

## PATENT COOPERATION TREATY

From the  
INTERNATIONAL SEARCHING AUTHORITY

To: FANG XIE  
GREENBERG TRAURIG LLP  
ONE INTERNATIONAL PLACE  
SUITE 2000  
BOSTON, MA 02110

# PCT

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing  
(day/month/year)

07 DEC 2018

Applicant's or agent's file reference  
165687010601

**FOR FURTHER ACTION**

See paragraph 2 below

International application No.

PCT/US2018/046477

International filing date (day/month/year)

13 August 2018

Priority date (day/month/year)

11 August 2017

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

IPC(8) - C07K 14/195; C12N 9/12; C12P 21/00 (2018.01)

CPC - C07K 14/195; C12N 1/06; C12N 9/1247; C12P 21/02 (2018.08)

Applicant SYNVTROBIO, INC.

1. This opinion contains indications relating to the following items:

- Box No. I Basis of the opinion
- Box No. II Priority
- Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- Box No. IV Lack of unity of invention
- Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- Box No. VI Certain documents cited
- Box No. VII Certain defects in the international application
- Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US  
Mail Stop PCT, Attn: ISA/US  
Commissioner for Patents  
P.O. Box 1450, Alexandria, VA 22313-1450  
Facsimile No. 571-273-8300

Date of completion of this opinion

30 October 2018

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300  
PCT OSP: 571-272-7774

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2018/046477

## Box No. 1 Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

- the international application in the language in which it was filed.  
 a translation of the international application into \_\_\_\_\_ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2.  This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3.  With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

- a.  forming part of the international application as filed:  
 in the form of an Annex C/ST.25 text file.  
 on paper or in the form of an image file.
- b.  furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c.  furnished subsequent to the international filing date for the purposes of international search only:  
 in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).  
 on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

4.  In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

ISA/225 mailed on 27 August 2018. No approved electronic sequence listing was submitted in response to the ISA/225.

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2018/046477

**Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

the entire international application.

claims Nos. 15, 16

because:

the said international application, or the said claims Nos. \_\_\_\_\_ relate to the following subject matter which does not require an international search (*specify*):

the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 15, 16 are so unclear that no meaningful opinion could be formed (*specify*):

Claims 15 and 16 are multiple dependent claims not drafted in accordance with the second and third sentences of Rule 6.4(a).

the claims, or said claims Nos. \_\_\_\_\_ are so inadequately supported by the description that no meaningful opinion could be formed (*specify*):

no international search report has been established for said claims Nos. 15, 16

a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

See Supplemental Box for further details.

**WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2018/046477

**Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

## 1. Statement

Novelty (N)	Claims	6-14	YES
	Claims	1-5	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-14	NO
Industrial applicability (IA)	Claims	1-14	YES
	Claims	None	NO

## 2. Citations and explanations:

Claims 1-5 lack novelty under PCT Article 33(2) as being anticipated by Sun et al. (hereinafter Sun).

Regarding Claim 1, Sun discloses a composition for in vitro gene expression (We describe the preparation and execution of an efficient endogenous E. coli based transcription-translation (TX-TL) cell-free expression system that can produce equivalent amounts of protein as T7-based systems at a 98% cost reduction to similar commercial systems, Abstract), comprising: a treated cell lysate derived from one or more host cells (A basic TX-TL reaction has 3 parts (tubes): crude cell extract, buffer, and DNA, Pg. 5, 5. Experimental Execution of a TX-TL Reaction, first paragraph; It utilizes Mg- and K- glutamate over Mg- and K- acetate for increased efficiency, removes 2-mercaptoethanol, and lyses cells using a beadbeater.... Bead-beating is chosen over homogenization, pressure-based methods, or sonication due to its lower cost and comparable yields to competing systems, Pg. 2, first paragraph; The protocol presented here is optimized for a BL21-Rosetta2 strain, but is generalizable to other E. coli strains, Pg. 12, final paragraph); a plurality of supplements for gene transcription and translation (Buffer Preparation requires the completion of Crude Cell Extract Preparation, Amino Acid Solution Preparation, and Energy Solution Preparation. Each buffer is unique to a batch of crude cell extract.... Thaw on ice 100 mM Mg-glutamate (4 °C), 3 M K-glutamate (4 °C), 6 mM Amino Acid Solution (26 µl, -80 °C), Energy Solution (7 µl, -80°C), 100 mM DTT (-20 °C), positive control DNA (-20 °C), 40% PEG-8000 (4 °C), crude cell extract (-80 °C), and water (4 °C), Pg. 4, 4. Buffer Preparation, first paragraph and step 2); an energy recycling system for providing and recycling adenosine triphosphate (ATP) (3-phosphoglyceric acid (3-PGA) is used as the energy source as it was found to give superior protein yields when compared to creatine phosphate and phosphoenolpyruvate, Pg. 2, first paragraph); and one or more exogenous additives selected from the group consisting of polar aprotic solvents, quaternary ammonium salts, sulfones, ectoines, glycols, amides, amines, sugar polymers, sugar alcohols, slow elongation-rate RNA polymerase (RNAP) and ribosomes (Final reaction conditions are:... 2% PEG-8000, Pg. 5, 5. Experimental Execution of a TX-TL Reaction, first paragraph).

Regarding Claim 2, Sun discloses the composition of claim 1, for use in expressing a metagenomically derived gene, a plurality of genes that together constitute a pathway, and/or synthetic proteins (Without limitations of cell division and metabolism, variability in synthetic circuits such as the repressilator or in metabolic engineered pathways such as those producing artemisinin can be reduced or better understood.... We have used these advantages to implement genetic switches, as well as to understand sigma factor sequestration.... Such technology can also form the backbone of "minimal" or "artificial" cells - small, well-characterized and self-sufficient embodied units of extract, Pg. 13, first paragraph).

Regarding Claim 3, Sun discloses the composition of claim 2, wherein the gene or pathway has not been optimized for in vitro gene expression (The protocol presented here is optimized for a BL21-Rosetta2 strain, but is generalizable to other E. coli strains.... Cell-free expression systems utilizing both endogenous and exogenous transcription-translation machinery and regulation mechanisms have wide applications in both protein and metabolite expression and in synthetic biology.... Instead of being limited to T7-regulated circuits, one can envision producing complex biomolecules in a user-controllable setting using a mix of native E. coli promoters and exogenously supplied transcription and regulation mechanisms, Pg. 12, final paragraph - Pg. 13, first paragraph).

Regarding Claim 4, Sun discloses the composition of claim 1, wherein the plurality of supplements comprise magnesium and potassium salts, ribonucleotides, amino acids, a starting energy substrate, and a pH buffer (Buffer Preparation requires the completion of Crude Cell Extract Preparation, Amino Acid Solution Preparation, and Energy Solution Preparation. Each buffer is unique to a batch of crude cell extract.... Thaw on ice 100 mM Mg-glutamate (4 °C), 3 M K-glutamate (4 °C), 6 mM Amino Acid Solution (26 µl, -80 °C), Energy Solution (7 µl, -80°C), 100 mM DTT (-20 °C), positive control DNA (-20 °C), 40% PEG-8000 (4 °C), crude cell extract (-80 °C), and water (4 °C), Pg. 4, 4. Buffer Preparation, first paragraph and step 2).

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2018/046477

**Box No. VIII Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-14 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 1-14 are indefinite for the following reason(s):

Claim 1 contains exemplary claim language (such as; preferably), which is not given any consideration. For the purpose of this Written Opinion, claim 1 has been interpreted without the exemplary claim language and to read as:

1. A composition for in vitro gene expression, comprising: a treated cell lysate derived from one or more host cells; a plurality of supplements for gene transcription and translation;  
an energy recycling system for providing and recycling adenosine triphosphate (ATP);  
and one or more exogenous additives selected from the group consisting of polar aprotic solvents, quaternary ammonium salts, sulfones, ectoines, glycols, amides, amines, sugar polymers, sugar alcohols, slow elongation-rate RNA polymerase (RNAP) and ribosomes.

Claim 6 contains exemplary claim language (such as), which is not given any consideration. For the purpose of this Written Opinion, claim 6 has been interpreted without the exemplary claim language and to read as:

6. The composition of claim 1, wherein the slow elongation-rate RNAP is homologous to the host cells.

Claim 7 contains exemplary claim language (such as), which is not given any consideration. For the purpose of this Written Opinion, claim 7 has been interpreted without the exemplary claim language and to read as:

7. The composition of claim 1, wherein the slow elongation-rate RNAP is heterologous to the host cells.

Claim 9 contains exemplary claim language (such as), which is not given any consideration. For the purpose of this Written Opinion, claim 9 has been interpreted without the exemplary claim language and to read as:

9. The composition of claim 1, wherein the slow elongation-rate RNAP is a synthetic RNAP.

Claim 13 is objected to as lacking clarity under PCT Rule 66.2(a)(v). Claim 13 contains exemplary claim language (preferably), which is not given any consideration. For the purpose of this Written Opinion, claim 13 has been interpreted without the exemplary claim language and to read as:

13. The composition of claim 1, wherein the ribosomes are sourced from the host cells, or from an organism different than the host cells.

Claim 14 contains exemplary claim language (preferably), which is not given any consideration. For the purpose of this Written Opinion, claim 14 has been interpreted without the exemplary claim language and to read as:

14. The composition of any one of claims 1-13, wherein the composition comprises both slow elongation-rate RNAP and exogenous ribosomes.

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2018/046477

## Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 5, Sun discloses the composition of claim 1.

Further, in regards to the limitation relating to "the one or more additives modulate nucleic acid secondary structure, improve RNAP processivity and/or stability, affect RNAP elongation rate, improve ribosome synergy with RNAP and/or stability, and/or improve stability of polypeptide being synthesized", a recitation of the intended use of the claimed invention must result in a structural difference between the claimed invention and the prior art in order to distinguish the claimed invention from the prior art. If the prior art is capable of performing the intended use, it meets the limitation for modulating nucleic acid secondary structure, improving RNAP processivity and/or stability, affecting RNAP elongation rate, improving ribosome synergy with RNAP and/or stability, and/or improving stability of polypeptide being synthesized. Specifically, the composition in Sun discloses PEG, and PEG improves RNA Polymerase processivity (see, e.g., "Cell-Free Protein Expression under Macromolecular Crowding Conditions" to Ge et al., Alternatively, the macromolecular crowding environments can be created experimentally by adding inert macromolecule such as PEG and Ficoll.... Both PEG and Ficoll display excellent biocompatibility and are attractive polymers to mimic those macromolecules present in living cells [...]. In the present study we compared the effects of PEG and two Ficoll polymers on in vitro transcription and translation using coupled and two step CFPE in order to find out which of these gave the best protein yields.... For the in vitro transcription, the enhanced association of T7 RNA polymerase (T7 RNAP) with DNA template under macromolecular crowding conditions could be seen on an agarose gel following electrophoresis of transcription samples (Figure S4). The formation of a large DNA-RNAP-RNA complex resulted from the binding of T7 RNAP to DNA template and the subsequent transcript was more obvious in crowding solutions than in dilute solutions, Pg. 6, left column, final partial paragraph continued onto Pg. 6, right column, first full paragraph; The enhanced association of these biomolecules could be attributed to the excluded volume effects of the crowding agents, which increased the effective concentrations of the enzymes and biomolecular reactants, Pg. 6, right column, top of final partial paragraph).

Claims 6, 7, 9, and 12 lack an inventive step under PCT Article 33(3) as being obvious over Sun et al. (hereinafter Sun) in view of Kornblihtt et al. (hereinafter Kornblihtt).

Regarding Claim 6, Sun discloses the composition of claim 1, wherein the RNAP is homologous to the host cells (We have developed an endogenous E. coli cell-free expression system that preserves the efficiency of protein expression demonstrated by previous systems but adds additional versatility by allowing expression and regulation based on both endogenous and exogenous (T7 or other) mechanisms, Pg. 1, final partial paragraph continued onto Pg. 2, first partial paragraph).

Sun fails to explicitly disclose said RNAP has a slow elongation-rate.

Kornblihtt teaches using slow elongation RNA polymerases (Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both in vitro and in vivo.... Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons, Pg. 6, right column, second full paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Kornblihtt for the purpose of slowing the elongation rate to maximize the accuracy of peptide synthesis in the system (see, e.g., "Engine out of the chassis: Cell-free protein synthesis and its uses" to Rosenblum et al., In Escherichia coli CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [...]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [...]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation, Pg. 261, left column, final partial paragraph continued onto Pg. 261, right column, first partial paragraph).

Regarding Claim 7, Sun discloses the composition of claim 1, wherein the RNAP is heterologous to the host cells (We have developed an endogenous E. coli cell-free expression system that preserves the efficiency of protein expression demonstrated by previous systems but adds additional versatility by allowing expression and regulation based on both endogenous and exogenous (T7 or other) mechanisms, Pg. 1, final partial paragraph continued onto Pg. 2, first partial paragraph).

Sun fails to explicitly disclose said RNAP has a slow elongation rate.

Kornblihtt teaches using slow elongation RNA polymerases (Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both in vitro and in vivo.... Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons, Pg. 6, right column, second full paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Kornblihtt for the purpose of slowing the elongation rate to maximize the accuracy of peptide synthesis in the system (see, e.g., "Engine out of the chassis: Cell-free protein synthesis and its uses" to Rosenblum et al., In Escherichia coli CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [...]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [...]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation, Pg. 261, left column, final partial paragraph continued onto Pg. 261, right column, first partial paragraph).

**WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY**

International application No.

PCT/US2018/046477

**Supplemental Box**

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Regarding Claim 9, Sun discloses the composition of claim 1, but Sun fails to explicitly disclose wherein the slow elongation-rate RNAP is a synthetic RNAP.

Kornblihtt teaches mutated slow elongation RNA polymerases (Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both in vitro and in vivo.... Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons, Pg. 6, right column, second full paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Kornblihtt for the purpose of slowing the elongation rate to maximize the accuracy of peptide synthesis in the system (see, e.g., "Engine out of the chassis: Cell-free protein synthesis and its uses" to Rosenblum et al., In Escherichia coli CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [...]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [...]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation, Pg. 261, left column, final partial paragraph continued onto Pg. 261, right column, first partial paragraph).

Regarding Claim 12, Sun discloses the composition of claim 1, further comprising exogenous nucleic acids to be expressed in the composition, wherein each exogenous nucleic acid comprises a promoter that is recognized by the RNAP (We have developed an endogenous E. coli cell-free expression system that preserves the efficiency of protein expression demonstrated by previous systems but adds additional versatility by allowing expression and regulation based on both endogenous and exogenous (T7 or other) mechanisms....

Our system can produce up to 0.75 mg/ml of reporter protein using either a sigma70-based promoter with lambda-phage operators or a T7-driven promoter, similar to yields from other commercial systems.... Instead of being limited to T7-regulated circuits, one can envision producing complex biomolecules in a user-controllable setting using a mix of native E. coli promoters and exogenously supplied transcription and regulation mechanisms, Pg. 1, final partial paragraph continued onto Pg. 2, first partial paragraph).

Sun fails to explicitly disclose said RNAP has a slow elongation-rate.

Kornblihtt teaches using slow elongation RNA polymerases (Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both in vitro and in vivo.... Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons, Pg. 6, right column, second full paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Kornblihtt for the purpose of slowing the elongation rate to maximize the accuracy of peptide synthesis in the system (see, e.g., "Engine out of the chassis: Cell-free protein synthesis and its uses" to Rosenblum et al., In Escherichia coli CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [...]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [...]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation, Pg. 261, left column, final partial paragraph continued onto Pg. 261, right column, first partial paragraph).

Claims 8 and 10 lack an inventive step under PCT Article 33(3) as being obvious over Sun et al. (hereinafter Sun) in view of Kornblihtt et al. (hereinafter Kornblihtt) and New England Biolabs, Inc. et al. (hereinafter New England Biolabs).

Regarding Claim 8, modified Sun discloses the composition of claim 7. Sun fails to explicitly disclose wherein the slow elongation-rate RNAP is sourced from a thermophile or psychrophile.

New England Biolabs teaches using an RNA Polymerase from a thermophilic source for use in a cell-free system (A bacteriophage RNA polymerase variant is provided. In some embodiments, the variant may have increased thermostability relative to the corresponding wild type bacteriophage RNA polymerase and/or wild type T7 RNA polymerase, Abstract; Individual mutations (see for example those in FIGs. 1A-1D) were screened in a novel cell-free assay based on the reconstituted translation system from Thermus thermophilus (Tth PURE system). Reconstitution of translation from Thermus thermophilus reveals a minimal set of components sufficient for protein synthesis at high temperatures and functional conservation of modern and ancient translation components, Pg. 19, Lns. 29-32).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of New England Biolabs for the purpose of using a thermophilic RNA polymerase functional at high temperatures in cell-free systems.

Regarding Claim 10, modified Sun discloses the composition of claim 9. Sun fails to explicitly disclose wherein the slow elongation-rate RNAP is engineered by directed evolution and/or rational design.

New England Biolabs teaches an engineered T7 RNA Polymerase variant for use in a cell-free system (A bacteriophage RNA polymerase variant is provided. In some embodiments, the variant may have increased thermostability relative to the corresponding wild type bacteriophage RNA polymerase and/or wild type T7 RNA polymerase, Abstract; Individual mutations (see for example those in FIGs. 1A-1D) were screened in a novel cell-free assay based on the reconstituted translation system from Thermus thermophilus (Tth PURE system). Reconstitution of translation from Thermus thermophilus reveals a minimal set of components sufficient for protein synthesis at high temperatures and functional conservation of modern and ancient translation components.... All variants shown have a detectable increase in thermostability, Pg. 19, Ln. 29 - Pg. 20, Ln. 4; The high stability of enzymes from thermophilic organisms enables technologies in molecular biology and diagnostics (the Polymerase Chain Reaction, for example). However, equivalent enzymes from thermophilic organisms are not always available. In these cases, directed evolution or computational methods can serve as a powerful tool to identify variants of mesophilic enzymes that confer thermostability, Pg. 1, Lns. 5-8).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of New England Biolabs for the purpose of identifying and generating an RNA polymerase variant functional at high temperatures in cell-free systems.

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2018/046477

## Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Claim 11 lacks an inventive step under PCT Article 33(3) as being obvious over Sun et al. (hereinafter Sun) in view of New England Biolabs, Inc. et al. (hereinafter New England Biolabs) and Kornblihtt et al. (hereinafter Kornblihtt).

Regarding Claim 11, Sun discloses the composition of claim 1, but Sun fails to explicitly disclose wherein the slow elongation-rate RNAP is provided as a purified protein or as a nucleic acid encoding the slow elongation-rate RNAP.

Kornblihtt teaches mutated slow elongation RNA polymerases (Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both in vitro and in vivo.... Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons, Pg. 6, right column, second full paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Kornblihtt for the purpose of slowing the elongation rate to maximize the accuracy of peptide synthesis in the system (see, e.g., "Engine out of the chassis: Cell-free protein synthesis and its uses" to Rosenblum et al., In Escherichia coli CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [...]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [...]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation, Pg. 261, left column, final partial paragraph continued onto Pg. 261, right column, first partial paragraph).

New England Biolabs teaches a nucleic acid encoding a modified RNA Polymerase II for use in a cell-free system (A bacteriophage RNA polymerase variant is provided. In some embodiments, the variant may have increased thermostability relative to the corresponding wild type bacteriophage RNA polymerase and/or wild type T7 RNA polymerase, Abstract; Individual mutations (see for example those in FIGs. 1A-1D) were screened in a novel cell-free assay based on the reconstituted translation system from Thermus thermophilus (Tth PURE system). Reconstitution of translation from Thermus thermophilus reveals a minimal set of components sufficient for protein synthesis at high temperatures and functional conservation of modern and ancient translation components (...). Genes encoding T7 RNAP variants were transcribed in vitro using SP6 RNA polymerase. 1 µl of in vitro transcription reaction was added to 10 µl of Tth PURE system with a fluorescent reporter gene (a GFP variant under the control of a T7 RNAP promoter). The activity of T7 RNA polymerase variants synthesized in Tth PURE system was coupled to the expression of a GFP gene under the control of a T7 promoter.... All variants shown have a detectable increase in thermostability, Pg. 19, Ln. 29 - Pg. 20, Ln. 4).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of New England Biolabs for the purpose of providing a nucleic acid specifically encoding an RNA polymerase variant functional at high temperatures in cell-free systems.

Claim 13 lacks an inventive step under PCT Article 33(3) as being obvious over Sun et al. (hereinafter Sun) in view of Shimizu et al. (hereinafter Shimizu).

Regarding Claim 13, Sun discloses the composition of claim 1, but Sun fails to explicitly disclose wherein the ribosomes are sourced from the host cells, or from an organism different than the host cells.

Shimizu teaches a cell-free translation system comprising exogenous ribosomes at a concentration between 0.1 to 100 µM (Here we describe a cell-free translation system reconstructed from purified histidine (His)-tagged translation factors and "programmed" by natural mRNA, Pg. 751, right column, first full paragraph; The PURE system includes 32 components that we purified individually: IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, RF1, RF3, RRF, 20 aminoacyl-tRNA synthetases (ARSs), methionyl-tRNA transformylase (MTF), T7 RNA polymerase, and ribosomes, Pg. 751, right column, final full paragraph; The DHFR template was translated for 1 h at 37°C in reaction mixtures containing 2.4 µM ribosome, Pg. 754, right column, final partial paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Shimizu for the purpose of using a purified ribosome at an optimized concentration for cell-free protein translation.



WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US2018/046477

## Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

Claim 14 lacks an inventive step under PCT Article 33(3) as being obvious over Sun et al. (hereinafter Sun) in view of Kornblihtt et al. (hereinafter Kornblihtt) and Rosenblum et al. (hereinafter Rosenblum).

Regarding Claim 14, Sun discloses the composition of claim 1, but Sun fails to explicitly disclose wherein the composition comprises both slow elongation-rate RNAP and exogenous ribosomes.

Kornblihtt teaches using slow elongation RNA polymerases (Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both in vitro and in vivo.... Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons, Pg. 6, right column, second full paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Kornblihtt for the purpose of slowing the elongation rate to maximize the accuracy of peptide synthesis in the system (see, e.g., "Engine out of the chassis: Cell-free protein synthesis and its uses" to Rosenblum et al., In Escherichia coli CFPS, the translation machinery is typically about 20-fold more dilute than in the cell, decreasing the rates of initiation, elongation and protein accumulation [...]. As well, the average distance between two adjacent ribosomes on a single mRNA strand increases and polysomes are less likely to form [...]. Despite these differences, CFPS can benefit from the relatively slower synthesis rate and the greater distance between ribosomes by allowing nascent polypeptide chains more time and space to form desirable intra-peptide chain contacts, while decreasing the probability of undesirable, non-specific inter-peptide chain contacts, thereby increasing the probability of proper folding and decreasing the probability of aggregation, Pg. 261, left column, final partial paragraph continued onto Pg. 261, right column, first partial paragraph).

Rosenblum teaches using exogenous ribosomes in cell-free systems (Extracting the cytoplasmic milieu from a cell affords a lysate capable of producing proteins in concentrations reaching to tens of micromolar. Such lysates, derivable from a variety of cells, allow the facile addition and subtraction of components that are directly or indirectly related to the translation machinery and/or the over-expressed protein. The flexible nature of such cell-free expression systems, when coupled with high throughput monitoring, can be especially suitable for protein engineering studies, allowing one to bypass multiple steps typically required using conventional in vivo protein expression, Abstract; Removal of endogenous ribosomes from CFPS and their replacement with exogenous ribosomes is easily performed. In lysates, an ultracentrifugation step is required [...], while in PURExpress a solution lacking ribosomes is used. Examples of such replacements include: fluorescently labeled ribosomes for single molecule experiments [...], and ribosomes containing mutations in ribosomal RNA (rRNA) [...] that may affect their activities, Pg. 265, left column, final partial paragraph).

It would have been obvious to one of ordinary skill in the art at the time of the invention to modify Sun with the teaching of Rosenblum for the purpose of creating a cell-free system for protein synthesis using exogenous ribosomes that can pair with modified RNA polymerases (The preparation of lysates for CFPS was significantly improved over the years in terms of buffer composition [...], energy recycling [...], the utilization of various mutated cell strains [...], the supplementation of small molecules [...], the addition of proteins such as T7 RNA polymerase (RNAP) to generate a transcription/translation coupled system [...] and chaperones to improve the yields of properly folded target proteins, Rosenblum, Pg. 261, left column, final full paragraph).

Claims 1-14 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.