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(71) Applicant: NORTHWESTERN UNIVERSITY

[US/US]; 633 Clark Street, Evanston, IL 60208 (US).

(72) Inventors: JEWETT, Michael, Christopher; 3135 Park

Place, Evanston, IL 60201 (US). DES SOYE, Benjamin,

James; 217D Dodge Avenue, Evanston, IL 60202 (US).

DAVIDSON, Samuel, Ryan; c/o Northwestern University,

633 Clark Street, Evanston, IL 60208 (US).

(74) Agent: MCBRIDE, Scott, M. et al.; Andrus Intellectual

Property Law, LLP, 100 East Wisconsin Avenue, Suite

1100, Milwaukee, WI 53202 (US).

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(54) Title: CELL-FREE PROTEIN SYNTHESIS PLATFORM DERIVED FROM CELLULAR EXTRACTS OF VIBRIO NATRIE-GENS

(57) Abstract: Disclosed are compositions, methods, and kits for performing cell-free RNA transcription and/or cell-free protein synthesis (CFPS). The disclosed compositions, methods, and kits include or utilize components prepared from *Vibrio* species such as cellular extracts from *Vibrio natriegens*.



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CELL-FREE PROTEIN SYNTHESIS PLATFORM DERIVED FROM CELLULAR EXTRACTS OF *VIBRIO NATRIEGENS*

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0001] This invention was made with government support under HR0011-15-C-0084 awarded by the Defense Advanced Research Projects Agency (DARPA). The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

[0002] This application claims the benefit of priority under 35 C.F.R. § 119(e) to U.S. Provisional Patent Application No. 62/584,406, filed on November 10, 2017, the content of which is incorporated herein by reference in its entirety.

BACKGROUND

[0003] The present invention generally relates to compositions, methods, and kits for performing cell-free RNA transcription and/or cell-free protein synthesis (CFPS). More specifically, the present invention relates to compositions, methods, and kits for performing cell-free RNA transcription and/or performing cell-free protein synthesis (CFPS) that include or utilize components prepared from naturally occurring or recombinant *Vibrio* species such as cellular extracts from *Vibrio natrie gens*.

[0004] Cell-free protein synthesis (CFPS) is fueling numerous applications as a powerful *in vitro* expression system. Yet, in spite of significant improvements to system productivity and a rapid expansion of platform capabilities, widespread adoption of these technologies for various applications has been slow to materialize. This may be due, at least in part, to the relative difficulty in producing efficacious lysates, as this generally requires specialized equipment and expertise to consistently succeed.

[0005] In an effort to alleviate these requirements and lower the entry barrier into the use of CFPS systems, here we describe the development and characterization of a

novel, facile CFPS platform based on lysates derived from the non-model bacterium *Vibrio natriegens*, which has been characterized as a fast-growing host for molecular biology and a genomic powerhouse. (See Weinstock *et al.*, Nature Methods 13, 849-851, doi:10.1038/nmeth.3970 (2016); and Lee *et al.*, bioRxiv, doi: 10.1101/058487 (June 12, 2016); the contents of which are incorporated by reference in their entireties. Working with the wildtype strain, we identified the optimal culture media, temperature, and harvest time-point for the generation of highly-productive lysates. Next, we analyzed lysis conditions via sonication and discovered that *V. natriegens* cells are relatively agnostic to both cell pellet resuspension volume as well as sonication energy. Once optimal practices for producing lysates were established, we next looked to optimize the CFPS reagent mix specifically for use with *V. natriegens* lysates. By varying the concentrations of critical CFPS reaction substrates including salts, amino acids, and phosphoenol pyruvate, we identified an optimized reagent mix for *V. natriegens* CFPS. Our final optimized system is capable of synthesizing 1.6 g/L of superfolder green fluorescent protein in batch mode CFPS, making it competitive with existing bacterial CFPS platforms.

[0006] We applied our new platform to the successful synthesis of complex eukaryotic proteins including luciferase, an inactivated mutant diphtheria toxin, and an antibody fragment. We also demonstrate that lyophilized *V. natriegens* CFPS reactions assembled with a cryoprotectant retain ~100% productivity after being stored for a week at room temperature. Finally, to further increase the productivity of the system, we explored a small knockout library in which putative negative effectors of CFPS were genomically removed from the source strain. Our *V. natriegens*-derived platform is versatile, and simple to prepare and use. We expect it will facilitate expansion of CFPS systems into new laboratories and fields.

SUMMARY

[0007] Disclosed are compositions, methods, and kits for performing cell-free RNA transcription and/or performing cell-free protein synthesis (CFPS). The disclosed compositions, methods, and kits include or utilize components prepared from naturally occurring or recombinant *Vibrio* species such as cellular extracts from *Vibrio natriegens*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figure 1. Simplified schematic of the production and utilization of crude lysates from bacterial chassis cells to catalyze cell-free protein synthesis (CFPS). Reactions are supplemented with enzymatic cofactors, energy, and other substrates required for protein synthesis as well as plasmid DNA template directing the system towards the production of a product of interest.

[0009] Figure 2. Optimization of *V. natriegens* harvest and lysis procedures. (a) sfGFP fluorescence *in vitro* from cell extracts derived from *V. natriegens* cultured in the specified liquid medium supplemented with v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14 mM MgCl₂). Cells were harvested at OD₆₀₀ = 4.5 (b) sfGFP fluorescence from cell extracts derived from *V. natriegens* harvested at the specified OD₆₀₀. Cell culture was performed using BHI medium plus v2 salts. For both (a) and (b), established *E. coli* protocols were used for lysate preparation. (c) Lysis optimization. *V. natriegens* cell pellets were suspended in the specified volume of S30 lysis buffer and subjected to the indicated number of sonication pulses to achieve lysis. sfGFP fluorescence *in vitro* from the resulting lysates is shown. All CFPS reactions used an existing *E. coli* reagent mix. For all conditions, three independent reactions were performed and one standard deviation is shown. * = statistically significant for $p < 0.05$. Bullet points: BHI medium is best for culturing cells for CFPS lysate preparation; the most productive *V. natriegens* lysates are derived from stationary phase cultures; and *V. natriegens* cells are agnostic to lysis conditions.

[0010] Figure 3. Optimization of CFPS reagent mix. A single *V. natriegens* lysate was prepared using optimized parameters identified in Figs. 1-2. Shown are sfGFP fluorescence values obtained using the lysates *in vitro* when the specified concentration of each of the following reagents is used: (a) Mg(GLU), (b) K(GLU), (c) amino acids, (d) PEP. For all conditions, three independent reactions were performed and one standard deviation is shown. * = statistically significant for $p < 0.05$. Bullet points: *V. natriegens* lysates perform best using a slightly different reagent mix than *E. coli* lysates; 6 mM Mg(GLU), 290 mM K(GLU), 3 mM AAs and 67 mM PEP are optimal for this system.

[0011] Figure 4. Summary of the development and optimization of *V. natriegens* CFPS. sfGFP yields from each of the best conditions identified in the indicated optimizations are shown. For each condition, three independent reactions were performed and one standard deviation is shown. Media opt = identification of preferred liquid culture medium; OD opt = identification of optimal OD₆₀₀ at harvest; Lysis opt = identification of optimal sonication procedure; Mg opt = optimization of Mg(GLU) in CFPS reaction mix; K opt = optimization of K(GLU) in CFPS reaction mix; AA opt = optimization of amino acids in CFPS reaction mix; PEP opt = optimization of PEP in CFPS reaction mix. Bullet point: Yields of the fully optimized system are comparable to popular systems based on *E. coli* lysates.

[0012] Figure 5. Demonstration of the capabilities of *V. natriegens* CFPS. (a) Luminescence generated from active firefly luciferase synthesized using *V. natriegens* CFPS. 12 independent reactions were performed, and one standard deviation is shown. (b) Autoradiograph depicting full-length, soluble CRM197 and ScFv synthesized in *V. natriegens* CFPS. (c) Yields of sfGFP from lyophilized *V. natriegens* CFPS reactions. Reactions were assembled both without (All) and with supplementation with 2.5% trehalose (All + trehalose) or 5% glycerol (All + glycerol). These reactions, along with samples of just *V. natriegens* lysate (Extract alone) were lyophilized overnight. Lyophilized samples were reconstituted either immediately (Same day) or after incubation at room temperature (~23°C) for 1 week. For each condition 3 independent reactions were performed, and one standard deviation is shown. Bullet points: *V. natriegens* CFPS can synthesize full-length eukaryotic proteins; in the case of luciferase, the product is enzymatically active; and *V. natriegens* CFPS reactions lyophilized in the presence of trehalose lose no efficacy even after a week of incubation at room temperature.

[0013] Figure 6. Comparison of *E. coli* and *V. natriegens* lysate preparation workflows. As compared to *E. coli*, *V. natriegens* cells are easier to handle both in the context of culture harvest and lysis. It is extremely difficult to spoil a *V. natriegens* extract preparation. *V. natriegens*' overall workflow is faster due to the organism's rapid growth rate and omission of any runoff reaction step. Finally, because *V. natriegens* pellets are gathered at stationary phase, they are significantly larger than those gathered for *E. coli* –

consequently, the volume of *V. natriegens* lysate derived from a single 1 L harvest is significantly higher. Bullet points: For basically every aspect of lysate preparation, *V. natriegens* is less temperamental than *E. coli* and thus easier to work with; *V. natriegens* workflow is faster than the *E. coli* workflow; and 1 L of *V. natriegens* cell culture yields an order of magnitude more lysate than 1 L of *E. coli* culture.

[0014] Figure 7. Characterization of *V. natriegens* growth rate. (a) Doubling time, in minutes, of *V. natriegens* grown in the specified media and salt conditions at 37°C. v2 salts = 204 mM NaCl, 4.2 mM KCl, 23.14 mM MgCl₂. (b) Timecourse of *V. natriegens* growth in liquid culture at 1L scale using the listed growth media. Cells were grown in a 2.5 L Tunair® shake flask and incubated at 37°C at 250 RPM.

[0015] Figure 8. CFPS yields from *V. natriegens* cells harvested at late stages of growth. (a) sfGFP fluorescence values from a lysate that was prepared from *V. natriegens* cells that remained in stationary phase for 7 hours, compared to a lysate prepared from cells gathered right at the onset of stationary phase. (b) sfGFP fluorescence values from a lysate that was prepared from *V. natriegens* cells that were inoculated near the end of the day and allowed to grow at 37°C overnight (total culture time = 16 hours). The result is compared to a lysate prepared from cells gathered right at the onset of stationary phase. For each condition 3 independent reactions were performed, and one standard deviation is shown.

[0016] Figure 9. Characterization of *V. natriegens* run-off reaction. sfGFP fluorescence from lysates prepared using run-off reactions performed under the specified conditions is shown. Run-off reactions, if performed, ran for 1 hr. Shaking = agitated at 250 RPM. For each condition 3 independent reactions were performed, and one standard deviation is shown.

[0017] Figure 10. *V. natriegens* Na(GLU) optimization. sfGFP fluorescence is shown from CFPS reactions supplemented with the indicated concentrations of Na(GLU). For each condition, 3 independent reactions were performed, and one standard deviation is shown.

[0018] Figure 11. Characterization of *V. natriegens* CFPS reaction incubation temperature. Shown are sfGFP fluorescence values for CFPS reactions left to incubate for 20 hrs at the indicated temperature. For each condition, 3 independent reactions were performed, and one standard deviation is shown. * = statistically significant for $p < 0.05$.

[0019] Figure 12. Comparison of cell-free protein synthesis of Cecropin P1 peptide utilizing an extract from *V. natriegens* versus an extract from *E. coli* strain B95ΔA (see Mukai *et al.*, “Highly reproductive Escherichia coli cells with no specific assignment to the UAG codon,” Sci. Rep. 2015 May 18;5:9699, the content of which is incorporated herein by reference in its entirety).

DETAILED DESCRIPTION

[0020] Definitions and Terminology

[0021] The disclosed subject matter may be further described using definitions and terminology as follows. The definitions and terminology used herein are for the purpose of describing particular embodiments only, and are not intended to be limiting.

[0022] As used in this specification and the claims, the singular forms “a,” “an,” and “the” include plural forms unless the context clearly dictates otherwise. For example, the term “a component” should be interpreted to mean “one or more components” unless the context clearly dictates otherwise. As used herein, the term “plurality” means “two or more.”

[0023] As used herein, “about”, “approximately,” “substantially,” and “significantly” will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, “about” and “approximately” will mean up to plus or minus 10% of the particular term and “substantially” and “significantly” will mean more than plus or minus 10% of the particular term.

[0024] As used herein, the terms “include” and “including” have the same meaning as the terms “comprise” and “comprising.” The terms “comprise” and “comprising” should be interpreted as being “open” transitional terms that permit the inclusion of additional components further to those components recited in the claims. The terms “consist” and “consisting of” should be interpreted as being “closed” transitional terms that do not permit the inclusion of additional components other than the components recited in the claims. The term “consisting essentially of” should be interpreted to be partially closed and allowing the inclusion only of additional components that do not fundamentally alter the nature of the claimed subject matter.

[0025] The phrase “such as” should be interpreted as “for example, including.” Moreover the use of any and all exemplary language, including but not limited to “such as”, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed.

[0026] Furthermore, in those instances where a convention analogous to “at least one of A, B and C, etc.” is used, in general such a construction is intended in the sense of one having ordinary skill in the art would understand the convention (e.g., “a system having at least one of A, B and C” would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description or figures, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase “A or B” will be understood to include the possibilities of “A” or “B” or “A and B.”

[0027] All language such as “up to,” “at least,” “greater than,” “less than,” and the like, include the number recited and refer to ranges which can subsequently be broken down into subranges as discussed above.

[0028] A range includes each individual member. Thus, for example, a group having 1-3 members refers to groups having 1, 2, or 3 members. Similarly, a group having 6 members refers to groups having 1, 2, 3, 4, or 6 members, and so forth.

[0029] The modal verb “may” refers to the preferred use or selection of one or more options or choices among the several described embodiments or features contained within the same. Where no options or choices are disclosed regarding a particular embodiment or feature contained in the same, the modal verb “may” refers to an affirmative act regarding how to make or use and aspect of a described embodiment or feature contained in the same, or a definitive decision to use a specific skill regarding a described embodiment or feature contained in the same. In this latter context, the modal verb “may” has the same meaning and connotation as the auxiliary verb “can.”

[0030] Polynucleotides and Synthesis Methods

[0031] The disclosed methods, devices, kits, and components may utilize and/or include polynucleotides. The terms “polynucleotide,” “polynucleotide sequence,” “nucleic acid” and “nucleic acid sequence” refer to a nucleotide, oligonucleotide, polynucleotide (which terms may be used interchangeably), or any fragment thereof. These phrases also refer to DNA or RNA of genomic, natural, or synthetic origin (which may be single-stranded or double-stranded and may represent the sense or the antisense strand).

[0032] 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 methods, 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.

[0033] Regarding polynucleotide sequences, the terms “percent identity” and “% identity” refer to the percentage of residue matches between at least two polynucleotide sequences aligned using a standardized algorithm. Such an algorithm may insert, in a standardized and reproducible way, gaps in the sequences being compared in order to optimize alignment between two sequences, and therefore achieve a more meaningful comparison of the two sequences. Percent identity for a nucleic acid sequence may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including “blastn,” that is used to align a known polynucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called “BLAST 2 Sequences” that is used for direct pairwise comparison of two nucleotide sequences. “BLAST 2 Sequences” can be accessed and used interactively at the NCBI website. The “BLAST 2 Sequences” tool can be used for both blastn and blastp (discussed above).

[0034] Regarding polynucleotide sequences, percent identity may be measured over the length of an entire defined polynucleotide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined sequence, for instance, a fragment of at least 20, at least 30, at least 40, at least 50, at least 70, at least 100, or at least 200 contiguous nucleotides. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures, or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

[0035] Regarding polynucleotide sequences, “variant,” “mutant,” or “derivative” may be defined as a nucleic acid sequence having at least 50% sequence identity to the particular nucleic acid sequence over a certain length of one of the nucleic acid sequences using blastn with the “BLAST 2 Sequences” tool available at the National Center for Biotechnology Information’s website. (See Tatiana A. Tatusova, Thomas L. Madden (1999), “Blast 2 sequences - a new tool for comparing protein and nucleotide sequences”, *FEMS Microbiol Lett.* 174:247-250). Such a pair of nucleic acids may show, for example, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or greater sequence identity over a certain defined length.

[0036] Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences due to the degeneracy of the genetic code where multiple codons may encode for a single amino acid. It is understood that changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequences that all encode substantially the same protein. For example, polynucleotide sequences as contemplated herein may encode a protein and may be codon-optimized for expression in a particular host. In the art, codon usage frequency tables have been prepared for a number of host organisms including humans, mouse, rat, pig, *E. coli*, plants, and other host cells.

[0037] 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.

[0038] 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.

[0039] 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.

[0040] 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, New York; 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).

[0041] 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.

[0042] A primer is preferably 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.

[0043] Primers can incorporate additional features which 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.

[0044] 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.

[0045] 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, RNA polymerases of bacteriophages (*e.g.* 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.

[0046] 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.

[0047] As used herein, the term “sequence defined biopolymer” refers to a biopolymer having a specific primary sequence. A sequence defined biopolymer can be equivalent to a genetically-encoded defined biopolymer in cases where a gene encodes the biopolymer having a specific primary sequence. As used herein, “expression” refers to the

process by which a polynucleotide is transcribed from a DNA template (such as into and mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as "gene product." If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0048] As used herein, "expression template" refers to a nucleic acid that serves as substrate for transcribing at least one RNA that can be translated into a sequence defined biopolymer (e.g., 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.

[0049] In certain exemplary embodiments, vectors such as, for example, expression vectors, containing a nucleic acid encoding one or more rRNAs or reporter polypeptides and/or proteins described herein are provided. 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" can be used interchangeably. However, the disclosed methods and compositions are intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[0050] In certain exemplary embodiments, the recombinant expression vectors comprise a nucleic acid sequence (e.g., a nucleic acid sequence encoding one or more rRNAs or reporter polypeptides and/or proteins described herein) in a form suitable for expression of the nucleic acid sequence in one or more of the methods described herein, which means that the recombinant expression vectors include one or more regulatory sequences which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, “operably linked” is intended to mean that the nucleotide sequence encoding one or more rRNAs or reporter polypeptides and/or proteins described herein is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an in vitro ribosomal assembly, transcription and/or translation system). The term “regulatory sequence” is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990).

[0051] The polynucleotide sequences contemplated herein may be present in expression vectors. For example, the vectors may comprise: (a) a polynucleotide encoding an ORF of a protein; (b) a polynucleotide that expresses an RNA that directs RNA-mediated binding, nicking, and/or cleaving of a target DNA sequence; and both (a) and (b). The polynucleotide present in the vector may be operably linked to a prokaryotic or eukaryotic promoter. “Operably linked” refers to the situation in which a first nucleic acid sequence is placed in a functional relationship with a second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences may be in close proximity or contiguous and, where necessary to join two protein coding regions, in the same reading frame. Vectors contemplated herein may comprise a heterologous promoter (e.g., a eukaryotic or prokaryotic promoter) operably linked to a polynucleotide that encodes a protein. A “heterologous promoter” refers to a promoter that is not the native or endogenous promoter for the protein or RNA that is being expressed. Vectors as disclosed herein may include plasmid vectors.

[0052] Oligonucleotides and polynucleotides may optionally include one or more non-standard nucleotide(s), nucleotide analog(s) and/or modified nucleotides. Examples of modified nucleotides include, but are not limited to diaminopurine, S²T, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-D46-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-diaminopurine and the like. Nucleic acid molecules may also be modified at the base moiety (e.g., at one or more atoms that typically are available to form a hydrogen bond with a complementary nucleotide and/or at one or more atoms that are not typically capable of forming a hydrogen bond with a complementary nucleotide), sugar moiety or phosphate backbone.

[0053] Peptides, Polypeptides, Proteins, and Synthesis Methods

[0054] The disclosed methods, devices, kits, and components may be utilized to synthesize proteins, polypeptides, and/or peptides. As used herein, the terms “protein” or “polypeptide” or “peptide” may be used interchangeably to refer to a polymer of amino acids. Typically, a “polypeptide” or “protein” is defined as a longer polymer of amino acids, of a length typically of greater than 50, 60, 70, 80, 90, or 100 amino acids. A “peptide” is defined as a short polymer of amino acids, of a length typically of 50, 40, 30, 20 or less amino acids.

[0055] As used herein, the terms “peptide,” “polypeptide,” and “protein,” refer to molecules comprising a chain a polymer of amino acid residues joined by amide linkages. The term “amino acid residue,” includes but is not limited to amino acid residues

contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues. The term “amino acid residue” also may include nonstandard, noncanonical, or unnatural amino acids, which optionally may include amino acids other than any of the following amino acids: alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, and tyrosine residues. The term “amino acid residue” may include alpha-, beta-, gamma-, and delta-amino acids.

[0056] In some embodiments, the term “amino acid residue” may include nonstandard, noncanonical, or unnatural amino acid residues contained in the group consisting of homocysteine, 2-Aminoadipic acid, N-Ethylasparagine, 3-Aminoadipic acid, Hydroxylysine, β -alanine, β -Amino-propionic acid, allo-Hydroxylysine acid, 2-Aminobutyric acid, 3-Hydroxyproline, 4-Aminobutyric acid, 4-Hydroxyproline, piperidinic acid, 6-Aminocaproic acid, Isodesmosine, 2-Aminoheptanoic acid, allo-Isoleucine, 2-Aminoisobutyric acid, N-Methylglycine, sarcosine, 3-Aminoisobutyric acid, N-Methylisoleucine, 2-Aminopimelic acid, 6-N-Methyllysine, 2,4-Diaminobutyric acid, N-Methylvaline, Desmosine, Norvaline, 2,2'-Diaminopimelic acid, Norleucine, 2,3-Diaminopropionic acid, Ornithine, and N-Ethylglycine. The term “amino acid residue” may include L isomers or D isomers of any of the aforementioned amino acids.

[0057] Other examples of nonstandard, noncanonical, or unnatural amino acids include, but are not limited, to a p-acetyl-L-phenylalanine, a p-iodo-L-phenylalanine, an O-methyl-L-tyrosine, a p-propargyloxyphenylalanine, a p-propargyl-phenylalanine, an L-3-(2-naphthyl)alanine, a 3-methyl-phenylalanine, an O-4-allyl-L-tyrosine, a 4-propyl-L-tyrosine, a tri-O-acetyl-GlcNAcp β -serine, an L-Dopa, a fluorinated phenylalanine, an isopropyl-L-phenylalanine, a p-azido-L-phenylalanine, a p-acyl-L-phenylalanine, a p-benzoyl-L-phenylalanine, an L-phosphoserine, a phosphoserine, a phosphotyrosine, a

p-bromophenylalanine, a p-amino-L-phenylalanine, an isopropyl-L-phenylalanine, an unnatural analogue of a tyrosine amino acid; an unnatural analogue of a glutamine amino acid; an unnatural analogue of a phenylalanine amino acid; an unnatural analogue of a serine amino acid; an unnatural analogue of a threonine amino acid; an unnatural analogue of a methionine amino acid; an unnatural analogue of a leucine amino acid; an unnatural analogue of a isoleucine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynyl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, 18ufl8hor, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or a combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged and/or photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol or polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an α -hydroxy containing acid; an amino thio acid; an α,α disubstituted amino acid; a β -amino acid; a γ -amino acid, a cyclic amino acid other than proline or histidine, and an aromatic amino acid other than phenylalanine, tyrosine or tryptophan.

[0058] As used herein, a “peptide” is defined as a short polymer of amino acids, of a length typically of 20 or less amino acids, and more typically of a length of 12 or less amino acids (Garrett & Grisham, Biochemistry, 2nd edition, 1999, Brooks/Cole, 110). In some embodiments, a peptide as contemplated herein may include no more than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids. A polypeptide, also referred to as a protein, is typically of length ≥ 100 amino acids (Garrett & Grisham, Biochemistry, 2nd edition, 1999, Brooks/Cole, 110). A polypeptide, as contemplated herein, may comprise, but is not limited to, 100, 101, 102, 103, 104, 105, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about

325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or more amino acid residues.

[0059] A peptide as contemplated herein may be further modified to include non-amino acid moieties. Modifications may include but are not limited to acylation (*e.g.*, O-acylation (esters), N-acylation (amides), S-acylation (thioesters)), acetylation (*e.g.*, the addition of an acetyl group, either at the N-terminus of the protein or at lysine residues), formylation lipoylation (*e.g.*, attachment of a lipoate, a C8 functional group), myristoylation (*e.g.*, attachment of myristate, a C14 saturated acid), palmitoylation (*e.g.*, attachment of palmitate, a C16 saturated acid), alkylation (*e.g.*, the addition of an alkyl group, such as an methyl at a lysine or arginine residue), isoprenylation or prenylation (*e.g.*, the addition of an isoprenoid group such as farnesol or geranylgeraniol), amidation at C-terminus, glycosylation (*e.g.*, the addition of a glycosyl group to either asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein). Distinct from glycation, which is regarded as a nonenzymatic attachment of sugars, polysialylation (*e.g.*, the addition of polysialic acid), glypiation (*e.g.*, glycosylphosphatidylinositol (GPI) anchor formation, hydroxylation, iodination (*e.g.*, of thyroid hormones), and phosphorylation (*e.g.*, the addition of a phosphate group, usually to serine, tyrosine, threonine or histidine).

[0060] The proteins disclosed herein may include “wild type” proteins and variants, mutants, and derivatives thereof. As used herein the term “wild type” is a term of the art understood by skilled persons and means the typical form of an organism, strain, gene or characteristic as it occurs in nature as distinguished from mutant or variant forms. As used herein, a “variant,” “mutant,” or “derivative” refers to a protein molecule having an amino acid sequence that differs from a reference protein or polypeptide molecule. A variant or mutant may have one or more insertions, deletions, or substitutions of an amino acid residue relative to a reference molecule. A variant or mutant may include a fragment of a reference molecule. For example, a mutant or variant molecule may one or more

insertions, deletions, or substitution of at least one amino acid residue relative to a reference polypeptide.

[0061] Regarding proteins, a “deletion” refers to a change in the amino acid sequence that results in the absence of one or more amino acid residues. A deletion may remove at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 200, or more amino acids residues. A deletion may include an internal deletion and/or a terminal deletion (*e.g.*, an N-terminal truncation, a C-terminal truncation or both of a reference polypeptide). A “variant,” “mutant,” or “derivative” of a reference polypeptide sequence may include a deletion relative to the reference polypeptide sequence.

[0062] Regarding proteins, “fragment” is a portion of an amino acid sequence which is identical in sequence to but shorter in length than a reference sequence. A fragment may comprise up to the entire length of the reference sequence, minus at least one amino acid residue. For example, a fragment may comprise from 5 to 1000 contiguous amino acid residues of a reference polypeptide, respectively. In some embodiments, a fragment may comprise at least 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150, 250, or 500 contiguous amino acid residues of a reference polypeptide. Fragments may be preferentially selected from certain regions of a molecule. The term “at least a fragment” encompasses the full-length polypeptide. A fragment may include an N-terminal truncation, a C-terminal truncation, or both truncations relative to the full-length protein. A “variant,” “mutant,” or “derivative” of a reference polypeptide sequence may include a fragment of the reference polypeptide sequence.

[0063] Regarding proteins, the words “insertion” and “addition” refer to changes in an amino acid sequence resulting in the addition of one or more amino acid residues. An insertion or addition may refer to 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, or more amino acid residues. A “variant,” “mutant,” or “derivative” of a reference polypeptide sequence may include an insertion or addition relative to the reference polypeptide sequence. A variant of a protein may have N-terminal insertions, C-terminal insertions, internal insertions, or any combination of N-terminal insertions, C-terminal insertions, and internal insertions.

[0064] Regarding proteins, the phrases “percent identity” and “% identity,” refer to the percentage of residue matches between at least two amino acid sequences aligned using a standardized algorithm. Methods of amino acid sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail below, generally preserve the charge and hydrophobicity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide. Percent identity for amino acid sequences may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including “blastp,” that is used to align a known amino acid sequence with other amino acids sequences from a variety of databases.

[0065] Regarding proteins, percent identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

[0066] Regarding proteins, the amino acid sequences of variants, mutants, or derivatives as contemplated herein may include conservative amino acid substitutions relative to a reference amino acid sequence. For example, a variant, mutant, or derivative protein may include conservative amino acid substitutions relative to a reference molecule. “Conservative amino acid substitutions” are those substitutions that are a substitution of an amino acid for a different amino acid where the substitution is predicted

to interfere least with the properties of the reference polypeptide. In other words, conservative amino acid substitutions substantially conserve the structure and the function of the reference polypeptide. The following table provides a list of exemplary conservative amino acid substitutions which are contemplated herein:

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

[0067]

[0068] Conservative amino acid substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain. Non-conservative amino acids typically disrupt (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain.

[0069] The disclosed proteins, mutants, variants, or described herein may have one or more functional or biological activities exhibited by a reference polypeptide (*e.g.*, one or more functional or biological activities exhibited by wild-type protein).

[0070] The disclosed proteins may be substantially isolated or purified. The term “substantially isolated or purified” refers to proteins that are removed from their natural environment, and are at least 60% free, preferably at least 75% free, and more preferably at least 90% free, even more preferably at least 95% free from other components with which they are naturally associated.

[0071] The proteins disclosed herein may be expressed from a “translation template.” As used herein, “translation template” refers to an RNA product of transcription from an expression template that can be used by ribosomes to synthesize polypeptides or proteins.

[0072] The proteins disclosed herein may be expressed in a “reaction mixture.” The term “reaction mixture,” as used herein, refers to a solution containing reagents necessary to carry out a given reaction. A reaction mixture is referred to as complete if it contains all reagents necessary to perform the reaction. Components for a reaction mixture may be stored separately in separate container, 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.

[0073] Cell-Free Protein Synthesis

[0074] Cell-free protein synthesis (CFPS) and methods for making cell extracts for use in CFPS are known in the art. (See, e.g., Carlson *et al.*, “Cell-free protein synthesis: Applications come of age,” Biotech. Adv. Vol. 30, Issue 5, Sept-Oct 2012, Pages 1185-1194; Hodgman *et al.*, “Cell-free synthetic biology: Thinking outside the cell,” Metabol. Eng. Vol. 14, Issue 3, May 2012, Pages 261-269; and Harris *et al.*, “Cell-free biology: exploiting the interface between synthetic biology and synthetic chemistry,” Curr. Op. Biotech. Vol. 23, Issue 5, October 2012, Pages 672-678; see also U.S. Patent Nos. 7,312,049; 7,008,651; and 6,994,986; see also U.S. Published Application Nos. 20170306320; 20160362708; 20160060301; 20120088269; 20090042244; 2008024821; 20080138857; 20070154983; 20070141661; 20050186655; 200501480461 20050064592;

20050032086; 20040209321; and 20040038332; the contents of which are incorporated herein by reference in their entireties).

[0075] The disclosed compositions may include platforms for preparing a sequence defined biopolymer of protein in vitro. The platforms for preparing a sequence defined polymer or protein in vitro comprises a cellular extract from an organism, and in particular a species of *Vibrio*, such as *Vibrio natriegens*. Because CFPS exploits an ensemble of catalytic proteins prepared from the crude lysate of cells, the cell extract (whose composition is sensitive to growth media, lysis method, and processing conditions) is an important component of extract-based CFPS reactions. A variety of methods exist for preparing an extract competent for cell-free protein synthesis, including those disclosed in U.S. Published Application No. 20140295492, published on Oct. 2, 2014, which is incorporated by reference.

[0076] The platform may comprise 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. In certain specific embodiments, the platform may be a coupled transcription/translation ("Tx/Tl") system where synthesis of translation template and a sequence defined biopolymer from the same cellular extract.

[0077] The platform 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.

[0078] The platform may comprise an orthogonal translation system. An orthogonal translation system may comprise one or more orthogonal components that are designed to operate parallel to and/or independent of the organism's orthogonal translation machinery. In certain embodiments, the orthogonal translation system and/or orthogonal components are configured to incorporation of unnatural amino acids. An orthogonal component may be an orthogonal protein or an orthogonal RNA. In certain embodiments, an orthogonal protein may be an orthogonal synthetase. In certain embodiments, the orthogonal RNA may be an orthogonal tRNA or an orthogonal rRNA. An example of an orthogonal rRNA component has been described in U.S. Published Application Nos. 20170073381 and 20160060301, the contents of which are incorporated by reference in their entireties. In certain embodiments, one or more orthogonal components may be prepared *in vivo* or *in vitro* by the expression of an oligonucleotide template. The one or more orthogonal components may be expressed from a plasmid present in the genomically recoded organism, expressed from an integration site in the genome of the genetically recoded organism, co-expressed from both a plasmid present in the genomically recoded organism and an integration site in the genome of the genetically recoded organism, express in the *in vitro* transcription and translation reaction, or added exogenously as a factor (e.g., a orthogonal tRNA or an orthogonal synthetase added to the platform or a reaction mixture).

[0079] Platforms Comprising Extracts from *Vibrio* Species

[0080] The disclosed compositions (or systems) may include platforms for preparing a sequence defined biopolymer or protein *in vitro*, where the platform comprising a cellular extract prepared from a cell culture of a species of *Vibrio*. In particular, the species of *Vibrio* may include *Vibrio natriegens*.

[0081] The species of *Vibrio* may be a naturally occurring isolate (*i.e.*, a wild-type species), or the species of *Vibrio* may be engineered. For example, the species of *Vibrio* may be engineered genetically to be deficient in a negative effector for cell-free protein synthesis (CFPS), for example via a knock-out mutation. Negative effectors for CFPS have been defined for *E. coli* and may include, but are not limited to, endA (SEQ ID

NO:7), lon (SEQ ID NO:8), mazF (SEQ ID NO:9), ompT (SEQ ID NO:10), rna (SEQ ID NO:11), rnb (SEQ ID NO:12), glpK (SEQ ID NO:13), gor (SEQ ID NO:14), gshA (SEQ ID NO:15), tnaA (SEQ ID NO:16), rne (SEQ ID NO:31), gdhA (SEQ ID NO:32), sdaA (SEQ ID NO:33), sdaB (SEQ ID NO:34), speA (SEQ ID NO:35), WaaL (SEQ ID NO:36), and any combination thereof.

[0082] The species of *Vibrio* contemplated herein may be engineered to be deficient in a gene encoding the corresponding homolog of any of *E. coli* endA, mazF, rna, rnb, rne, gor, lon, ompT, gdhA, gshA, sdaA, sdaB, speA, WaaL, tnaA, glpK, and any combination thereof. For example, the species of *Vibrio* contemplated herein may be deficient in a gene which encodes the corresponding homolog of any of *E. coli* endA, mazF, rna, rnb, rne, gor, lon, ompT, gdhA, gshA, sdaA, sdaB, speA, WaaL, tnaA, glpK, which homolog has at least about 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to one or more of endA (SEQ ID NO:7), lon (SEQ ID NO:8), mazF (SEQ ID NO:9), ompT (SEQ ID NO:10), rna (SEQ ID NO:11), rnb (SEQ ID NO:12), glpK (SEQ ID NO:13), gor (SEQ ID NO:14), gshA (SEQ ID NO:15), tnaA (SEQ ID NO:16), rne (SEQ ID NO:31), gdhA (SEQ ID NO:32), sdaA (SEQ ID NO:33), sdaB (SEQ ID NO:34), speA (SEQ ID NO:35), WaaL (SEQ ID NO:36), and any combination thereof.

[0083] In some embodiments, the species of *Vibrio* contemplated herein may be engineered to be deficient in one or more of *Vnat_endA* (SEQ ID NO:17), *Vnat_lon* (SEQ ID NO:18), *Vnat_rnb* (SEQ ID NO:19), *Vnat_glpK* (SEQ ID NO:20), *Vnat_gor* (SEQ ID NO:21), *Vnat_gshA* (SEQ ID NO:22), *Vnat_tnaA* (SEQ ID NO:23), or any combination thereof. In other embodiments, the species of *Vibrio* contemplated herein may be deficient in a gene encoding one or more of *Vnat_endA* (SEQ ID NO:24), *Vnat_lon* (SEQ ID NO:25), *Vnat_rnb* (SEQ ID NO:26), *Vnat_glpK* (SEQ ID NO:27), *Vnat_gor* (SEQ ID NO:28), *Vnat_gshA* (SEQ ID NO:29), *Vnat_tnaA* (SEQ ID NO:30), or any combination thereof.

[0084] In addition or in the alternative, the species of *Vibrio* may be engineered to express an upregulated gene product that is a positive effector for CFPS. Positive

effectors for CFPS have been defined for *E. coli* and may include, but are not limited to *ackA*, *ndk*, *pykF*, *cdd*, *dsbC*, *dnaK*, *dnaJ*, *crpE*, *tig*, *groS*, *groL*, *infA*, *infB*, *fusA*, *efp*, *lepA*, *tufB*, *hslR*, *ffr*, and any combination thereof. The species of *Vibrio* may be engineered genomically, for example by recombinantly introducing heterologous DNA into the genome of the species of *Vibrio*, and or the species of *Vibrio* may be engineered by introducing an episomal vector (*e.g.*, a plasmid) to the species of *Vibrio* in order to create an engineered species of *Vibrio* that expresses an upregulated gene product that is the corresponding homolog of any of *E. coli* *ackA*, *ndk*, *pykF*, *cdd*, *dsbC*, *dnaK*, *dnaJ*, *crpE*, *tig*, *groS*, *groL*, *infA*, *infB*, *fusA*, *efp*, *lepA*, *tufB*, *hslR*, *ffr*, and any combination thereof. For example, the species of *Vibrio* contemplated herein may be engineered to express an upregulated gene product that has an amino acid sequence having at least about 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid sequence identity to one or more of *E. coli* *ackA*, *ndk*, *pykF*, *cdd*, *dsbC*, *dnaK*, *dnaJ*, *crpE*, *tig*, *groS*, *groL*, *infA*, *infB*, *fusA*, *efp*, *lepA*, *tufB*, *hslR*, *ffr*, and any combination thereof.

[0085] The species of *Vibrio* may be engineered to be deficient specifically in a release factor of translation. Release factors for translation may include, but are not limited to release factor 1 (RF-1).

[0086] The species of *Vibrio* may be genomically-recoded. For example, the species of *Vibrio* may be genomically-recoded to replace one or more stop codons with a different codon, optionally where all of one stop codon is replaced in the genome of the species of *Vibrio* with a different codon.

[0087] The species of *Vibrio* may be engineered to express a non-native or heterologous RNA polymerase, for example, by recombinantly introducing heterologous DNA encoding the RNA polymerase into the genome of the species of *Vibrio*, and or the species of *Vibrio* may be engineered by introducing an episomal vector that expresses the RNA polymerase (*e.g.*, a plasmid) to the species of *Vibrio*. Suitable RNA polymerases may include, but are not limited to T7 RNA polymerase.

[0088] The cellular extract of the platform is prepared from a cell culture of a species of *Vibrio*. In some embodiments, the cell culture is in stationary phase. In some embodiments, stationary phase may be defined as the cell culture having an OD₆₀₀ of greater than about 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, or having an OD₆₀₀ within a range bounded by any of these values.

[0089] The cell extract may be prepared by lysing the cells of the cell culture and isolating a fraction from the lysed cells. For example, the cell extract may be prepared by lysing the cells of the cell culture and subjecting the lysed cells to centrifugal force, and isolating a fraction after centrifugation (*e.g.*, where the S12 fraction and/or S30 fraction is isolated).

[0090] The platforms disclosed herein may include additional components, for example, one or more components for performing CFPS. Components may include, but are not limited to amino acids which optionally may include noncanonical amino acids, NTPs, salts (*e.g.*, sodium salts, potassium salts, and/or magnesium salts), cofactors (*e.g.*, nicotinamide adenine dinucleotide (NAD) and/or coenzyme-A (CoA)), an energy source and optionally an energy source comprising a phosphate group (*e.g.*, phosphoenol pyruvate (PEP), ATP, or creatine phosphate), a translation template (*e.g.*, a non-native mRNA that is translated in the platform) and/or a transcription template (*e.g.*, a template DNA for synthesizing a non-native mRNA that is translated in the platform), and any combination thereof.

[0091] In some embodiments, the platform may comprise an energy source and optionally an energy source comprising a phosphate group (*e.g.*, phosphoenol pyruvate (PEP), ATP, or creatine phosphate), where the energy source is present in the platform at a concentration of greater than about 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, or 90 mM (preferably greater than about 67 mM), but less than about 100 mM, or within a concentration range bounded by of these values.

[0092] In some embodiments, the platform further comprises a source of potassium (K⁺)(such as a potassium salt such as potassium glutamate), where the platform

comprises potassium at a concentration greater than about 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, or 450 mM (preferably about 300 mM), but less than about 500 mM, or within a concentration range bounded by of these values,

[0093] The disclosed platforms and cell extracts may be utilized in methods for preparing a sequence defined biopolymer or protein *in vitro*. The disclosed methods typically include translating *in vitro* a translation template (*e.g.*, mRNA) encoding the sequence defined biopolymer or protein in the platform of any of the foregoing claims. Optionally, the disclosed methods may include transcribing a transcription template (*e.g.*, DNA) in the platform to provide the translation template.

[0094] The disclosed methods may be performed under conditions that are suitable for cellular extracts prepared from a species of *Vibrio*. In some embodiments, the disclosed methods are performed at a temperature between about 20-40 °C, and preferably at a temperature of about 30 °C.

[0095] The disclosed methods may be performed to synthesize any sequence defined biopolymer or protein. In some embodiments, the sequence defined polymer or protein is a therapeutic protein and/or the method may be utilized to identify therapeutic proteins or biomaterials by translating a library of transcription templates. In some embodiments, the disclosed methods may be performed to optimize *in vitro* translation conditions for a cellular extract prepared from a species of *Vibrio*.

[0096] Kits also are contemplated herein. In some embodiments, the contemplated kits comprise as components: (a) a cellular extract prepared from a cell culture of a species of *Vibrio* (*e.g.*, *Vibrio natriegens*); and (b) a reaction mixture for translating an mRNA. Suitable components for the reaction mixture of the disclosed kits may include, but are not limited to, amino acids which optionally may include noncanonical amino acids, NTPs, salts (*e.g.*, sodium salts, potassium salts, and/or magnesium salts), cofactors (*e.g.*, nicotinamide adenine dinucleotide (NAD) and/or coenzyme-A (CoA)), an energy source and optionally an energy source comprising a phosphate group (*e.g.*, ATP or creatine phosphate).

[0097] Knock-Out Mutations

[0098] The species of *Vibrio* disclosed herein may include a genetic knock-out mutation, preferably a knock-out mutation that downregulates or eliminates a negative protein effector for CFPS. In certain embodiments, the at least one additional genetic knock-out mutation improves DNA stability, RNA stability, protein stability, amino acid stability, energy supply, or any combination thereof. In certain embodiments, the at least one additional genetic knock-out mutation comprises 1, 2, 3, 4, or more than 4 genetic knock-out mutations. In embodiments where the strain comprises 2 or more genetic knock-out mutations, at least 2 of the genetic knock-out mutations may both improve the same attribute, improved DNA stability, improved RNA stability, improved protein stability, improved amino acid stability, improved energy supply, or may both improve different attributes.

[0099] To improve DNA or RNA stability, the at least one additional genetic knock-out mutation may target the functional inactivation of nucleases. *In vivo*, nucleases play important roles in regulating DNA and mRNA turnover. However, their presence in crude cell extracts is expected to be deleterious, leading to template instability and reaction termination. A nonexhaustive list of potential negative effectors that have been identified in *E.coli* follow: RNase A (encoded by *ma*) degrades RNA by catalyzing the cleavage of phosphodiester bonds, and identification of strains (e.g., MRE600, A19) lacking *ma* was important for early studies in *in vitro* translation. RNase II (encoded by *rnb*) is responsible for mRNA decay by 3' to 5' exonuclease activity, and cell extracts lacking RNase II exhibit a 70% increase in CFPS efficiency. RNase E (encoded by *me*) is part of a cold shock degradosome that induces mRNA decay in cold shock, which the cells experience during harvest prior to extract generation. MazF (encoded by *mazF*) is a toxin that degrades mRNA by sequence-specific (ACA) endoribonuclease activity, which could affect transcript stability. CsdA (encoded by *csdA*) is part of a cold shock degradosome along with RNase E and induces mRNA decay in cold shock, which the cells experience during harvest prior to extract generation. DNA-specific endonuclease I (encoded by *endA*) breaks double-stranded DNA, and its deletion has previously shown to be important for extending the duration of CFPS reactions. The corresponding *Vibrio* homolog of these

and other nucleases may be functionally inactivated by the at least on additional genetic knock-out mutation.

[00100] To improve protein stability, the at least one additional genetic knock-out mutation may target the functional inactivation of proteases. *In vivo*, these proteases play important roles in regulating protein turnover. However, their presence in CFPS reactions is expected to be deleterious, leading to protein instability issues. A nonexhaustive list of potential negative effectors identified in *E. coli* follow: Glutathione reductase (encoded by *gor*) reduces oxidized glutathione to maintain a reducing environment in the cytoplasm of a cell, making synthesis of disulfide-bonded proteins problematic. Lon (encoded by *lon*) is an ATP-dependent protease that demonstrated improved protein production in cell-free systems in BL21 strains upon transcriptional down regulation. Outer membrane protease VII (encoded by *ompT*) demonstrates specificity for paired basic residues and has been shown to stabilize proteins during CFPS upon removal. The corresponding *Vibrio* homolog of these and other proteases may be functionally inactivated by the at least on additional genetic knock-out mutation.

[00101] The at least one additional genetic knock-out mutation may target proteins known to negatively affect amino acid or energy supply. *In vivo*, these proteins play important roles in metabolism and substrate turnover. However, their presence in crude cell extracts is expected to be deleterious, leading to decreased amino acid and energy supply to support translation. A nonexhaustive list of potential negative effectors identified in *E. coli* follow. Glutamate dehydrogenase (encoded by *gdhA*) catalyzes the deamination of glutamate, which may affect glutamate's stability. Glutamate-cysteine-ligase (encoded by *gshA*) catalyzes the first step of glutathione synthesis and may decrease the stability of cysteine. Serine deaminase I (encoded by *sdaA*) and serine deaminase II (encoded by *sdaB*) are two of the three enzymes involved in serine degradation. Arginine decarboxylase (encoded by *speA*) consumes arginine in the biosynthetic production of putrescine. Tryptophanase (encoded by *tnaA*) consumes tryptophan in the production of indole. Lastly, glycerol kinase (encoded by *glpK*) consumes ATP to phosphorylate glycerol, which could help deplete the energy supply required for cell-free reactions. The

corresponding *Vibrio* homolog of these and other proteins may be functionally inactivated by the at least on additional genetic knock-out mutation.

[00102] Strains having at least one additional genetic knock-out mutation, may be prepared by any method of engineering a strain to functionally inactivate the negative effector to lessen or eliminate the negative effector from a lysate prepared from the strain. In certain embodiments, the genetic knock-out mutations may be prepared by inserting either a nonsense mutation and/or a frameshift mutation into the genome of the strain as well as deleting a vital portion of a gene coding sequence. In certain embodiments, the genetic knock-out mutations may be prepared by removing regulatory sequences (i.e. promoter, ribosome binding site) or otherwise changing these sequences in the genome as to render them non-functional. In certain embodiments, negative effectors can be functionally knocked out in lysates by introducing a unique affinity tag and subsequently using the tag to selectively remove the effector protein from the lysates. In certain embodiments a strain having at least one additional genetic knock-out mutation may be prepared by multiplex automated genome engineering (MAGE), λ -Red recombinase-mediated recombination (Datsenko-Wanner), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9), and any other commonly used recombineering and genome engineering tools.

[00103] Upregulated Gene Products

[00104] The species of *Vibrio* disclosed herein may be engineered to express an additional upregulated gene product. The at least one additional upregulated gene product is preferably an upregulated gene product that is a positive effector for CFPS. In certain embodiments, the at least one additional upregulated gene product improves energy supply, chaperone levels, translations function, ribosome recycling, or any combination thereof. In certain embodiments, the at least on additional upregulated gene product comprises 1, 2, 3, 4, or more than 4 upregulated gene products. In embodiments where the strain comprises 2 or more upregulated gene products, at least 2 of the upregulated gene products may both improve the same attribute, improved energy supply, improved

chaperone levels, improved translation function, or improved ribosome recycling, or may both improve different attributes.

[00105] To improve energy supply, the at least one additional upregulated gene product may target the upregulation of kinases. *In vivo*, these proteins play important roles in metabolism and the transfer of phosphate groups. The upregulated presence in crude cell extracts is expected to improve energy supply to support translation. A nonexhaustive list of potential positive effectors identified in *E. coli* follow. Acetate kinase (encoded by *ackA*) increases the overall metabolic flux of metabolites toward substrate-level ATP generation. Nucleoside-diphosphate kinase (encoded by *ndk*) facilitates the synthesis of NTPs from their corresponding NDPs. Pyruvate kinase monomer (encoded by *pykF*) helps drive ATP generation. The corresponding *Vibrio* homolog of these and other kinases may be the at least one additional upregulated gene product.

[00106] To improve energy supply, the at least one additional upregulated gene product may target the upregulate of deaminases. *In vivo*, these proteins may play important roles in metabolism and preparing metabolites. A nonexhaustive list of potential positive effectors identified in *E. coli* follow. Cytidine deaminase (encoded by *cdd*) initiates the deamination of cytidine which may lead to the synthesis of UTP. The corresponding *Vibrio* homolog of these and other deaminases may be the at least one additional upregulated gene product.

[00107] To improve chaperone levels, the at least one upregulated gene product may target the upregulation of isomerases, foldases and/or holdases. *In vivo*, these proteins may play important roles in the assisting proteins to adopt functionally active conformations. The upregulated presence in crude cell extracts is expected to improve chaperone levels to support protein production into soluble and/or active confirmations. A nonexhaustive list of potential positive effectors identified in *E. coli* follow. Disulfide bond isomerase (encoded by *dsbC*) shuffles disulfide bonds into correct positions. Chaperone protein DnaK (encoded by *dnaK*) aids the folding of nascent polypeptide chains and the rescue of misfolded proteins. Chaperone protein DnaJ (encoded by *dnaJ*) stimulates the ATPase activity of DnaK. Protein GrpE (encoded by *grpE*) stimulates the

ATPas activity of DnaK. Trigger Factor (encoded by *tig*) aids the folding of nascent polypeptides. The 10 kDa chaperonin subunit (encoded by *groS*) forms part of the GroEL-GroES chaperonin complex that aids in protein folding. The 60 kDa chaperonin subunit (encoded by *groL*) forms part of the GroEL-GroES chaperonin complex that aids in protein folding. The corresponding *Vibrio* homolog of these and other isomerases, foldases, and/or holdases may be the at least one additional upregulated gene product.

[00108] To improve translation function, the at least one upregulated gene product may target the upregulation of initiation factors and/or elongation factors. *In vivo*, these proteins play important roles in the translation function. The upregulated presence in crude cell extracts is expected to improve translation function. A nonexhaustive list of potential positive effectors identified in *E. coli* follow. Translation initiation factor IF-1 (encoded by *infA*) interacts with the 30S ribosomal subunit to initiate translations. Translation initiation factor IF-2 (encoded by *infB*) has a role in the proper placement of the charged initiator fMet-tRNA via a GTP-dependent mechanism. Elongation factor G (encoded by *fusA*) facilitates translocation of the ribosome by one codon along a mRNA. Elongation factor P (encoded by *efp*) stimulates the synthesis of peptide bonds. Elongation factor 4 (encoded by *lepA*) can alter the rate of translation, leading to increases in the rate of translation under certain stress conditions. Elongation factor TU 2 (encoded by *tufB*) helps shuttle charged tRNAs to ribosomes. The corresponding *Vibrio* homolog of these and other initiation factors and/or elongation factors may be the at least one additional upregulated gene product.

[00109] To improve translation function, the at least one upregulated gene product may target the upregulation of recycling factors. *In vivo*, these proteins play important roles in the ribosome recycling. The upregulated presence in crude cell extracts is expected to improve ribosome recycling. A nonexhaustive list of potential positive effectors identified in *E. coli* follow. Heat shock protein 15 (encoded by *hslR*) is involved with the recycling of free 50S ribosomal subunits. Ribosome-recycling factor (encoded by *frr*) promotes rapid recycling of ribosomal subunits after the release of the polypeptide chain. The corresponding *Vibrio* homolog of these and other recycling factors may be the at least one additional upregulated gene product.

[00110] Strains having at least one additional genetic knock-out mutation, may be prepared by any method of engineering a strain to functionally increase a positive effector to increase the presence of the positive effector in the lysate prepared from the strain. In certain embodiments, the upregulated gene product is expressed from a plasmid present in the GRO and/or expressed from an integration site in GRO genome. Additionally, gene upregulation may be enhanced by engineering the promoter and/or ribosome binding site in front of your gene of interest located either on a plasmid or on the genome. A stronger promoter/ribosome binding site would lead to an increase in transcriptional activity. Techniques commonly employed to integrate a plasmid overexpressing a positive effector into a strain includes transformation. Techniques commonly employed to integrate a gene cassette containing a positive effector into the genome for overexpression includes X-Red recombinase-mediated recombination (Datsenko-Wanner).

[00111] Genomically Recoded Organisms

[00112] An aspect of the present invention is a genomically recoded organism (GRO) which optionally may be a strain deficient in release factor 1 (RF1) or a genetic homolog thereof. GROs may be prepared by any method of strain engineering. In certain embodiments, a strain deficient in RF1 is prepared by replacing in the strain all instances of the UAG codon, permitting the deletion of release factor 1 (RF1; terminates translation at UAG and UAA) and, hence, eliminating translational termination at UAG codons. This GRO allows for the reintroduction of UAG codons, along with orthogonal translation machinery to permit efficient and site-specific incorporation of nonstandard or noncanonical amino acids into proteins. That is, UAG may be transformed from a nonsense codon (terminates translation) to a sense codon (incorporates amino acid of choice), provided the appropriate translation machinery is present.

ILLUSTRATIVE EMBODIMENTS

[00113] The following embodiments are illustrative and should not be interpreted to limit the scope of the claimed subject matter.

[00114] Embodiment 1. A platform for preparing mRNA, a sequence defined biopolymer, a peptide (*e.g.*, an amino acid sequence of 5-100 residues), or a polypeptide *in vitro*, the platform comprising a cellular extract prepared from a cell culture of a species of *Vibrio*.

[00115] Embodiment 2. The platform of embodiment 1, wherein the species of *Vibrio* is *Vibrio natriegens*.

[00116] Embodiment 3. The platform of embodiment 1 or 2, wherein the species of *Vibrio* is engineered to be deficient in a negative effector for cell-free protein synthesis (CFPS).

[00117] Embodiment 4. The platform of embodiment 3, wherein the negative effector for CFPS is selected from the group consisting of the *Vibrio* homolog of *E. coli* *endA*, *mazF*, *rna*, *rnb*, *rne*, *gor*, *lon*, *ompT*, *gdhA*, *gshA*, *sdaA*, *sdaB*, *speA*, *WaaL*, *tnaA*, *glpK*, and any combination thereof.

[00118] Embodiment 5. The platform of any of the foregoing embodiments, wherein the species of *Vibrio* is engineered to express an upregulated gene product that is a positive effector for CFPS.

[00119] Embodiment 6. The platform of embodiment 5, wherein the positive effector for CFPS is selected from the group consisting of the *Vibrio* homolog of *E. coli* *ackA*, *ndk*, *pykF*, *cdd*, *dsbC*, *dnaK*, *dnaJ*, *crpE*, *tig*, *groS*, *groL*, *infA*, *infB*, *fusA*, *efp*, *lepA*, *tufB*, *hslR*, *ffr*, and any combination thereof.

[00120] Embodiment 7. The platform of any of the foregoing embodiments, wherein the species of *Vibrio* is engineered to be deficient in a release factor 1.

[00121] Embodiment 8. The platform of any of the foregoing embodiments, wherein the species of *Vibrio* has been engineered to express T7 RNA polymerase.

[00122] Embodiment 9. The platform of any of the foregoing embodiments, wherein the cell culture are in stationary phase.

[00123] Embodiment 10. The platform of embodiment 9, wherein stationary phase is defined as the cell culture having an OD₆₀₀ of greater than about 3.0.

[00124] Embodiment 11. The platform of any of the foregoing embodiments, wherein the cellular extract comprises an S12 fraction and/or S30 fraction of the cell culture.

[00125] Embodiment 12. The platform of any of the foregoing embodiments further comprising one or more components selected from the group consisting of amino acids which optionally may include non-canonical amino acids, NTPs, salts, cofactors, an energy source and optionally an energy source comprising a phosphate group (such as phosphoenol pyruvate (PEP)), a translation template, a transcription template, and any combination thereof.

[00126] Embodiment 13. The platform of any of the foregoing embodiments further comprising an energy source and optionally an energy source comprising a phosphate group (such as phosphoenol pyruvate (PEP)), wherein the energy source is present at a concentration of greater than about 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, or 90 mM (preferably greater than about 67 mM), but less than about 100 mM, or within a concentration range bounded by of these values.

[00127] Embodiment 14. The platform of any of the foregoing embodiments further comprising a source of potassium (K⁺), wherein the platform comprises potassium at a concentration greater than about 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, or 450 mM (preferably about 300 mM), but less than about 500 mM, or within a concentration range bounded by of these values; and/or the platform of any of the foregoing embodiments further comprising a source of magnesium (Mg⁺), wherein the platform comprises magnesium at a concentration greater than about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 8mM, 12 mM, 16 mM or 20mM (preferably about 8 mM), but less than about 30 mM, or within a concentration range bounded by of these values.

[00128] Embodiment 15. The platform of any of the foregoing embodiments, wherein the platform or one or more components thereof are preserved by freeze-drying.

[00129] Embodiment 16. A method for preparing mRNA, a sequence defined biopolymer, a peptide (*e.g.*, an amino acid sequence having 5-100 residues), or a polypeptide *in vitro*, the method comprising transcribing the mRNA from a transcription template and/or translating an mRNA in the platform of any of the foregoing embodiments, wherein the mRNA encodes the sequence defined biopolymer, the peptide, or the polypeptide.

[00130] Embodiment 17. The method of embodiment 16, wherein the method comprises transcribing a DNA template in the platform to provide the translated mRNA.

[00131] Embodiment 18. The method of embodiment 16 or 17, wherein the method is performed at a temperature between about 20-40 °C.

[00132] Embodiment 19. The method of any of the embodiments 16-18, wherein the sequence defined biopolymer or protein is a therapeutic protein and/or the method is utilized to identify therapeutic proteins or biomaterials.

[00133] Embodiment 20. A method of detecting a target molecule in a biological or environmental sample, the method comprising detecting the target molecule in a cell-free protein synthesis system with *Vibrio*-based cell lysates.

[00134] Embodiment 21. A kit comprising as components: (a) a cellular extract prepared from a cell culture of a species of *Vibrio*; and (b) a reaction mixture for translating an mRNA, optionally wherein the species of *Vibrio* is *Vibrio natriegens*.

[00135] Embodiment 22. The kit of embodiment 21 or 22, wherein the reaction mixture comprises one or more components selected from the group consisting of amino acids which optionally may include non-canonical amino acids, NTPs, salts, cofactors, an energy source and optionally an energy source comprising a phosphate group (such as phosphoenol pyruvate (PEP)).

[00136] Embodiment 23. A recombinant species of *Vibrio*, optionally *Vibrio natriegens*, wherein the species of *Vibrio* is engineered to be deficient in a negative effector for cell-free protein synthesis (CFPS).

[00137] Embodiment 24. The recombinant species of *Vibrio* of embodiment 23, wherein the negative effector for CFPS is selected from the group consisting of the *Vibrio* homolog of *E. coli* *endA*, *mazF*, *rna*, *rnb*, *rne*, *gor*, *lon*, *ompT*, *gdhA*, *gshA*, *sdaA*, *sdaB*, *speA*, *WaaL*, *tnaA*, *glpK*, and any combination thereof.

[00138] Embodiment 25. The recombinant species of *Vibrio* of any of the foregoing embodiments, wherein the species of *Vibrio* is engineered to express an upregulated gene product that is a positive effector for CFPS.

[00139] Embodiment 26. The recombinant species of *Vibrio* of embodiment 25, wherein the positive effector for CFPS is selected from the group consisting of the *Vibrio* homolog of *E. coli* *ackA*, *ndk*, *pykF*, *cdd*, *dsbC*, *dnaK*, *dnaJ*, *crpE*, *tig*, *groS*, *groL*, *infA*, *infB*, *fusA*, *efp*, *lepA*, *tufB*, *hslR*, *ffr*, and any combination thereof.

[00140] Embodiment 27. The recombinant species of *Vibrio* of any of the foregoing embodiments, wherein the species of *Vibrio* is engineered to be deficient in a release factor 1.

[00141] Embodiment 28. The recombinant species of *Vibrio* of any of the foregoing embodiments, wherein the species of *Vibrio* has been genomically-recoded to replace one or more stop codons with a different codon.

[00142] Embodiment 29. The recombinant species of *Vibrio* of any of the foregoing embodiments, wherein the species of *Vibrio* has been engineered to express T7 RNA polymerase.

EXAMPLES

[00143] The following Examples are illustrative and should not be interpreted to limit the scope of the claimed subject matter.

[00144] Example 1 – Establishing a high-yielding cell-free protein synthesis platform derived from *Vibrio natriegens*

[00145] Reference is made to Des Soye *et al.*, “Establishing a High-Yielding Cell-Free Protein Synthesis Platform Derived from *Vibrio natriegens*,” ACS Synth. Biol. 2018 Sep. 21;7(9):2245-2255, the content of which is incorporated herein by reference in its entirety.

[00146] Abstract

[00147] A new wave of interest in cell-free protein synthesis (CFPS) systems has shown their utility for producing proteins at high titers, establishing genetic regulatory element libraries (*e.g.*, promoters, ribosome binding sites) in non-model organisms, optimizing biosynthetic pathways before implementation in cells, and sensing biomarkers for diagnostic applications. Unfortunately, most previous efforts have focused on a select few model systems, such as *Escherichia coli*. Broadening the spectrum of organisms used for CFPS promises to better mimic host cell processes in prototyping applications and open up new areas of research. Here, we describe the development and characterization of a facile CFPS platform based on lysates derived from the fast-growing bacterium *Vibrio natriegens*, which is an emerging host organism for biotechnology. We demonstrate robust preparation of highly active extracts using sonication, without specialized and costly equipment. After optimizing the extract preparation procedure and cell-free reaction conditions, we show synthesis of 1.6 ± 0.05 g/L of superfolder green fluorescent protein in batch mode CFPS, making it competitive with existing *E. coli* CFPS platforms. To showcase the flexibility of the system, we demonstrate that it can be lyophilized and retain biosynthesis capability, that it is capable of producing antimicrobial peptides, and that our extract preparation procedure can be coupled with the recently described Vmax™ Express strain in a one-pot system. Finally, to further increase system productivity, we explore a knockout library in which putative negative effectors of CFPS are genetically removed from the source strain. Our *V. natriegens*-derived CFPS platform is versatile, and simple to prepare and use. We expect it will facilitate expansion of CFPS systems into new laboratories and fields for compelling applications in synthetic biology.

[00148] Introduction

[00149] Cell-free systems have recently enjoyed a technical renaissance that has transformed them into robust platforms for the synthesis of a wide variety of useful and interesting products¹⁻⁴. Such platforms combine crude cell lysates or purified components with substrates in a test tube, enabling the activation and use of cellular processes *in vitro* (Fig. 1). Cell-free protein synthesis (CFPS) systems in particular have made significant advances in reaction volume, duration, and productivity, now reaching g/L quantities of protein^{3,5-12}. These systems provide several unique advantages for understanding, harnessing, and expanding the capabilities of natural biological systems. Reactions are open, and are therefore easily accessible for sample extraction and substrate feeding. Dilute reaction environments facilitate the folding of complex eukaryotic protein products which may otherwise express poorly in bacterial systems⁴. Importantly, the removal of genomic material from the chassis organism directs reaction substrates and machinery towards the desired synthesis reaction at high rates. Exploiting these features, CFPS platforms enjoy increasingly widespread use as a complement to *in vivo* expression for applications including biomolecular breadboarding¹³⁻¹⁶, expression of toxic products¹⁷⁻²⁰, production of complex protein products that are poorly soluble *in vivo*^{7,21-23}, manufacture of glycoproteins²⁴⁻²⁷, detection of disease²⁸⁻³⁰, on demand biomanufacturing^{21,31-35}, and education^{36,37}.

[00150] Despite the emergence of cell-free systems as a prominent research tool for fundamental and applied biology, the vast majority of previous efforts have focused on a select few model systems such as *Escherichia coli*, *Saccharomyces cerevisiae*, and Chinese Hamster Ovary cells, among others^{2,5,11,12,38,39}. However, we and others hypothesize that developing cell-free systems composed of extracts derived from relevant chassis organisms that better mimic the natural physicochemical environment might enhance predictive power for synthetic biology applications. This idea motivates the development of new cell-free systems. In this context, several new CFPS systems have been developed, including some from *Streptomyces* species and *Bacillus* ⁴⁰⁻⁴⁴. For example, an elegant study by Freemont and colleagues characterized new DNA parts from

the non-model bacterium *Bacillus megaterium* by combining automated CFPS and Bayesian models⁴⁴.

[00151] A particularly exciting chassis organism for developing a new cell-free system is the fast-growing halophilic marine bacterium *Vibrio natriegens*. First discovered in a Georgia salt marsh in 1958, *V. natriegens* (originally classified as *Pseudomonas natriegens*) was identified as the fastest-growing bacterium known to date when it was discovered that cell populations in liquid culture double approximately once every 10 minutes^{45,46}. Despite this noteworthy trait, *V. natriegens* went largely unstudied for decades. Recently, interest in this organism has been renewed, largely out of a desire to leverage its rapid generational time to accelerate molecular biology efforts and improve recombinant protein production^{47,48}.

[00152] *V. natriegens*' rapid doubling time is particularly interesting for potential CFPS system development, as in the context of CFPS it is generally accepted that lysate productivity loosely correlates to chassis organism growth rate^{49,50}. After all, proper cell division relies on the coordinated activities of a large suite of proteins, so it is reasonable to infer that rapidly dividing cells require high protein synthesis rates, and by extension possess highly active protein translation machinery. This is very likely the case for *V. natriegens*—the species features 12 rRNA operons as compared to the 7 found in *E. coli* strain MG1655⁴⁷. Furthermore, it has been suggested that exponentially-growing *V. natriegens* cells contain ~115,000 ribosomes/cell, significantly higher than the ~70,000 ribosomes/cell observed in *E. coli*⁵¹. Considering these advantages, we hypothesized that lysates derived from *V. natriegens* would be enriched in active protein translation machinery and thus compose a highly productive CFPS platform.

[00153] In this study, we describe the development of a facile CFPS platform derived from *V. natriegens*. Key design criteria were to make the system robust, easy to use, and accessible to all. Therefore, since cell lysis procedures using homogenization or French press can be expensive, time and labor intensive, and hard to standardize, we focused on developing an extract preparation procedure using standard sonication equipment⁵². Previously, we have shown that sonication offers a simple strategy to reduce

cost and variability in crude *E. coli* extract preparation, while eliminating the need for specialized and expensive growth and lysis equipment⁵². We first showed the ability to create a protein synthesis competent cell-free system. We then optimized the extract preparation process by modifying growth media, cultivation time, cell disruption conditions, and lysate clarification conditions. This led to a 400% increase from the non-optimized case, resulting in a cell-free system capable of synthesizing ~1 mg/mL of superfolder green fluorescent protein (sfGFP) using a typical *E. coli* CFPS reagent mix. Further optimization of key reagent concentrations increased the productivity of the system to ~1.6 mg/mL. We lyophilized fully-assembled *V. natriegens* CFPS reactions and found that, if prepared in the presence of trehalose, reactions retain 100% productivity after one week of storage at room temperature. Next, we applied homologous recombination-based genome engineering to prepare a small library of knockout strains in which the genes encoding putative negative effectors of CFPS have been removed. Lysates were prepared from each of these knockout strains in an attempt to identify a strain background with improved productivity *in vitro*. The *V. natriegens* CFPS system described here is productive, robust, and facile to prepare. We expect it will lower the barrier for entry into the use of CFPS systems.

[00154] Results and Discussion

[00155] Identifying extract preparation conditions for *V. natriegens* CFPS. We began our study by trying to develop extract preparation procedures. Because CFPS exploits an ensemble of catalytic proteins prepared from the crude lysate of cells, the cell extract (whose composition is sensitive to growth media, lysis method, and processing conditions) is the most critical component of extract-based CFPS reactions. In recent years, systematic optimization of each step in extract preparation for *E. coli* CFPS has improved extract robustness and productivity^{1,52}. Similar advances have been made in *S. cerevisiae*, *Streptomyces*, and *Pseudomonas* CFPS systems^{38,41,53}. Based on these successes, we chose to vary extract preparation conditions in search of parameters that might improve reproducibility between extract preps, increase the level of protein synthesized, and allow for potential downstream scalability. A key focus was to generate a

large volumetric yield of lysate even when chassis cells are cultured at volumes that can be accommodated by shake flasks.

[00156] Generally, the extract preparation process includes the following major steps: cell cultivation, cell disruption, lysate clarification, and some optional steps like run-off reaction and dialysis. We decided to explore each of these steps. First, we wanted to confirm the previously reported doubling times for *V. natriegens* (*Vnat*). To accomplish this, we prepared liquid cultures of wild type *Vnat* cells in several different liquid growth media. Each growth medium was tested with and without v2 salt supplementation⁴⁷, and culture growth was monitored via plate reader over a 20 hour incubation (Fig. 7A). Under the conditions tested, *Vnat* was found to double approximately every 12-14 minutes in salt-supplemented growth medium. This range aligns with previously reported data and outpaces standard *E. coli* laboratory strains^{47,48}. Unsurprisingly, in most of the media investigated, the growth of *Vnat* is severely inhibited in the absence of salt. This held true at culturing volumes of 1 L incubated in shake flasks, which are typical volumes and conditions used to culture cells for lysate preparation (Fig. 7B).

[00157] We next set out to establish the growth medium that is optimal for culturing cells for *Vnat* CFPS lysate preparation. 1 L cultures of *Vnat* were grown in 2xYTPG (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 7 g/L K₂HPO₄, 3 g/L KH₂PO₄, 18 g/L glucose), BHI (brain-heart infusion), and a BHI variant supplemented with 1.8% (m/v) glucose (BHIG), in all cases supplemented with v2 salts. Cell pellets were collected at OD₆₀₀ = 4.5 to mimic the best practice of harvesting *E. coli* cultures during mid-exponential phase, and protocols previously established for *E. coli* were used for lysate preparation and CFPS synthesis of sfGFP. Specifically, we assembled CFPS reactions with the sfGFP template and carried out 15 µL batch reactions for 20 h at 30°C. Lysates derived from cells cultured in BHI were significantly more productive than those from cells grown in either of the other two media types (Fig. 2A). Based on these cumulative results, we selected BHI for use in *Vnat* cultures going forward.

[00158] Next, we investigated the ideal time point at which to collect *Vnat* cell pellets for CFPS lysate production. As previously mentioned, in *E. coli*-based systems, the

most productive lysates are derived from cells gathered during mid-exponential phase growth^{3,52,54}. It is generally accepted that this is because the pool of active ribosomes is most enriched during this phase of the growth cycle. We therefore hypothesized that the most productive *Vnat* lysates would similarly be derived from cells harvested in mid-exponential phase. To test this, we harvested *Vnat* cultures at a range of optical densities ranging from lag phase, through exponential phase, and even into stationary phase. Lysates were prepared from each cell pellet and directed to synthesize sfGFP in CFPS (Fig. 2B). We were surprised to observe that *Vnat* lysate productivity increases with increasing OD₆₀₀ past the exponential phase of growth—indeed, the most productive lysate identified was prepared from cells in early stationary phase. Lysates retain ~85% productivity when prepared from cultures at stationary phase for several hours (Fig. 8A) and ~75% productivity when prepared from overnight cultures (Fig. 8B). These surprising results run counter to what is typically observed with other CFPS chassis organisms. In *E. coli*, for instance, it is believed that stationary phase cells experience a reduction in active ribosomes in response to the reduced demand for protein synthesis; this effect propagates to lysates, resulting in a severe reduction in CFPS productivity⁵⁵. Going forward, *Vnat* cultures were harvested for lysate preparation immediately upon entry into stationary phase (OD₆₀₀ ~ 7.5).

[00159] Identification of optimal procedures for preparation of *V. natriegens* lysates. Having established *Vnat*-specific cell culture and harvest parameters, we proceeded to identify conditions for preparing *Vnat* lysates via sonication that maximized CFPS yields. We focused our investigation on two key factors pertaining to lysate preparation. The first of these, cell pellet resuspension volume, describes the volume of lysis buffer used to resuspend a cell pellet prior to lysis. Modulating this volume has a direct influence on the concentration of cellular components in the final lysate, which in turn affects lysate productivity. The second factor considered was energy delivery to the cells during sonication. During lysis, enough energy must be delivered to ensure adequate rupturing of cellular compartments but must then be constrained to prevent denaturation of ribosomes, enzymes, and other fragile cellular components required for robust protein synthesis. Both of these parameters were simultaneously varied for lysis optimization, as

we have done before when developing a similar protocol for *E. coli*⁵². *Vnat* cell pellets were resuspended in 0.5, 0.8, or 1.0 mL of S30 buffer per gram of wet cell mass. Each suspension was then sonicated, with lysis achieved using one, two, or three 45-second pulses. Each resulting lysate was directed in CFPS to synthesize sfGFP (Fig. 2C).

[00160] Collectively, the results reveal that *Vnat* cells are relatively agnostic to both resuspension volume and lysis energy. Productivities of all nine lysates tested were within 10% of one another. The more concentrated suspensions demonstrate a modest (but statistically significant) increase in productivity relative to the samples prepared from cells resuspended in 1.0 mL buffer/g cells, likely a consequence of slightly more concentrated translation components. There is no appreciable difference between samples suspended in 0.5 mL buffer/g cells vs. 0.8 mL buffer/g cells. Due to the relative difficulty of resuspending cells in 0.5 mL buffer/g as well as the accompanying reduction in the volume of lysate yielded under that condition, we selected 0.8 mL buffer/g cells as our resuspension density going forward. Due to the insignificant differences in productivity when two or three sonication pulses are used for lysis, we settled on the use of three pulses to remain consistent with several recently-reported *E. coli* lysis protocols^{3,54}.

[00161] After we defined a reproducible cell lysis strategy to generate highly active extracts, we decided to investigate a post-lysis extract preparation step. Specifically, we evaluated the effect of the run-off reaction. Lysates derived from some strains of *E. coli* benefit tremendously from a run-off reaction, whereby clarified lysate is shaken in an incubator followed by a second clarifying spin to yield the final extract^{3,52,54}. It is believed that this incubation allows ribosomes to complete translation of native mRNAs that they were bound to at the moment of lysis and subsequent degradation of those mRNAs by native RNAses. In this way, ribosomes are made available for synthesis of a target CFPS product^{56,57}. To see if *Vnat* lysate productivity could be improved in this way, we prepared a panel of lysates subjected to run-off reactions at either 30°C or 37°C, both with and without shaking at 250 RPM (Fig. 9). This analysis revealed no benefit to performing any sort of run-off reaction. Indeed, overall productivity suffers when *Vnat* lysates are subjected to prolonged agitation or elevated temperatures. Since pre-incubation is not

necessary, we chose to not include the run-off reaction step in our *Vnat* cell extract preparation procedure.

[00162] Optimization of reagent concentrations and reaction conditions in *Vibrio* CFPS. With extraction preparation procedures in hand, we turned our attention to optimizing several reaction conditions which are known to impact CFPS yields. These optimizations were essential, since all reactions performed up until this point had been done using reagent and substrate conditions previously developed for *E. coli* CFPS. Because *Vnat* in nature are found in a different environment than *E. coli*, we reasoned that *Vnat* lysates may have different demands for small molecules and other reagents. Previous studies have demonstrated that CFPS performance can be improved by supplying reagents at concentrations similar to what is encountered by the chassis organism in nature^{5,58}. Thus, we hypothesized that yields of the system could be increased by modifying the existing *E. coli* reagent mix to have a more *Vnat*-specific composition. To test this, we varied the concentrations of several key components and observed how each change affected the yields of the system in CFPS.

[00163] One notable difference between *E. coli* and *Vnat* is that the latter is halophilic⁴⁵⁻⁴⁸. Indeed, exponential phase *Vnat* requires significant amounts of cations including Mg^{2+} , K^+ , and Na^+ ⁵⁹. In CFPS, Mg^{2+} is a particularly important reagent as it is a critical cation required for proper ribosome assembly⁶⁰. Thus, we reasoned that the salt content of the extant reagent mix could be improved for use with *Vnat* lysates. Our approach to address this was twofold. First, we varied the concentration of magnesium glutamate [Mg(GLU)] in the reaction mix (Fig. 3A). The system performed best when supplemented with 6 mM Mg(GLU). We also varied the concentration of potassium glutamate [K(GLU)] in the reaction mix (Fig. 3B). System productivity increased steadily with increasing K(GLU) concentration, up to a maximum beginning at 290 mM K(GLU). This is more than double the 130 mM K(GLU) used in the *E. coli* mix, and is in agreement with a previous study documenting the higher demand for K^+ in *Vnat* growth⁵⁹. We also tested various concentrations of sodium glutamate, but observed no significant improvements to the system (Fig. 10).

[00164] Next, we looked at optimizing the concentration of amino acids in the reaction mix. As the monomeric building blocks of proteins, amino acids are a key CFPS reagent. Besides their central involvement in protein synthesis, some amino acids are also active participants in central metabolic pathways – consequently, CFPS productivity can be impaired by any non-productive consumption of amino acids by metabolic pathways still active in the lysate^{3,61}. We therefore reasoned that due to potential differences in central metabolism, *Vnat* lysates may have different amino acid demands than *E. coli* systems. To test this, we varied the concentrations of all 20 amino acids added to the CFPS reagent mix (Fig. 3C). Increasing the concentration of each amino acid from 2 mM to 3 mM yielded a modest, but significant increase in the productivity of the *Vnat* CFPS system.

[00165] The final reagent examined was phosphoenol pyruvate (PEP). A secondary metabolite used in the latter half of glycolysis, PEP is the primary source of ATP in the PANox-SP energy regeneration system⁵. Similar to amino acids, ATP is utilized by a plethora of cellular processes, and in cell-free systems is consumed to an extent by non-productive side pathways. Thus, as with amino acids, we reasoned that *Vnat* lysates may have different PEP requirements than *E. coli* lysates. To test this, we varied the concentration of PEP added to *Vnat* CFPS reactions (Fig. 3D). We found that doubling the PEP concentration from 33 mM (the optimal concentration previously reported for *E. coli*-based CFPS) to 66 mM yielded a 34% increase in the productivity of the system.

[00166] Cell-free reaction temperature is another key factor that can be optimized, because it affects enzyme activities and protein folding. We therefore next sought to establish the optimal reaction incubation temperature for *Vnat* CFPS. All prior reactions up to this point had been incubated at 30°C as per *E. coli* protocols^{3,54}. However, as *V. natriegens* evolved in an environment with an ambient temperature of only ~23°C, we reasoned that its cellular components may operate more efficiently at lower temperatures. To test this, we incubated *Vnat* CFPS reactions at several temperatures ranging from 16-37°C (Fig. 11). We observed that *Vnat* CFPS reactions are most productive at 30°C, which could also represent the optimum for our reporter protein sfGFP to fold into an active

confirmation. Still, the system only experiences a 10% reduction in productivity when incubated at room temperature (~23°C), and retains >60% productivity at 16°C.

[00167] The final, optimized *Vnat* CFPS platform described here is capable of synthesizing ~1.6 mg/mL of sfGFP in 20-hour batch mode reactions. Figure 4 captures the step wise yield increases achieved per process optimization. Overall, the yield is comparable to state-of-the-art systems derived from *E. coli*, which have been improved over the last two decades^{1,3,6}, and to our knowledge is the highest-yielding CFPS system derived from this relatively understudied non-model organism.

[00168] Assessing the capabilities of *V. natriegens* CFPS. After a systematic optimization of the *V. natriegens*-based CFPS system, we wanted to assess its capabilities. The ability to lyophilize CFPS reactions for storage at room temperature greatly expands the potential user base for a CFPS platform by removing the requirement of storing the materials in freezers at cold temperatures³³⁻³⁵. In *E. coli*-based systems, this flexibility often comes at the expense of productivity, as even in the presence of cryoprotectants the productivity of lyophilized samples generally decreases^{33,62}. To assess the ability of our *Vnat* CFPS platform to support robust protein synthesis even after lyophilization, we freeze-dried fully assembled reactions both with and without cryoprotectant supplementation (Fig. 5A). Samples reconstituted with water immediately following lyophilization performed quite well, experiencing only a small loss of activity likely as a result of the lyophilization process. Addition of 5% trehalose to reactions fully preserves reaction efficacy after a week of room temperature storage, with samples experiencing no detectable loss in productivity after this time. These data suggest freeze-dried strategies developed in other CFPS systems could be applied to our *V. natriegens*-based CFPS system.

[00169] To further demonstrate the ease-of-use of our system and to demonstrate possible applications, we tested CFPS reactions using lysates derived from a strain of *Vnat* recently developed and commercialized by Synthetic Genomics, Inc. that includes a genomic insert encoding the T7 RNA polymerase under the control of an IPTG-inducible promoter (Vmax™ Express)⁴⁷. Such a chassis strain circumvents the need to supply this

polymerase to CFPS reactions in purified form, partially addressing limitations imposed on reaction volume scale-up related to high costs of reaction substrates⁶¹. To test the ability of this strain to compose a one-pot CFPS platform, we prepared lysates from cells in which polymerase expression was induced and performed synthesis reactions both with and without supplementation with purified polymerase (Fig. 5B). This analysis revealed that, while overall productivity of the engineered strain is reduced relative to the wild type strain (~21% reduction), the lysates were enriched with enough T7 polymerase to catalyze more than 1 mg/mL of sfGFP synthesis.

[00170] Next, we aimed to expand the targets of our CFPS reactions beyond our reporter protein (sfGFP). Short peptides (<10 kDa) have emerged as important agents in biological engineering and synthetic biology. These small biomolecules are widely used as protein mimics for interrogating protein-protein interactions and assessing enzyme substrate preferences^{24,63-65}, and bacteria-killing antimicrobial peptides (AMPs) are increasingly being considered for use as next-generation antibiotics as we rapidly approach a post-antibiotic era^{31,66,67}. The use and study of peptides has historically been limited by our ability to synthesize usable amounts of these molecules – solid phase peptide synthesis is generally applicable only to peptides shorter than 30 amino acids⁶⁸, and recombinant expression in bacterial hosts is opposed by the degradation of peptide products by host proteases^{31,67}. We reasoned that protease activity in *Vnat* may be reduced since unwanted proteins could simply be diluted out by rapid cell divisions, which in turn may make this organism well suited for peptide synthesis. We thus set out to see if our *Vnat* CFPS platform could catalyze robust expression of peptides. To test this, *Vnat* CFPS was applied towards the synthesis of the AMPs cecropin A⁶⁹ (SEQ ID NO:1 and 2), cecropin P1³¹ (SEQ ID NO:3 and 4), and opisthporin I³¹ (SEQ ID NO:5 and 6) (Table 1). Opisthporin I expression in particular surpassed 250 µg/mL, suggesting that our *Vnat* CFPS platform might have utility for the recombinant expression of peptides. Indeed, we show that the vibrio system is better at manufacturing peptides than an *E. coli* based cell-free protein synthesis systems (Figure 12).

[00171] Table 1. Antimicrobial peptides used in this study.

Name	DNA Sequence and Peptide Sequence	Source
Cecropin A	(SEQ ID NO:1) atgAAGTGGA AATTGTTTAA AAAGATCGAA AAGGTGGGGC AAAATATCCG CGACGGGATC ATTAAGGCAG GTCCGGCTGT GGCGGTCGTT GGTCAAGCAA CGCAAATCGC AAAATAA (SEQ ID NO:2) MKWKLFKKIE KVGQNIRDGI IKAGPAVAVV GQATQIAK	⁷³
Cecropin P1	(SEQ ID NO:3) atgAGCTGGC TGAGCAAAAC CGCGAAAAAA CTGGAAAACA GCGCGAAAAA ACGCATTAGC GAAGGCATTG CGATTGCGAT TCAGGGCGGC CCGCGCTAA (SEQ ID NO:4) MSWLSKTAKK LENSARKRIS EGIAIAIQGG PR	⁷⁴
Opisthporin I	(SEQ ID NO:5) atgGGCAAAG TGTGGGATTG GATTAAAAGC ACCGCGAAAA AACTGTGGAA CAGCGAACCG GTGAAAGAAC TGAAAAACAC CGCGCTGAAC GCGGCGAAAA ACCTGGTGGC GGAAAAAATT GGCGCGACCC CGAGCTAA (SEQ ID NO:6) MGKVWDWIKS TAKKLWNSEP VKELKNTALN AAKNLVAEKI GATPS	⁷⁴

[00172] Conclusions

[00173] In this study, we present the development of a novel CFPS platform composed of crude lysates derived from the non-model bacterium *V. natriegens* (Fig. 6). We identified optimal culture harvest conditions for this organism, demonstrating that culturing in BHI media supplemented with v2 salts and harvesting at stationary phase is optimal for the generation of productive lysates. This feature is convenient, as it eliminates the need to collect cell pellets within a tightly specific OD₆₀₀ window in order to preserve lysate quality. Researchers can “set and forget” *V. natriegens* cultures and harvest whenever it is convenient without worrying about spoiling the resulting extracts. Attempts to optimize lysis parameters revealed that *V. natriegens* lysates are highly-resistant to damage via overlysis, and overall the system is relatively agnostic to both lysis buffer resuspension volume and lysis energy delivery. Surprisingly, the system is much less sensitive to overlysis during lysate preparation as compared to *E. coli*-based systems, a huge benefit for first-time or inexperienced CFPS users.

[00174] Optimization of the CFPS components specifically for use with *V. natriegens* lysates significantly increased the overall productivity of the system to ~1.6 mg/mL sfGFP, comparable to popular platforms based on lysates derived from *E. coli* and the highest-yielding CFPS system derived from a non-model organism, to our knowledge. This is also higher than a recent study published during the preparation of this manuscript which demonstrated expression titers of 0.4 mg/mL of green fluorescent protein in a *V. natriegens* CFPS system⁷¹. Further, our system is stable at room temperature if lyophilized in the presence of trehalose, is capable of synthesizing small peptide products, and can be carried out in a one-pot system with the use of the Vmax™ Express chassis strain.

[00175] Looking forward, we anticipate that the CFPS system described here will find use in the identification and characterization of *V. natriegens* genetic parts. Recent publications have developed a suite of tools for using *V. natriegens* for cloning and recombinant protein expression^{47,48}, but the knowledge base for this organism still lags behind the more established *E. coli* with regards to regulatory sequences such as promoters, terminators, and ribosome binding sites (RBSs). Due to its open and easily-

accessible nature, the CFPS platform developed here could be used to interrogate many such parts in parallel for rapid characterization. The resulting data could in turn be used to inform construct design for use *in vivo*, supporting the increasing interest in using this organism as an alternative to *E. coli* for molecular biological applications. This idea has already been briefly explored using a *V. natriegens*-based CFPS platform⁷¹.

[00176] Perhaps the most surprising finding to arise from this effort was that the most productive *V. natriegens* extracts are derived from stationary phase cells. This not only contradicts what is usually found in other bacterial CFPS systems, but is also overall difficult to rationalize alongside the generally-accepted notion that ribosomes are downregulated in stationary phase⁵⁵. It is possible that sequestration of ribosomes by native mRNAs in lysates derived from exponential phase cells accounts for the relatively low productivity of these lysates, despite the presence of a larger ribosome pool. In other systems this issue is alleviated by subjecting the lysate to a run-off reaction^{3,52,54,56,57}, but this approach was not successful here.

[00177] Going forward, we expect the efficacy of *V. natriegens* CFPS to improve rapidly. Indeed, the system development and characterization described here accomplished in a very short amount of time what took decades of research and development in *E. coli*. Exploration of the use of alternative energy regeneration systems is one obvious future direction. Indeed, development of an entirely novel energy regeneration system (perhaps using sucrose as the starting substrate⁴⁸) might ultimately be required to fully optimize *V. natriegens* CFPS. Another fruitful direction is in the screening of a larger, more comprehensive library of negative effector knockout strains. This could perhaps be informed by a time course analysis of small molecule concentrations in CFPS reactions, with knockouts targeted towards metabolic pathways that might be siphoning away critical substrates.

[00178] In conclusion, the *Vnat* cell-free platform is excellent for early forays into the use of CFPS systems, as the cells are fast and easy to grow, easy to lyse, and a high volume of active lysate is generated from as little as 1 L of cell culture. Collectively, these features reduce the need for specialized knowledge and equipment that have limited the

use of CFPS. We expect that this reduced entry barrier will facilitate the spread of these systems into new areas for use on exciting, novel applications in synthetic biology.

[00179] Methods

[00180] Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 2. *V. natriegens* was purchased from the American Type Culture Collection (ATCC® 14048TM). VmaxTM Express was purchased from SGI-DNA, a subsidiary of Synthetic Genomics, Inc. pJL1 plasmids encoding the antimicrobial peptides were synthesized and assembled by Twist Bioscience. Assembled plasmids were submitted to the NUSeq Core facility along with forward primers, and sequences were confirmed using traditional Sanger sequencing. Kanamycin (50 µg/mL) was used for maintaining pJL1-based plasmids. Chloramphenicol (34 µg/mL) was used to select for all negative effector knockout mutants.

[00181] Table 2. Strains and plasmids used in this study. Km^R and Cm^R are kanamycin and chloramphenicol resistance, respectively.

Strains and plasmids	Genotype/relevant characteristics	Source
Strains		
BL21 Star TM (DE3)	F ⁻ <i>ompT hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm rne131</i> (DE3)	Life Technologies
<i>V. natriegens</i>	ATCC® 14048 TM	ATCC
Vmax TM Express	Δ <i>Dns</i> *, IPTG-inducible T7 RNAP (Cat. No. CL1100)	Synthetic Genomics, Inc.
Plasmids		
pJL1-sfGFP	Km ^R , <i>P_{T7}</i> , super folder green fluorescent protein (sfGFP), C-terminal strep-tag	89
pJL1-cecropinA	Km ^R , <i>P_{T7}</i> , cecropin A	This study
pJL1-cecropinP1	Km ^R , <i>P_{T7}</i> , cecropin P1	This study
pJL1-opistoporinI	Km ^R , <i>P_{T7}</i> , opistoporin I	This study
pJL1-pyrrhocoricin	Km ^R , <i>P_{T7}</i> , pyrrhocoricin	This study

**Dns* is the conventional name for the *endA* homolog in *Vibrio* species

[00182] Cell culture. *V. natriegens* cells were grown in BHI media supplemented with v2 salts (204 mM NaCl, 4.2 mM KCl, 23.14 mM MgCl₂) unless noted otherwise. For confirmation of *V. natriegens* growth rate, 100 μ L cultures were assembled in a clear 96-well plate (Costar 3370; Corning, Corning, NY) and shaken at 250 RPM at 37°C for 20 hr in a Synergy H1 plate reader (BioTek, Winooski, VT) which continuously monitored the OD₆₀₀ of each sample. To minimize sample evaporation, plates were covered and sealed with Parafilm®. Doubling times were calculated using timepoints corresponding to OD₆₀₀ values between 0.02 and 0.2. For cultures performed at 1 L scale, cells were grown in a 2.5 L Tunair® shake flask and incubated at 37°C at 250 RPM. Except for experiments performed to identify optimal harvest OD₆₀₀, cultures were grown until the onset of stationary phase (an approx. OD₆₀₀ of 6.5-7.5). 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to cultures of Vmax™ Express cells between OD₆₀₀ 0.6-0.8 to induce expression of T7 RNA polymerase. In all cases, cells were pelleted by centrifuging for 15 min at 5000 $\times$ g at 4°C, washed three times with cold S30 buffer (10 mM tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 2 mM dithiothreitol)⁷², and stored at -80°C until lysed.

[00183] Extract preparation. Unless otherwise noted, cell pellets were thawed and suspended in 0.8 mL of S30 buffer per gram of wet cell mass. Prior to the optimization of lysis parameters, cell pellets were instead resuspended in 1.0 mL buffer per gram of cells. Following suspension, 1.4 mL of cell slurry was transferred into 1.5 mL microtubes. The cells were lysed using a Q125 Sonicator (Qsonica, Newtown, CT) with 3.175 mm diameter probe at a 20 kHz frequency and 50% amplitude. Sonication was continued for three cycles of 45s ON/59s OFF unless stated otherwise. To minimize heat damage during sonication, samples were placed in an ice-water bath. For each 1.4 mL sample, the input energy was ~270 Joules/sonication cycle. The lysate was then centrifuged at 12,000 $\times$ g at 4°C for 10 min. The supernatant was flash-frozen using liquid nitrogen and stored at -80°C until use. For preparations including a runoff reaction, following the first clarifying spin supernatant was transferred to a new tube and subjected to a 1 hr incubation at 30°C or

37°C, either stationary or with shaking at 250 RPM. Following this incubation, samples were centrifuged at 10,000 x g at 4°C for 10 min after which supernatant was flash-frozen and stored at -80°C until use.

[00184] CFPS reaction. A modified PANOx-SP system was utilized for CFPS reactions. Briefly, a 15 µL CFPS reaction in a 2.0 mL microtube was prepared by mixing the following components: 1.2 mM ATP; 0.85 mM each of GTP, UTP, and CTP; 34 µg/mL folinic acid; 170 µg/mL of *E. coli* tRNA mixture; 13.3 µg/mL plasmid; 16 µg/mL T7 RNA polymerase; 3 mM for each of the 20 standard amino acids; 0.33 mM nicotinamide adenine dinucleotide (NAD); 0.27 mM coenzyme-A (CoA); 1.5 mM spermidine; 1 mM putrescine; 4 mM sodium oxalate; 290 mM potassium glutamate; 10 mM ammonium glutamate; 6 mM magnesium glutamate; 57 mM HEPES, pH 7.2; 67 mM phosphoenolpyruvate (PEP), and 4 µL (27% v/v) of cell extract. Each CFPS reaction was incubated for 20 hr at 30°C unless noted otherwise. Experiments performed prior to determining an optimal reagent mix for *V. natriegens* lysates used the above mix with the following changes: 2 mM for each of the 20 standard amino acids, 130 mM potassium glutamate, 10 mM magnesium glutamate, and 33 mM PEP were used instead. As individual reagent concentrations were optimized, their optimal value listed above were used for all reactions from that point onward. *E. coli* total tRNA mixture (from strain MRE600) and phosphoenolpyruvate was purchased from Roche Applied Science (Indianapolis, IN). ATP, GTP, CTP, UTP, 20 amino acids and other materials were purchased from Sigma (St. Louis, MO) without further purification. T7RNAP was purified in house as described previously³. To direct synthesis of a specific product, 200 ng of pJL1 template plasmid encoding the product was added to each reaction.

[00185] Quantification of active sfGFP. CFPS reactions were diluted 1:25 in nanopure water and active full-length sfGFP protein yields were quantified by measuring fluorescence using a Synergy 2 plate reader (BioTek, Winooski, VT) with excitation at 485 nm, emission at 528 nm, and cut-off at 510 nm in 96-well half area black plates (Costar 3694; Corning, Corning, NY). sfGFP fluorescence units were converted to concentration using a standard curve established with ¹⁴C-Leu quantified sfGFP as described previously⁵⁴.

[00186] CFPS Lyophilization. Samples were assembled in 2 mL microtubes and lyophilized overnight using a VirTis BenchTop Pro Freeze Dryer (SP Scientific, Warminster, PA). Lyophilized samples were stored at room temperature under vacuum in a dessicator with Drierite dessicant. For reconstitution of fully-assembled reactions, template plasmid DNA plus nuclease-free water were added to each sample. Samples consisting of only lyophilized lysate were reconstituted with the complete CFPS reagent mix. Where indicated, lyophilized reactions were supplemented with 2.5% (m/v) trehalose.

[00187] Quantification of antimicrobial peptide yield in CFPS. Radioactive ^{14}C -Leucine was added into 15 μL CFPS reactions to a final concentration of 25 μM . After incubation, yields were quantified by determining radioactive ^{14}C -Leu incorporation into peptides precipitated in 15% (m/v) trichloroacetic acid (TCA)⁷². Radioactivity of TCA-precipitated samples was measured using liquid scintillation counting (MicroBeta2, PerkinElmer, Waltham, MA).

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[00278] In the foregoing description, it will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention. Thus, it should be understood that although the present invention has been illustrated by specific embodiments and optional features, modification and/or variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00279] All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[00280] Citations to a number of patent and non-patent references are made herein. The cited references are incorporated by reference herein in their entireties. In the event that there is an inconsistency between a definition of a term in the specification as compared to a definition of the term in a cited reference, the term should be interpreted based on the definition in the specification.

CLAIMS

We claim:

1. A platform for *in vitro* transcription of mRNA and/or translation of peptides, polypeptides, or sequence defined polymers, the platform comprising a cellular extract prepared from a cell culture of a species of *Vibrio*.
2. The platform of claim 1, wherein the species of *Vibrio* is *Vibrio natriegens*.
3. The platform of claim 1, wherein the species of *Vibrio* is engineered to be deficient in a negative effector for cell-free protein synthesis (CFPS).
4. The platform of claim 3, wherein the negative effector for CFPS is a selected from the group consisting of a homolog of *E. coli* endA, mazF, rna, rnb, rne, gor, lon, ompT, gdhA, gshA, sdaA, sdaB, speA, WaaL, tnaA, glpK, and any combination thereof.
5. The platform of claim 1, wherein the species of *Vibrio* is engineered to express an upregulated gene product that is a positive effector for CFPS.
6. The platform of claim 5, wherein the positive effector for CFPS is selected from the group consisting of a homolog of *E. coli* ackA, ndk, pykF, cdd, dsbC, dnaK, dnaJ, crpE, tig, groS, groL, infA, infB, fusA, efp, lepA, tufB, hslR, ffr, and any combination thereof.
7. The platform of claim 1, wherein the species of *Vibrio* is engineered to be deficient in a release factor 1.
8. The platform of claim 1, wherein the species of *Vibrio* has been genomically-recoded to replace one or more stop codons with a different codon.
9. The platform of claim 1, wherein the species of *Vibrio* has been engineered to express T7 RNA polymerase.

10. The platform of claim 1, wherein the *Vibrio* cellular extract is prepared from cell cultures that are in stationary phase, optionally wherein stationary phase is defined as the cell culture having an OD₆₀₀ of greater than about 3.0.

11. The platform of claim 1, wherein the cellular extract comprises an S12 and/or S30 fraction of the cell culture.

12. The platform of claim 1, further comprising one or more components selected from the group consisting of amino acids which optionally may include non-canonical amino acids, NTPs, salts, cofactors, an energy source and optionally an energy source comprising a phosphate group (such as phosphoenol pyruvate (PEP)) or non-phosphorylated energy group (*e.g.*, glucose, pyruvate), a translation template, a transcription template, and any combination thereof.

13. The platform of claim 1, further comprising an energy source and optionally an energy source comprising a phosphate group (such as phosphoenol pyruvate (PEP)), wherein the energy source is present at a concentration of greater than about 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, or 90 mM (preferably greater than about 67 mM), but less than about 100 mM, or within a concentration range bounded by of these values.

14. The platform of claim 1, further comprising a source of potassium (K⁺), wherein the platform comprises potassium at a concentration greater than about 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, or 450 mM (preferably about 300 mM), but less than about 500 mM, or within a concentration range bounded by of these values; and/or further comprising a source of magnesium (Mg⁺), wherein the platform comprises magnesium at a concentration greater than about 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 8mM, 12 mM, 16 mM or 20mM (preferably about 8 mM), but less than about 30 mM, or within a concentration range bounded by of these values.

15. The platform of claim 1, wherein the platform or one or more components of the platform are preserved such as through freeze-drying.

16. A method for *in vitro* transcription of mRNA and/or translation of a peptide, a polypeptide, or a sequence defined polymer, the method comprising transcribing a transcription templated encoding the mRNA and/or translating *in vitro* an mRNA encoding the sequence defined polymer, polypeptide, or peptide in the platform of any of the foregoing claims.

17. The method of claim 16, wherein the method comprises transcribing a DNA template in the platform to provide the translated mRNA.

18. The method of claim 16, wherein the method is performed at a temperature between about 20-40 °C.

19. The method of claim 16, wherein the sequence defined biopolymer or polypeptide is a therapeutic protein and/or the method is utilized to identify therapeutic proteins or biomaterials.

20. The method of claim 16, wherein the peptide has an amino acid sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, or 80 amino acids, but less than about 100 amino acids, or within a range bounded by of these values.

21. A kit comprising as components: (a) a cellular extract prepared from a cell culture of a species of *Vibrio*; and (b) a reaction mixture for translating an mRNA, optionally wherein the species of *Vibrio* is *Vibrio natriegens*.

22. The kit of claim 20, wherein the reaction mixture comprises one or more components selected from the group consisting of amino acids which optionally may include non-canonical amino acids, NTPs, salts, cofactors, an energy source and optionally an energy source comprising a phosphate group (such as phosphoenol pyruvate (PEP)).

23. A recombinant species of *Vibrio*, optionally *Vibrio natriegens*, wherein the species of *Vibrio* is engineered to be deficient in a negative effector for cell-free protein synthesis (CFPS).

24. The recombinant species of *Vibrio* of claim 23, wherein the negative effector for CFPS is selected from the group consisting of a homolog of *E.coli* endA, mazF, rna, rnb, rne, gor, lon, ompT, gdhA, gshA, sdaA, sdaB, speA, WaaL, tnaA, glpK, and any combination thereof.

25. The recombinant species of *Vibrio* of claim 23, wherein the species of *Vibrio* is engineered to express an upregulated gene product that is a positive effector for CFPS.

26. The recombinant species of *Vibrio* claim 25, wherein the positive effector for CFPS is selected from the group consisting of a homolog of *E. coli* ackA, ndk, pykF, cdd, dsbC, dnaK, dnaJ, crpE, tig, groS, groL, infA, infB, fusA, efp, lepA, tufB, hslR, ffr, and any combination thereof.

27. The recombinant species of *Vibrio* of claim 23, wherein the species of *Vibrio* is engineered to be deficient in a release factor 1.

28. The recombinant species of *Vibrio* of claim 23, wherein the species of *Vibrio* has been genomically-recoded to replace one or more stop codons with a different codon.

29. The recombinant species of *Vibrio* of claim 23, wherein the species of *Vibrio* has been engineered to express T7 RNA polymerase.

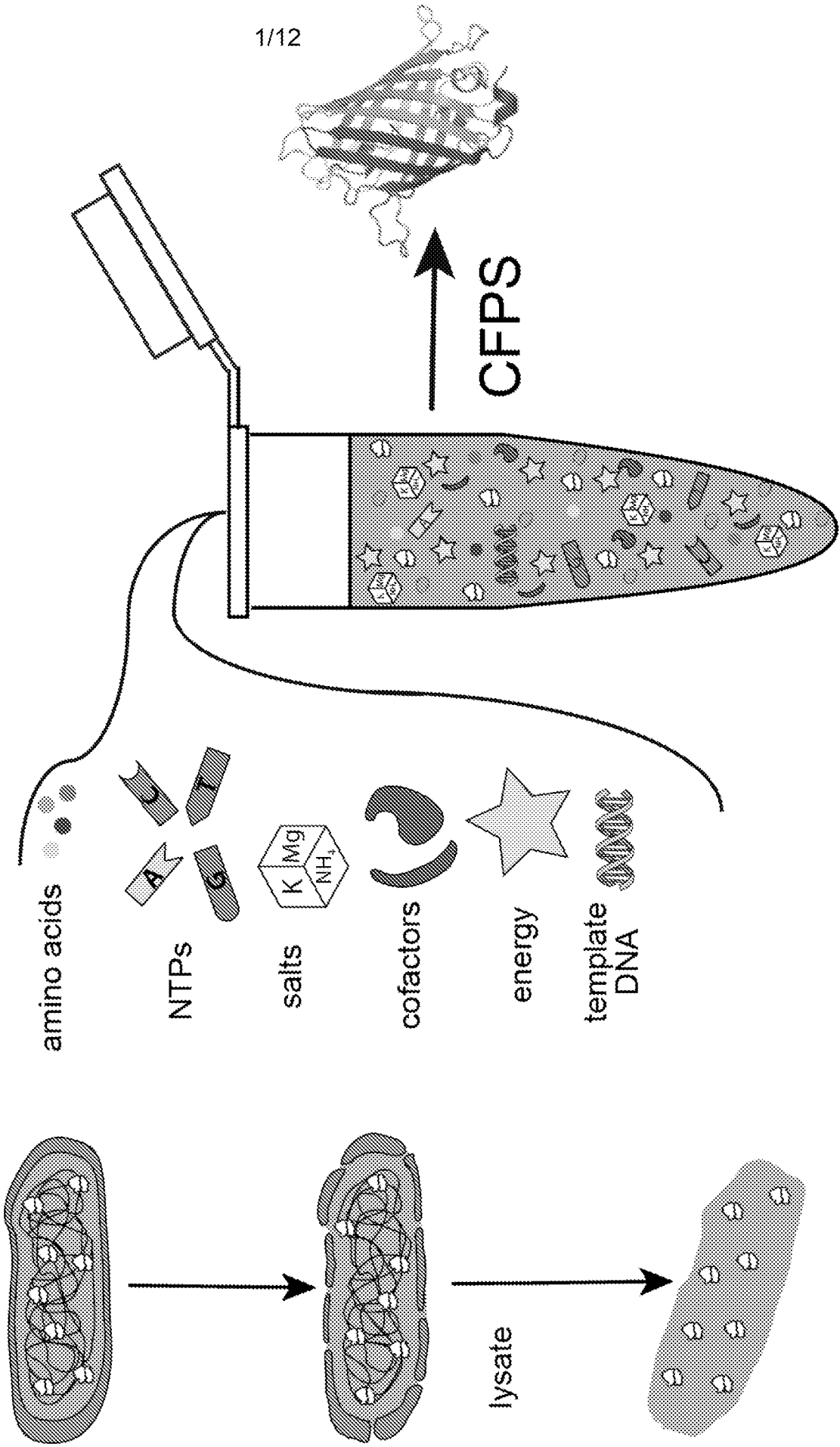


FIG. 1

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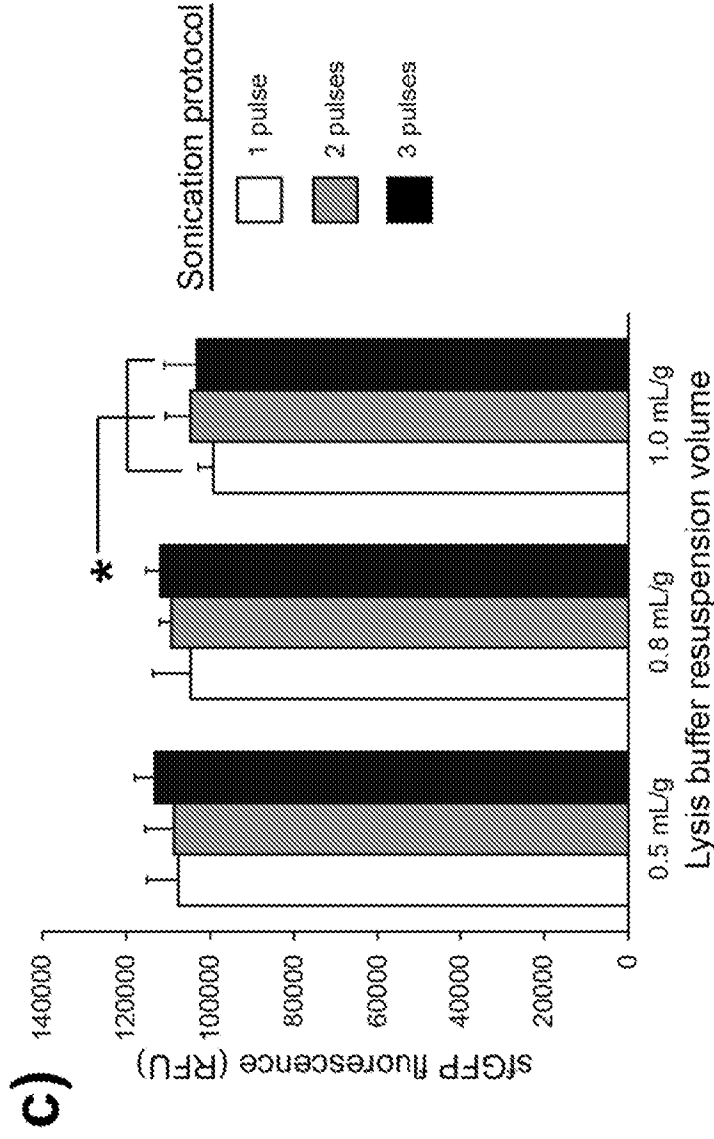
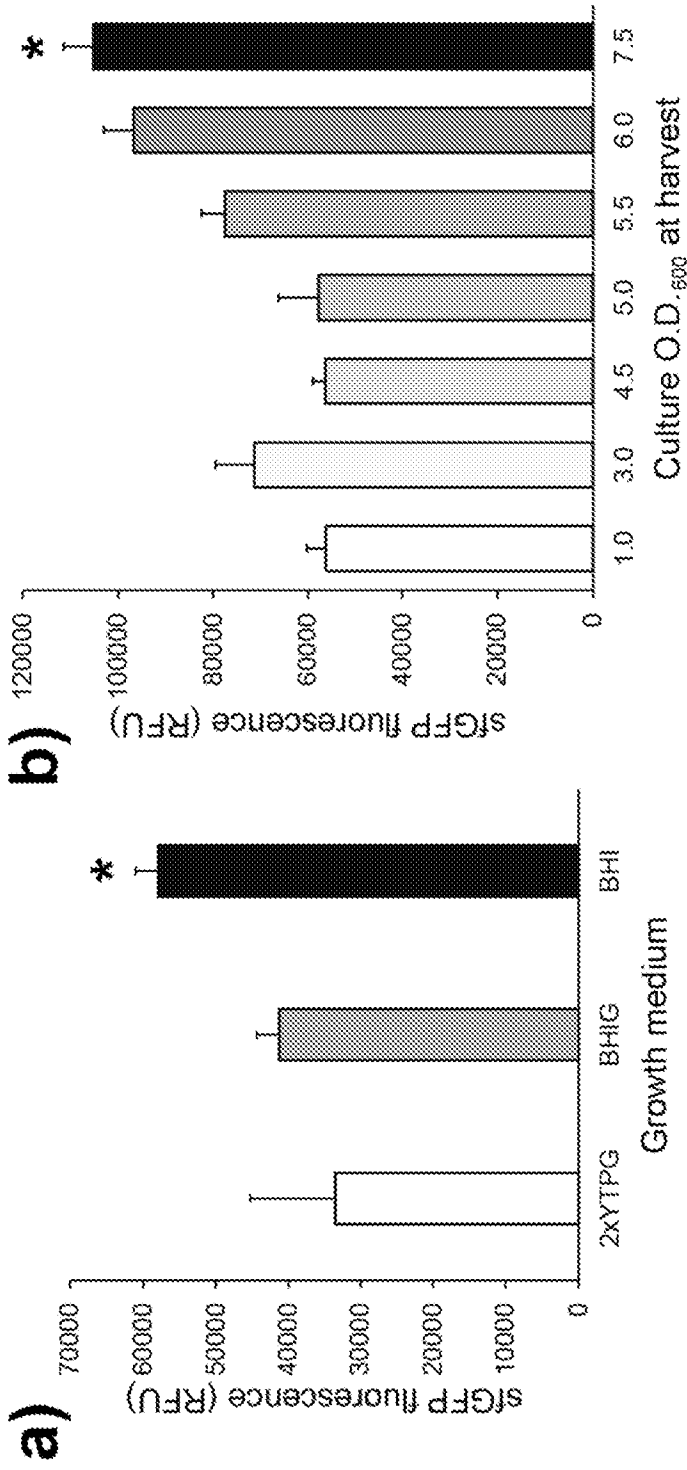


FIG. 2

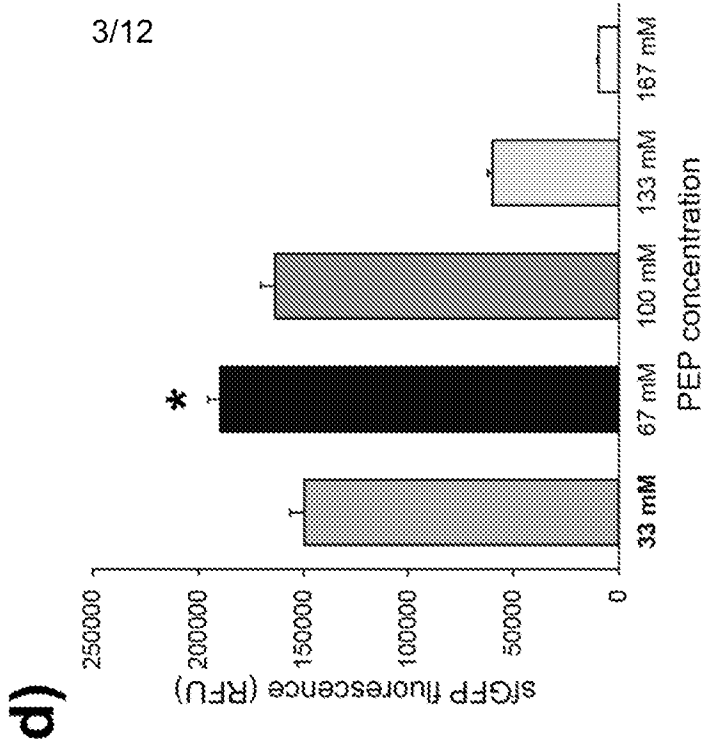
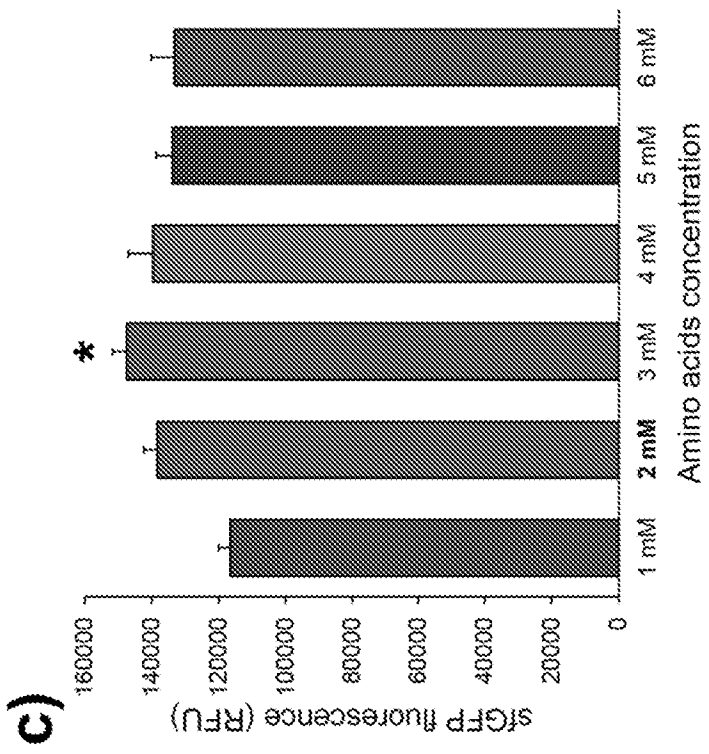
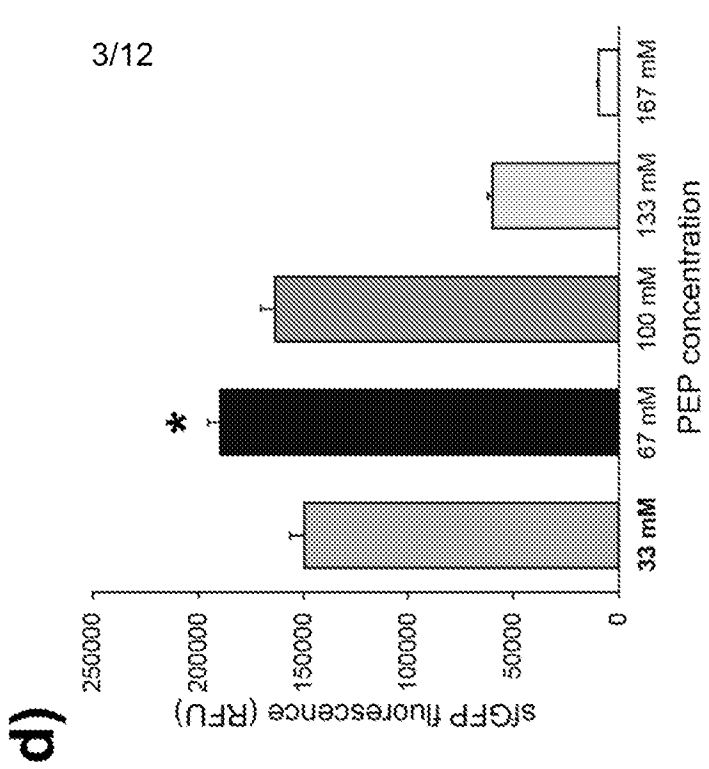
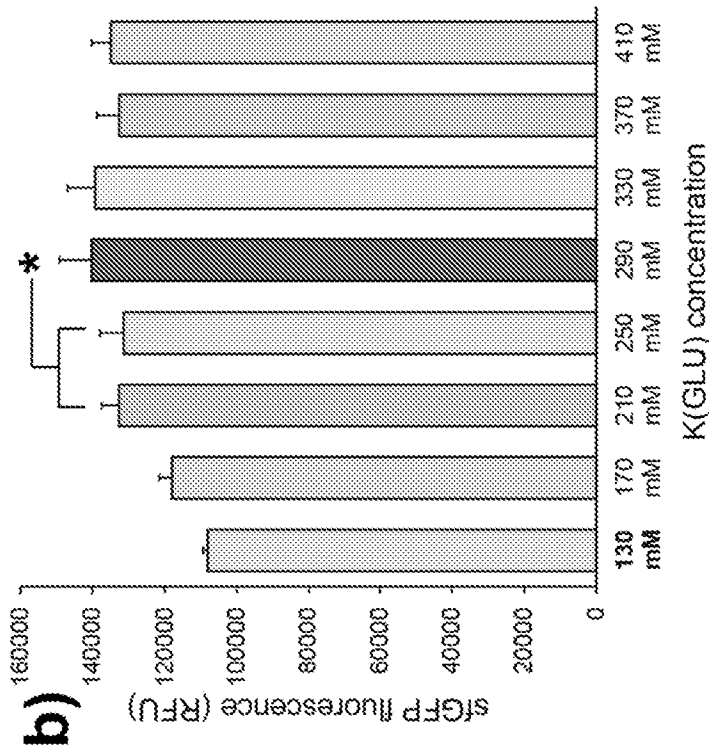


FIG. 3

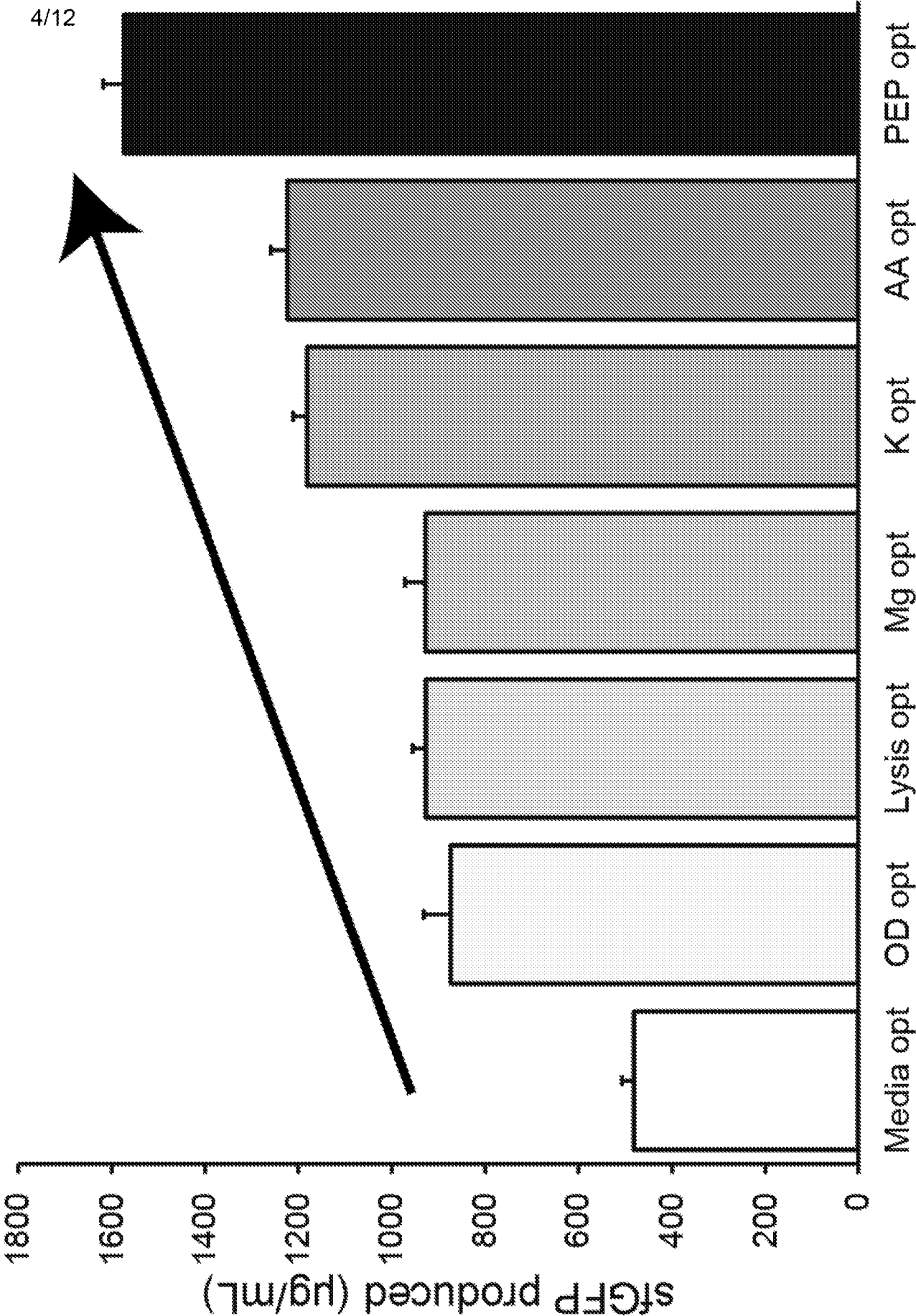


FIG. 4

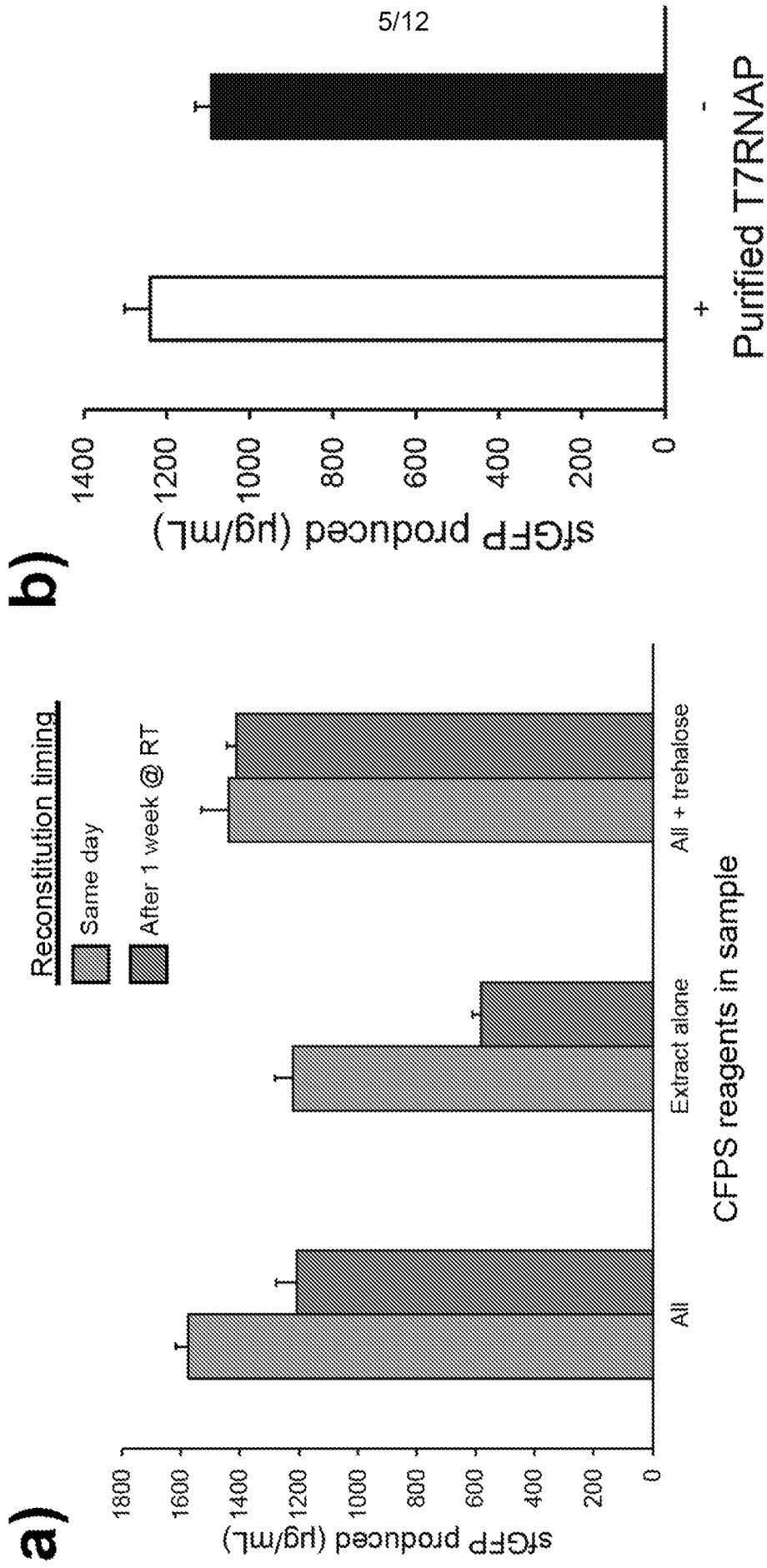


FIG. 5

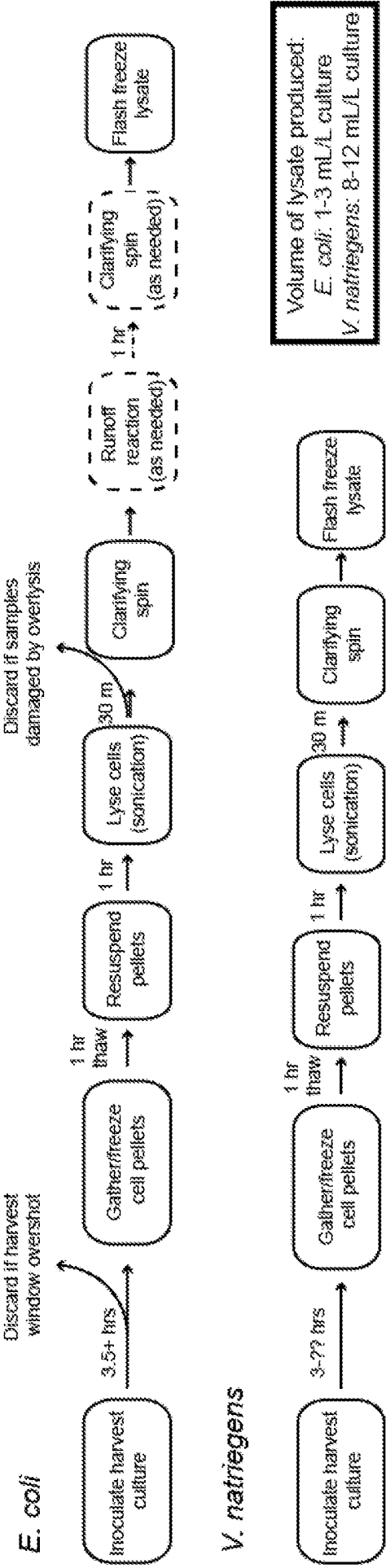


FIG. 6

a)

Growth media		+ v2 salts	- v2 salts
LB		14.1	no growth
BHI		12.4	no growth
2xYTPG		14.5	25.6

b)

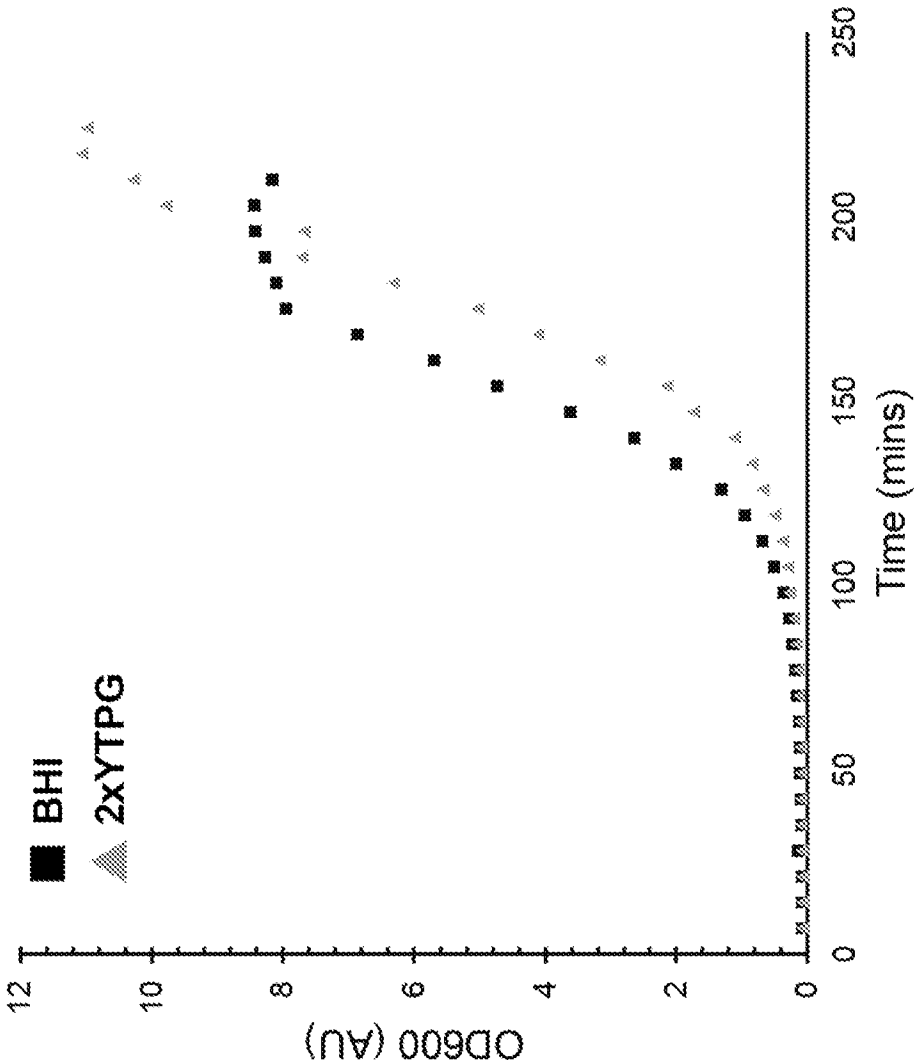


FIG. 7

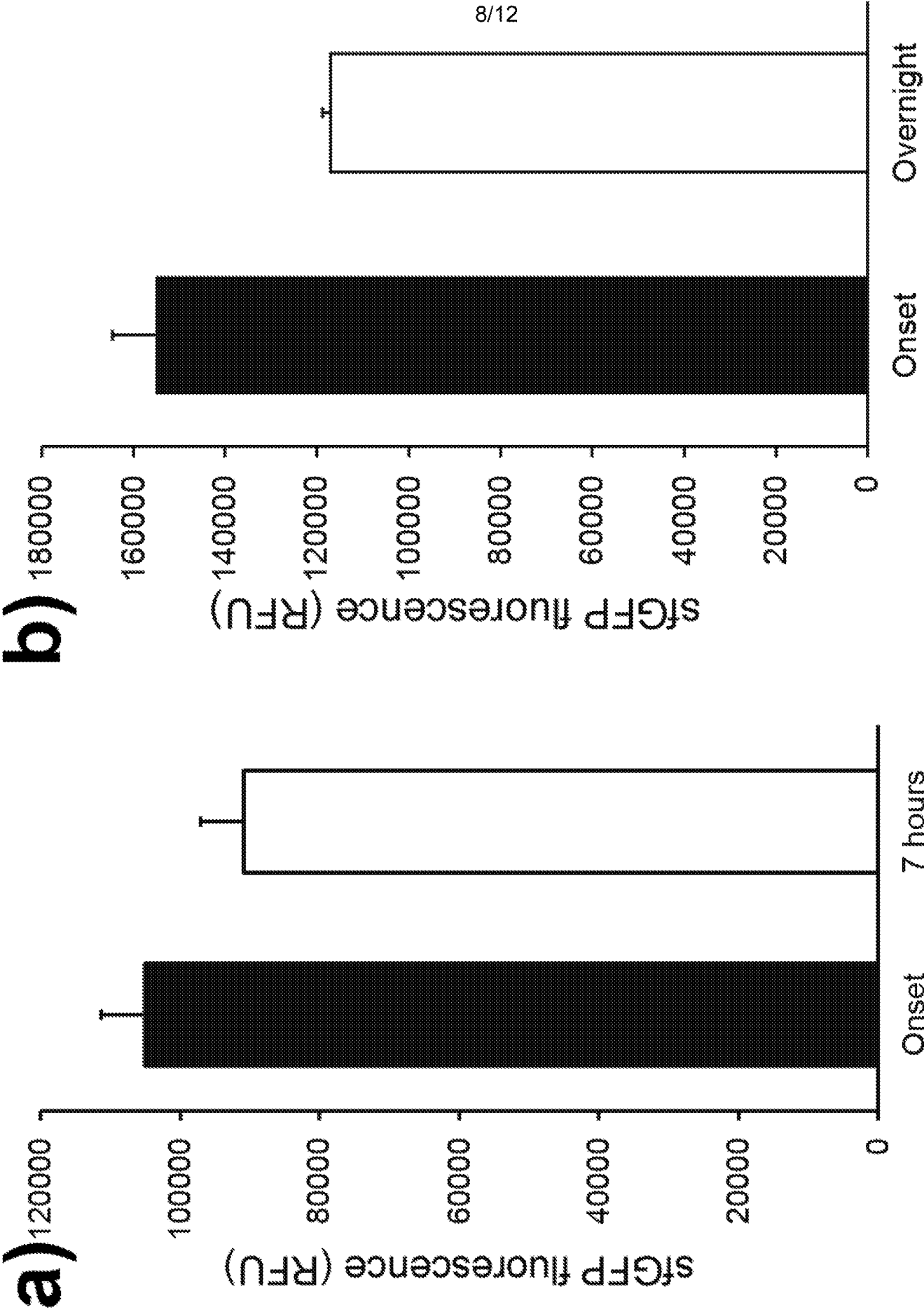


FIG. 8

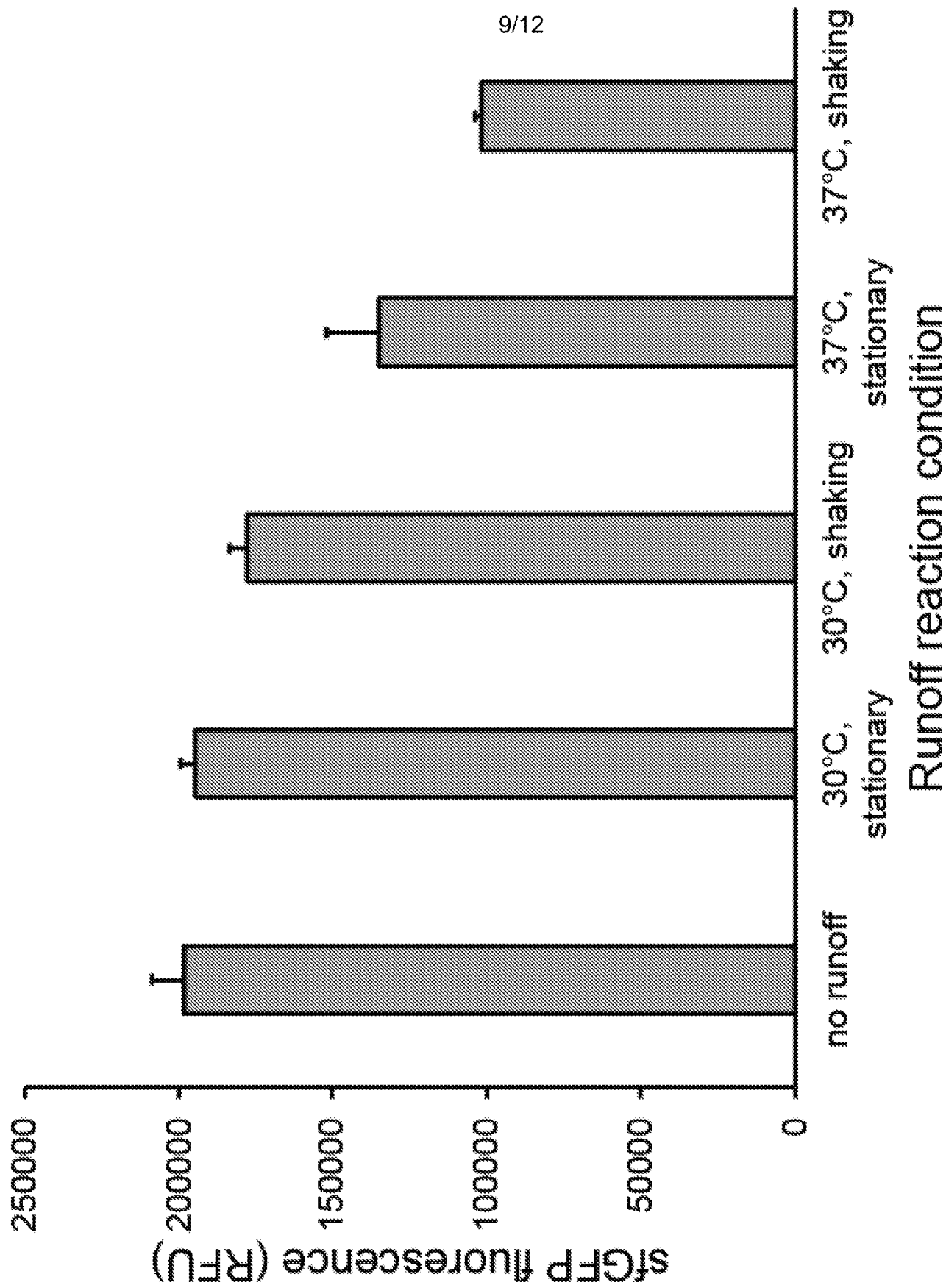


FIG. 9

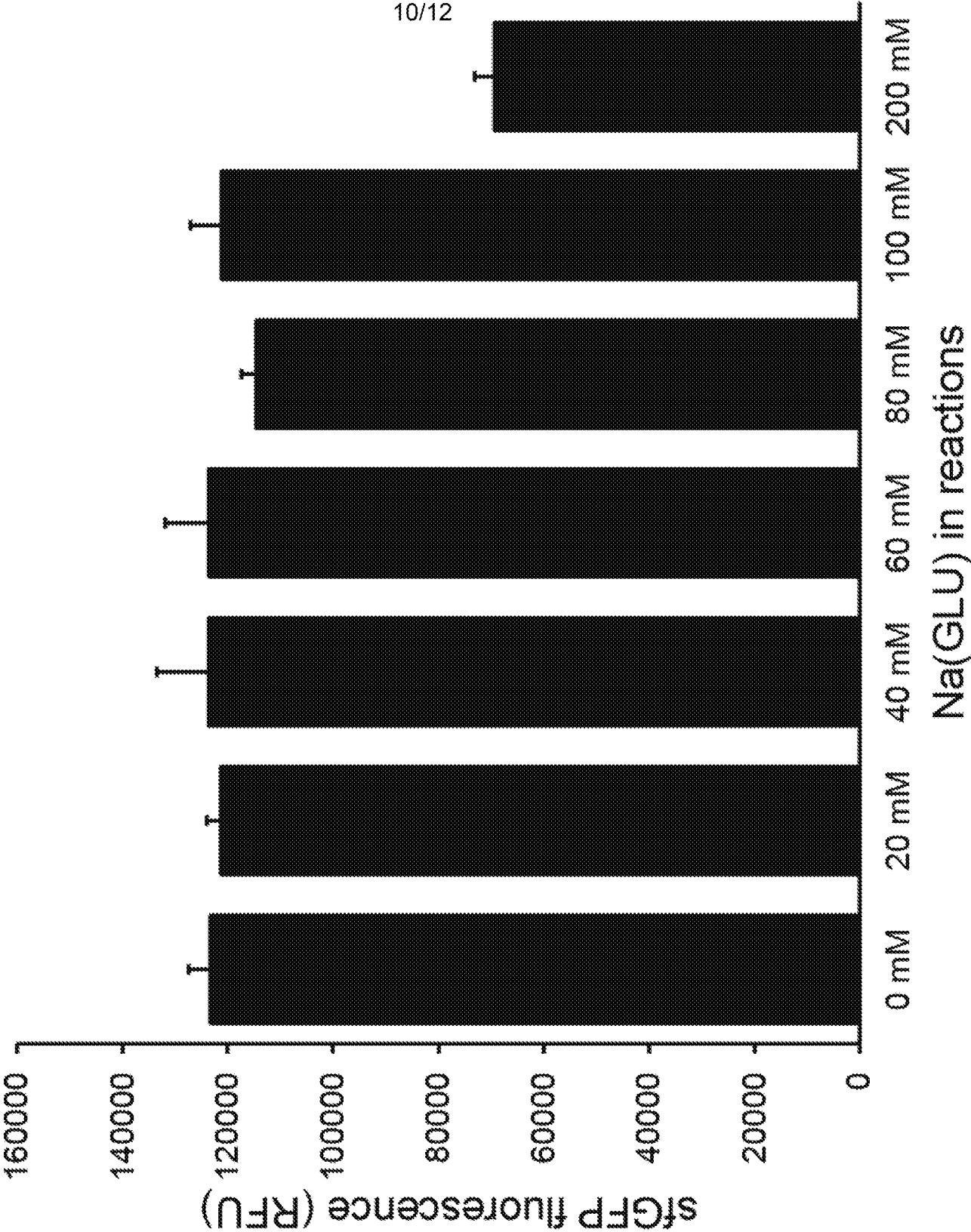


FIG. 10

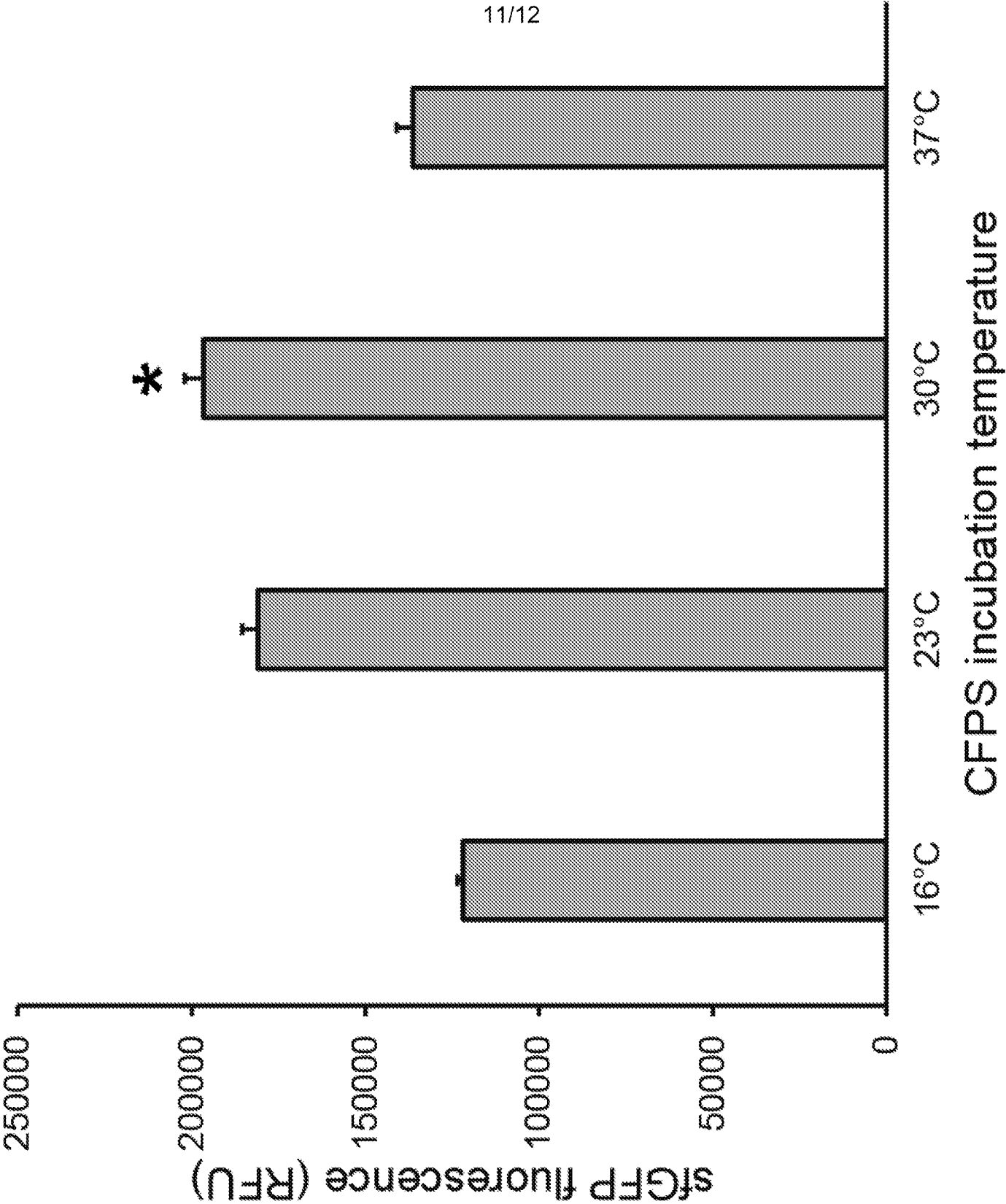


FIG. 11

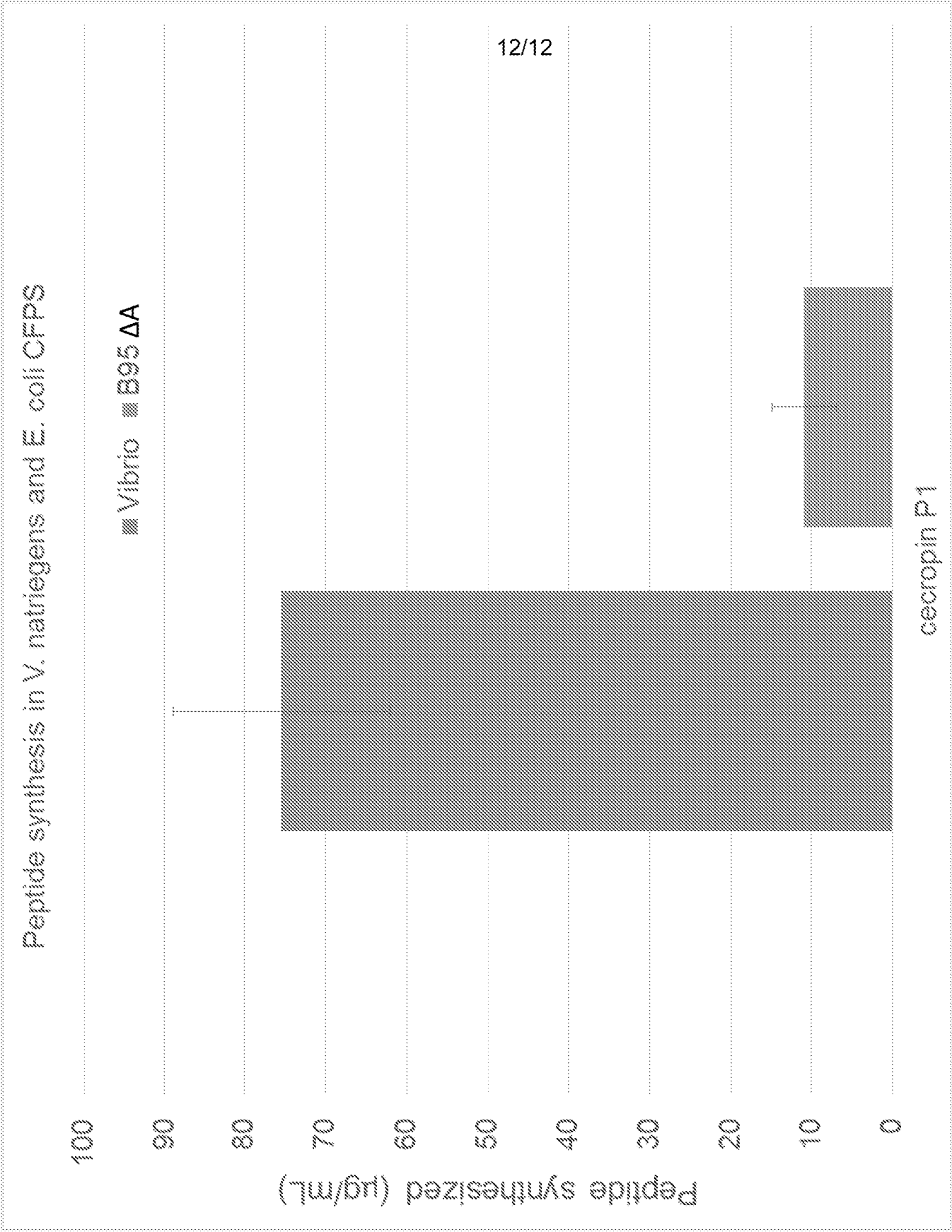


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2018/060279

A. CLASSIFICATION OF SUBJECT MATTER <i>C12P 21/02 (2006.01)</i> <i>C12N 1/21 (2006.01)</i> <i>C12N 1/38 (2006.01)</i> <i>C12Q 1/6869 (2018.01)</i> <i>C12Q 1/6874 (2018.01)</i> <i>C12R 1/63 (2006.01)</i> According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12P 21/02, C12N 1/21, 1/38, C12Q 1/6869, 1/6874, C12R 1/63 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatSearch (RUPTO Internal), USPTO, PAJ, Espacenet, Information Retrieval System of FIPS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0060301 A1 (NORTHWESTERN UNIVERSITY) 03.03.2016, claims, abstract, paragraphs [0006], [0095], [0102] - [0107], [0128] - [0129], [0220], examples 1-4	1-29
Y	WO 2016/196940 A1 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 08.12.2016, paragraphs "Summary of the invention", example 1	1-29
Y	WO 2017/172994 A1 (GELTOR, INC.) 05.10.2017, claims	1-29
Y	US 2017/0292139 A1 (B.G. NEGEV TECHNOLOGIES AND APPLICATIONS LTD. et al.) 12.10.2017, paragraph [0136]	15
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 07 February 2019 (07.02.2019)		Date of mailing of the international search report 28 February 2019 (28.02.2019)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhtkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37		Authorized officer I. Goretova Telephone No. 8(495) 531-64-81