-40, wherein the promoter is a T7 phage promoter, a lac promoter, a trp promoter, a recA promoter, a ribosomal RNA promoter, a Sp6 promoter, a araBad promoter, a pTac promoter, or a J23119 promoter.

42. The cell-free expression vector of 41, wherein the promoter is a T7 phage promoter.

43. The cell-free expression vector of claim 43, wherein the vector comprises a backbone.

44. The cell-free expression vector of any one of claims 38-42, wherein the backbone is from one of the following vectors: pJL1, pY71, p70a, pBEST, pEXP5, or pT7CFE.

45. The cell-free expression vector of claim 44, wherein the backbone is from a pJL1 plasmid.

46. The cell-free expression of any one of claims 38-45, wherein at least a portion of the cell-free expression vector comprises the following sequence: gggcggagcctatggaaaaacgccagcaacgcggccttttacggttcctggccttttccggcttatcggtcagtttcacctgattt acgtaaaaacccgcttcggcgggtttttgcttttggaggggcagaaagatgaatgactgtccacgacgctatacccaaaagaaa gctggccttttgctcacatgttcttatcccgcgaaattaatacgactcactatag (SEQ ID NO: 818).

47. The cell-free expression vector of any one of claims 38-46, comprising, consisting of, or consisting essentially of the features shown in FIG. 5.

48. The cell-free expression vector of any one of claims 38-47, wherein the insulating terminator is located between 0 and 10,000 nucleotides in a 5' direction from the promoter

49. The cell-free expression vector of any one of claims 38-48, wherein the insulating terminator sequence is located 27 to 37 nucleotides in the 5' direction from the promoter.

50. The cell-free expression vector of any one of claims 38-49, wherein the insulating terminator sequence is located 35 to 37 nucleotides in the 5' direction from the promoter.

51. The cell-free expression vector of any one of claims 38-50, wherein the insulating terminator sequence is located 37 nucleotides in the 5' direction from the promoter.

52. The cell-free expression vector of any one of claims 38-51, wherein the insulating terminator sequence is 0 to 10,000 nucleotides in a 3' direction of the ori.

53. The cell-free expression vector of any one of claims 38-52, wherein the insulating terminator sequence is located 30 to 40 nucleotides in the 3' direction of the ori.

54. The cell-free expression vector of any one of claims 38-53, wherein the insulating terminator sequence is located 40 nucleotides in the 3' direction of the ori.

55. The cell-free expression vector of any one of claims 38-54, wherein the cell-free expression vector further comprises a Ribosome-binding site (RBS).

56. The cell-free expression vector of any one of claims 38-55, wherein the cell-free expression vector further comprises an Open Reading Frame (ORF).

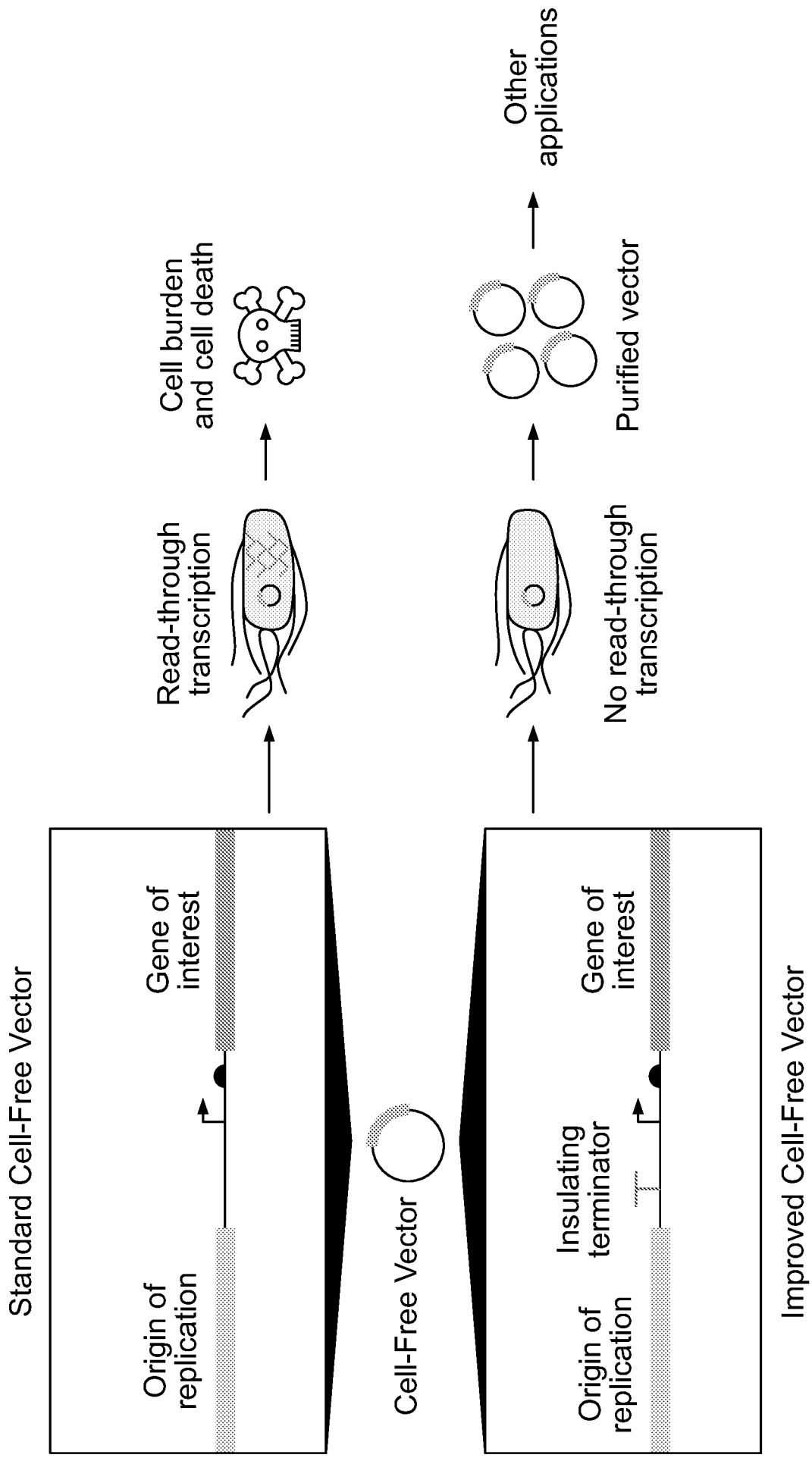
Standard Cell-Free Vector
Origin of replication
Gene of interest
Cell-Free Vector
Improved Cell-Free Vector
Origin of replication
Insulating terminator
Gene of interest
Read-through transcription
Cell burden and cell death
No read-through transcription
Purified vector
Other applications

FIG. 1

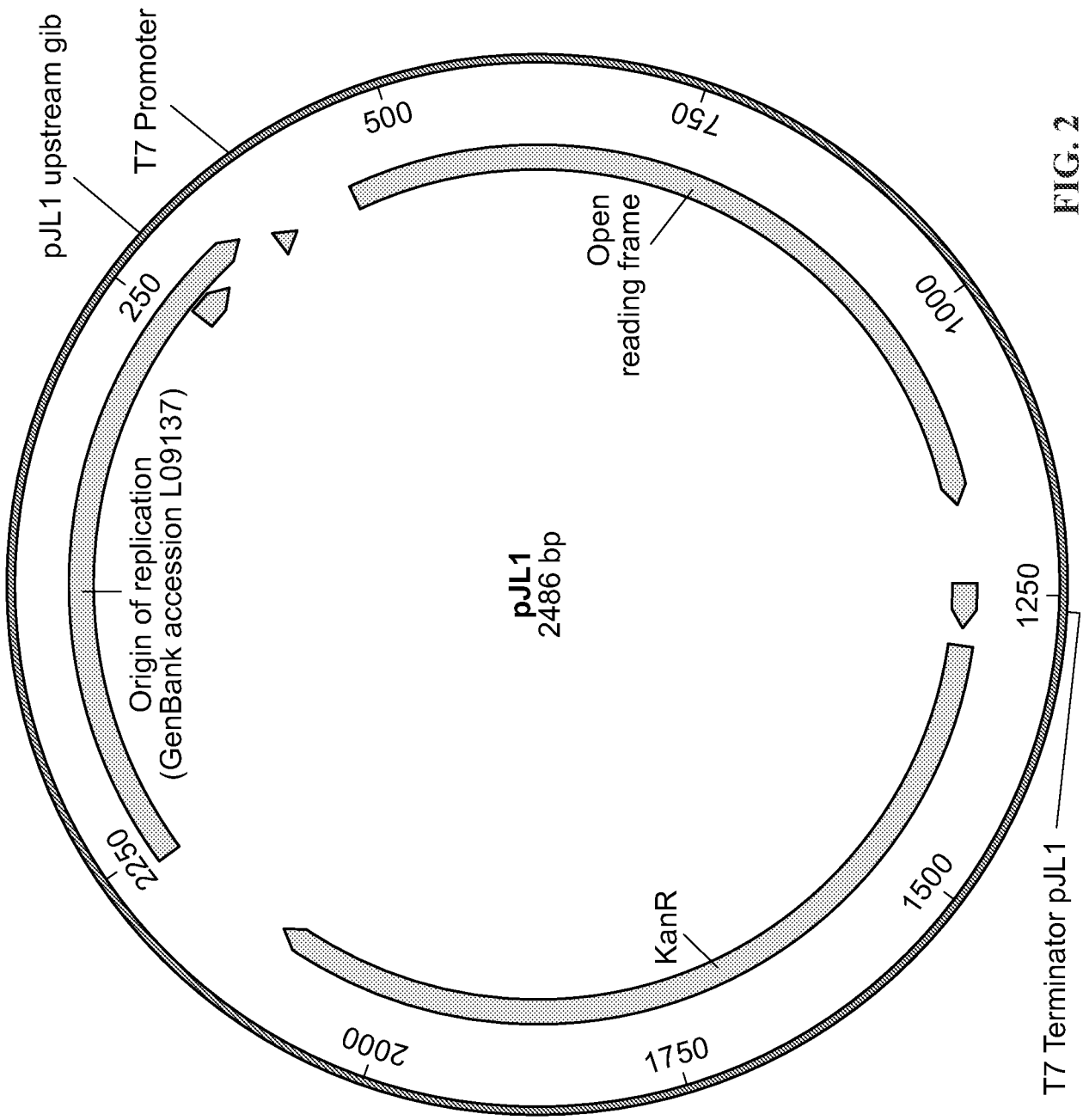
pJL1 upstream gib
T7 Promoter
500
750
Open
reading frame
1000
250
Origin of replication
(GenBank accession L09137)
pJL1
2486 bp
1250
T7 Terminator pJL1
2250
KanR
1500
2000
1750

FIG. 2

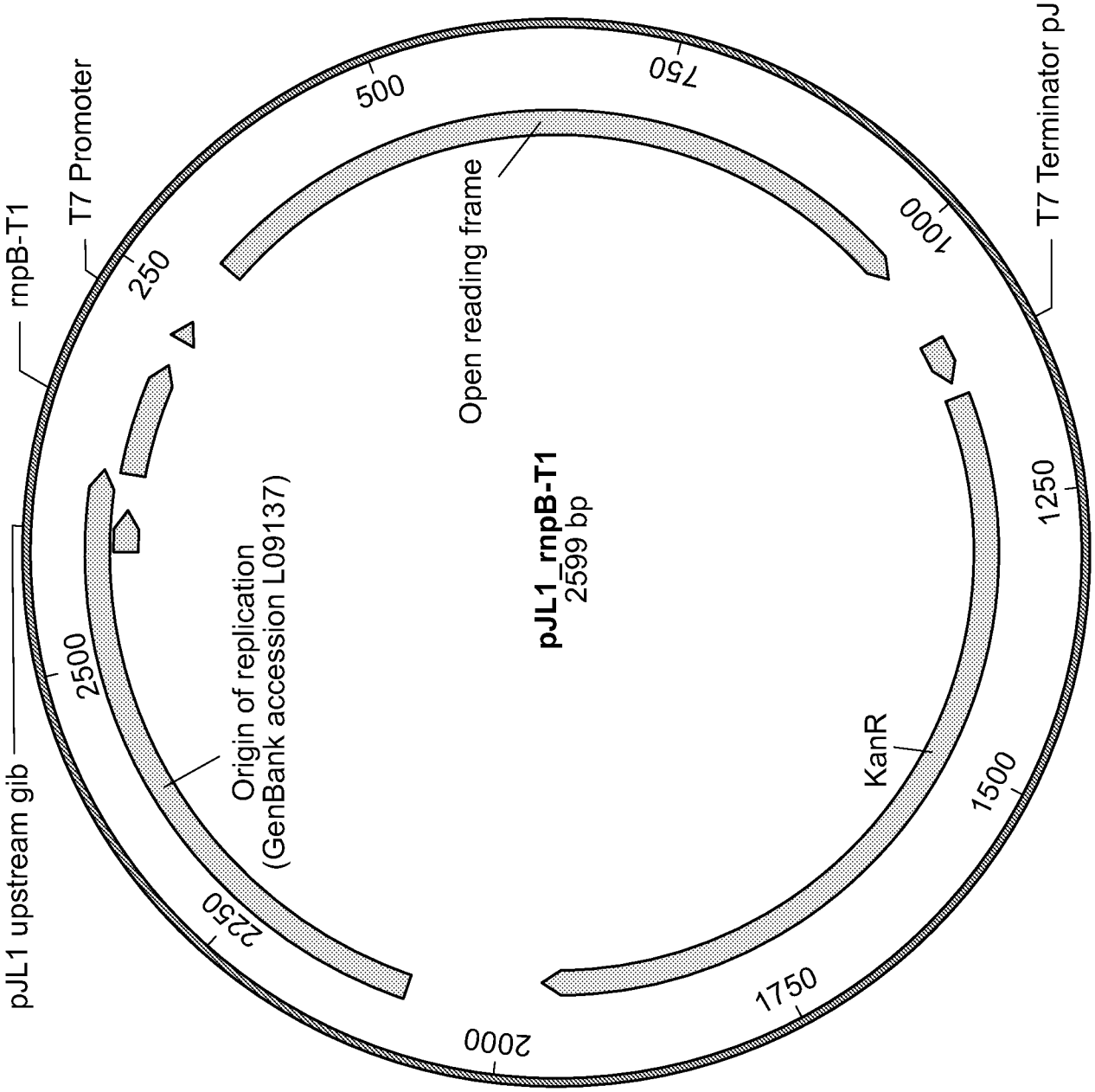
pJL1_rnpB-T1
2599 bp
rnpB-T1
T7 Promoter
T7 Terminator pJL1
pJL1 upstream gib
Open reading frame
Origin of replication
(GenBank accession L09137)
KanR
250
500
750
1000
1250
1500
1750
2000
2250
2500

FIG. 3

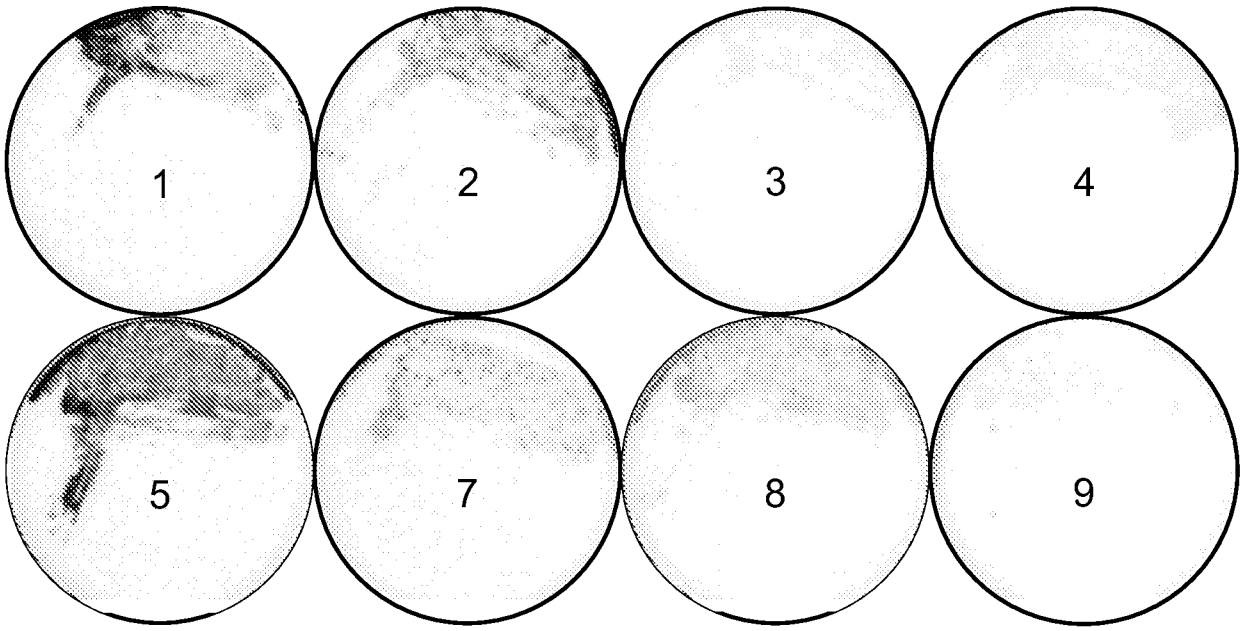

FIG. 4A

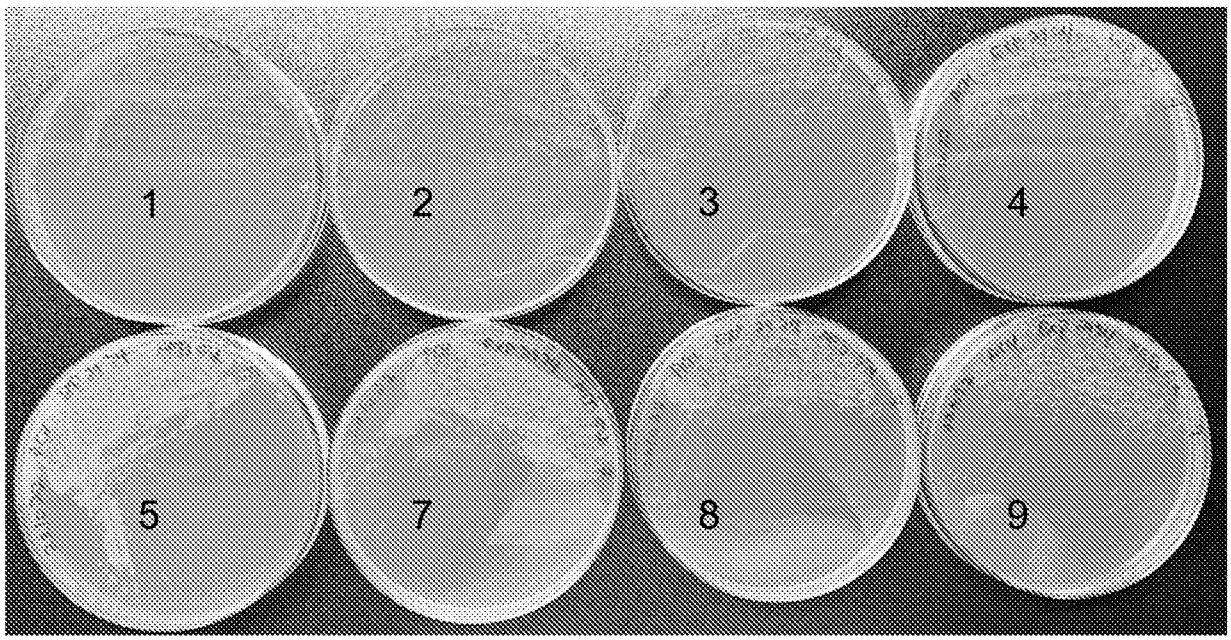

FIG. 4B

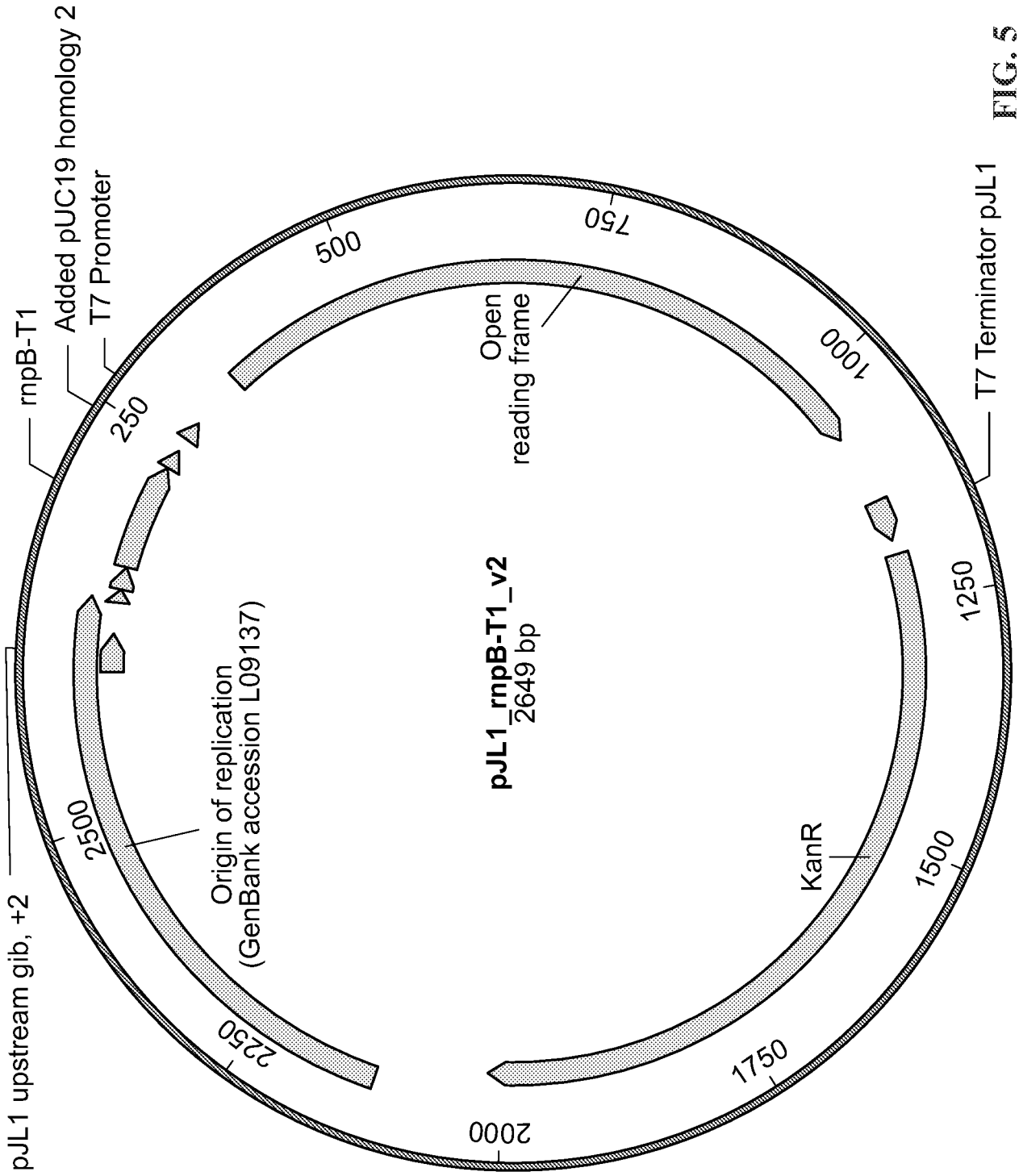
pJL1_rnpB-T1_v2
2649 bp
rnpB-T1
Added pUC19 homology 2
T7 Promoter
Open reading frame
T7 Terminator pJL1
Origin of replication (GenBank accession L09137)
KanR
pJL1 upstream gib, +2
250
500
750
1000
1250
1500
1750
2000
2250
2500

FIG. 5

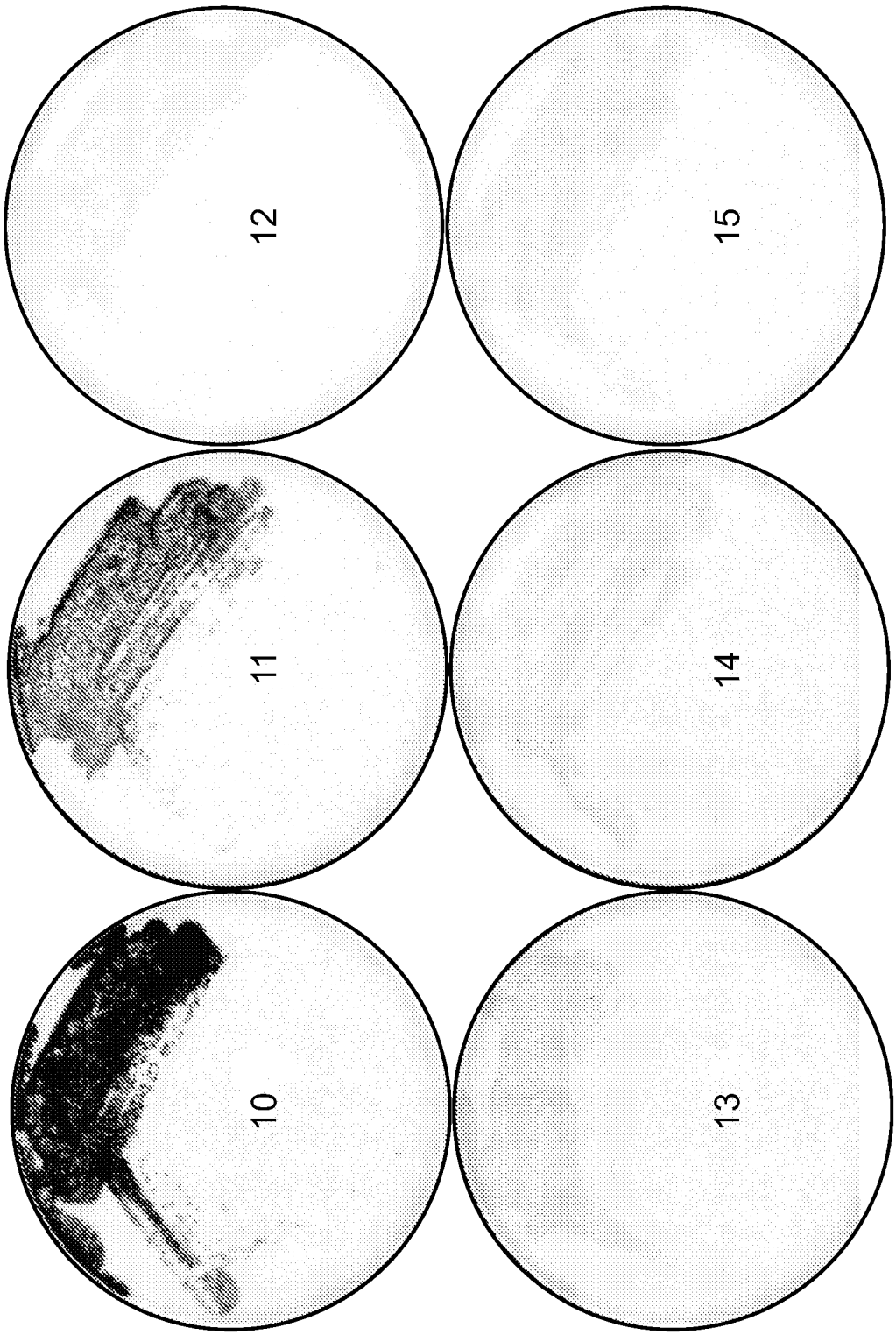

FIG. 6A

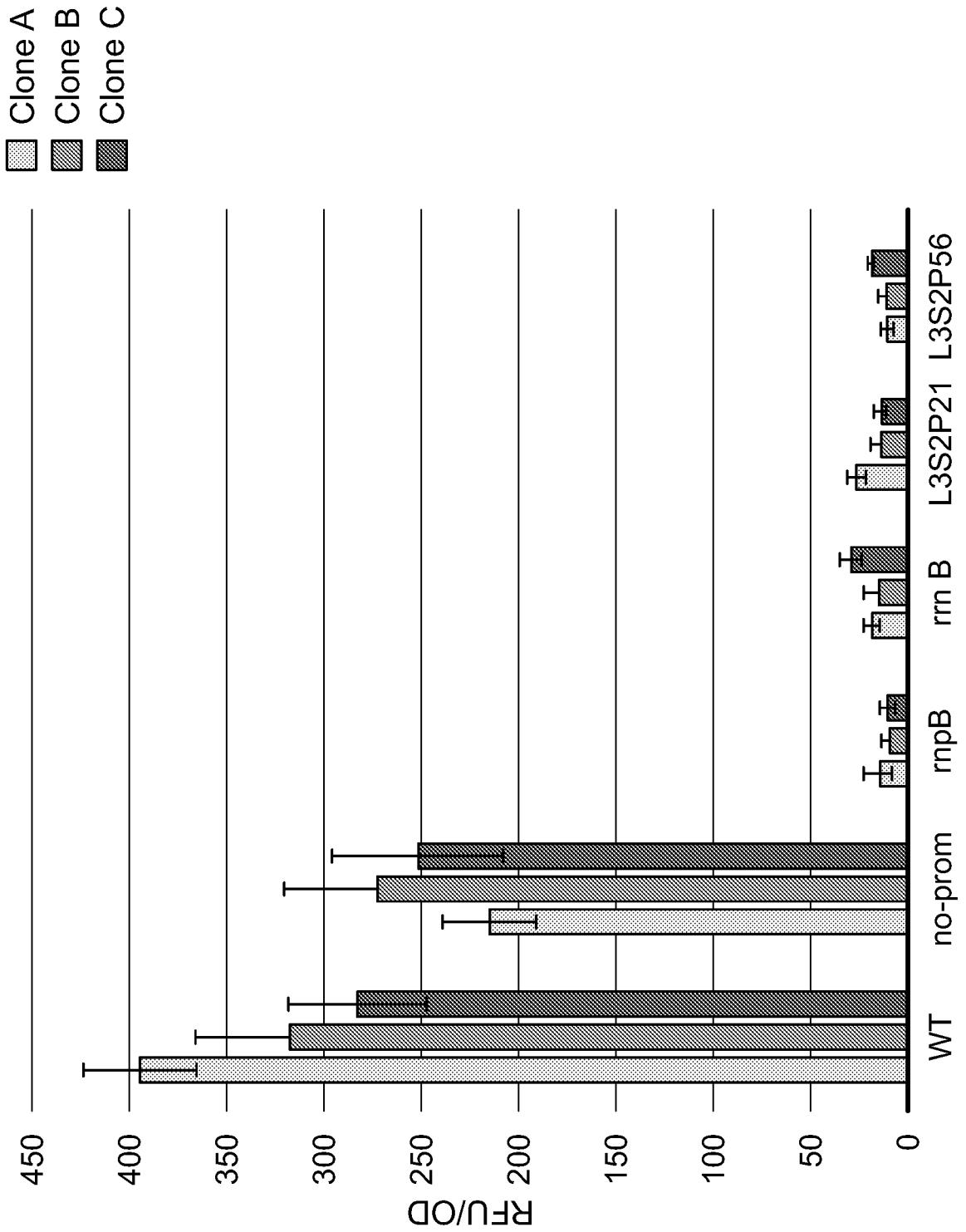
Clone A
Clone B
Clone C
RFU/OD
450
400
350
300
250
200
150
100
50
0
WT
no-prom
rnpB
rrn B
L3S2P21
L3S2P56

FIG. 6B

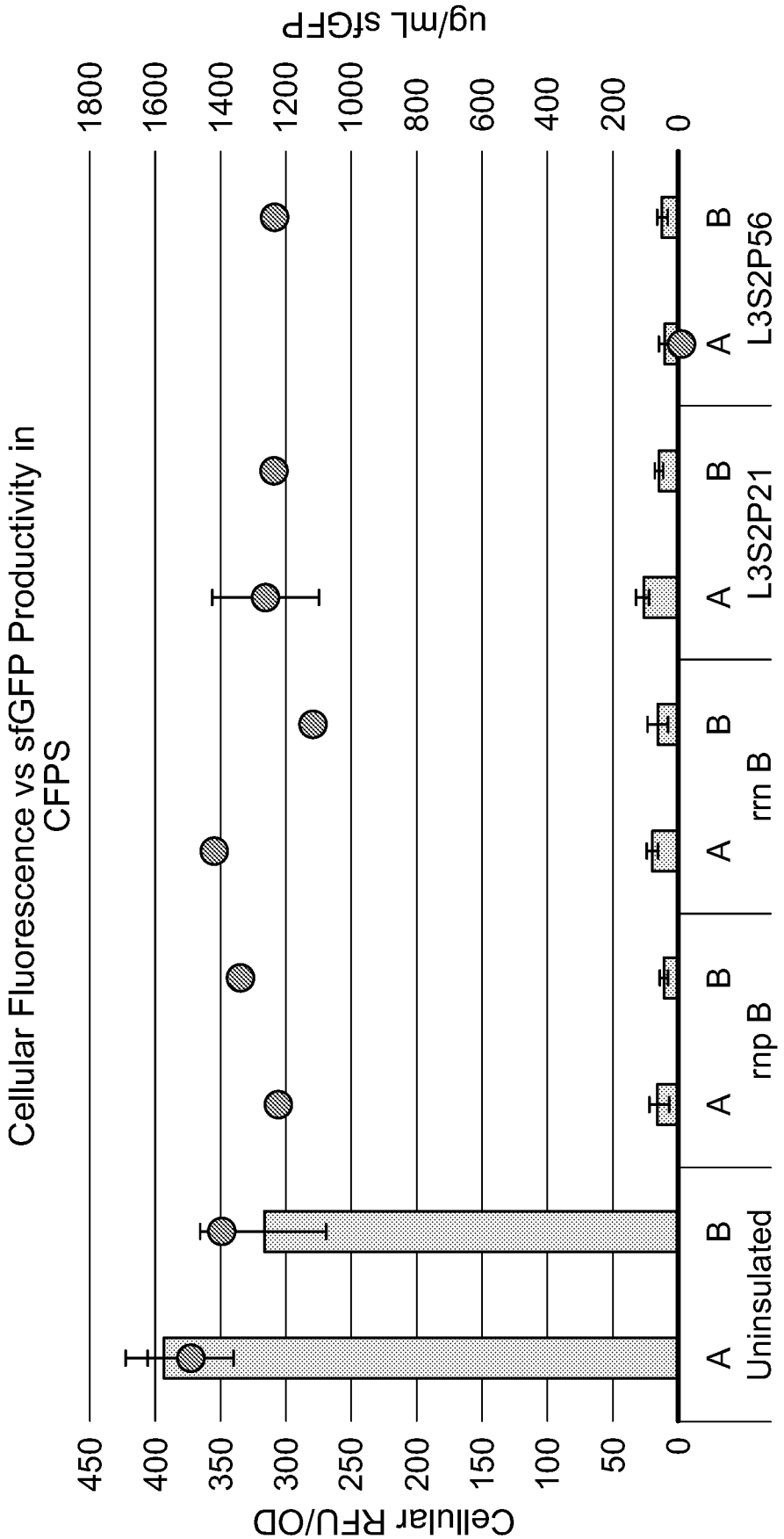
Cellular Fluorescence vs sfGFP Productivity in CFPS
Cellular RFU/OD
ug/mL sfGFP
0
50
100
150
200
250
300
350
400
450
0
200
400
600
800
1000
1200
1400
1600
1800
A
B
Uninsulated
A
B
rnp B
A
B
rrn B
A
B
L3S2P21
A
B
L3S2P56
RFU/OD
ug/mL sfGFP

FIG. 7