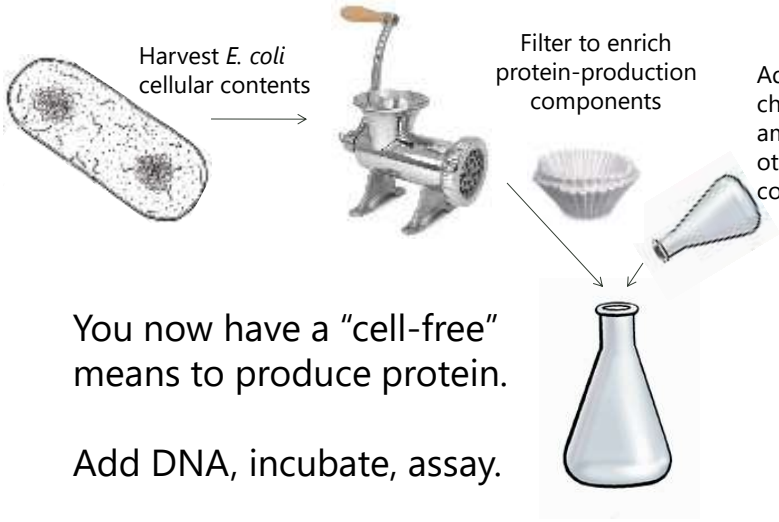
U.S. ARMY COMBAT CAPABILITIES DEVELOPMENT COMMAND

Cell Free Transcription and Translation – Faster, Safer

Aleksandr Miklos, Ph.D.
Research Biologist
R&T BioSciences / BioChemistry Branch

20 April 2022

In Vitro Transcription and Translation

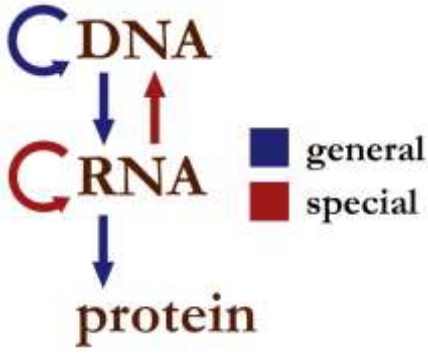


You now have a "cell-free" means to produce protein.

Add DNA, incubate, assay.

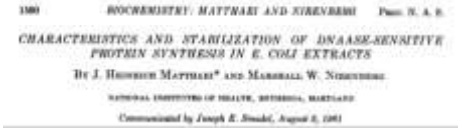
Cellular Lysates – Early Work

Establishing the Central Dogma of Molecular Biology



3

Cracking the Code



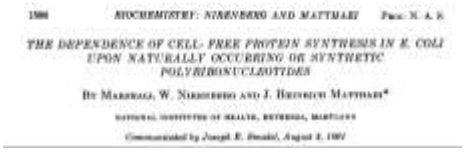
1508 BIOCHEMISTRY: MATTHAEI AND NIRENBERG Proc. N. A. S.

CHARACTERISTICS AND STABILIZATION OF DNAASE-SENSITIVE PROTEIN SYNTHESIS IN E. COLI EXTRACTS

By J. Heinrich Matthaei* and Marshall W. Nirenberg

Communicated by Joseph E. Smadel, August 8, 1961

J.H. Matthaei and M.W. Nirenberg
Proc Natl Acad Sci Unit States Am, 47 (1961), pp. 1508-1588, 10.1073/pnas.47.10.1580



1588 BIOCHEMISTRY: NIRENBERG AND MATTHAEI Proc. N. A. S.

THE DEPENDENCE OF CELL-FREE PROTEIN SYNTHESIS IN E. COLI UPON NATURALLY OCCURRING OR SYNTHETIC POLYRIBONUCLEOTIDES

By Marshall W. Nirenberg and J. Heinrich Matthaei*

Communicated by Joseph E. Smadel, August 8, 1961

M.W. Nirenberg and J.H. Matthaei
Proc Natl Acad Sci Unit States Am, 47 (1961), pp. 1588-1602, 10.1073/pnas.47.10.1588

4

Shining light in the black box; increasing productivity

Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 1803–1807, May 1974

Prolonged Transcription in a Cell-Free System Involving Nuclei and Cytoplasm

(transcription nuclei/RNA synthesis/protein synthesis)

GUANG-JER WU AND GEOFFREY ZUBAY

Department of Biological Sciences, Columbia University, New York City, N.Y. 10027

Communicated by Donald D. Brown, February 15, 1974

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 264, No. 11, Issue of April 15, pp. 6259–6264, 1989
Printed in U.S.A.

Possible Involvement of the 90-kDa Heat Shock Protein in the Regulation of Protein Synthesis*

(Received for publication, May 3, 1988)

David W. Rose‡, William J. Welch§, Gisela Kramer, and Boyd Hardesty¶

From the Clayton Foundation Biochemical Institute and the Department of Chemistry, University of Texas, Austin, Texas 78712 and the §Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

5

PRODUCTIVITY BREAKTHROUGH

A Continuous Cell-Free Translation System Capable of Producing Polypeptides in High Yield

ALEXANDER S. SPIRIN, VLADIMIR I. BARANOV, LUBOV' A. RYABOVA, SERGEY YU. OVODOV, YULY B. ALAKHOV

A cell-free translation system has been constructed that uses a continuous flow of the feeding buffer [including amino acids, adenosine triphosphate (ATP), and guanosine triphosphate (GTP)] through the reaction mixture and a continuous removal of a polypeptide product. Both prokaryotic (*Escherichia coli*) and eukaryotic (wheat embryos, *Triticum sp.*) versions of the system have been tested. In both cases the system has proven active for long times, synthesizing polypeptides at a high constant rate for tens of hours. With the use of MS2 phage RNA or brome mosaic virus RNA 4 as templates, 100 copies of viral coat proteins per RNA were synthesized for 20 hours in the prokaryotic or eukaryotic system, respectively. With synthetic calcitonin messenger RNA, 150 to 300 copies of calcitonin polypeptide were produced per messenger RNA in both types of continuous translation systems for 40 hours.

EXPRESSION OF ALIEN GENES IN LIVING cells is often subject to a number of limitations. The product polypeptide can be unstable in a given cell, and in some cases the product is toxic to the cell. ed if translation were possible in cell-free systems. Unfortunately, however, different versions of cell-free translation systems that have been previously described have a general shortcoming, namely, a low yield of the

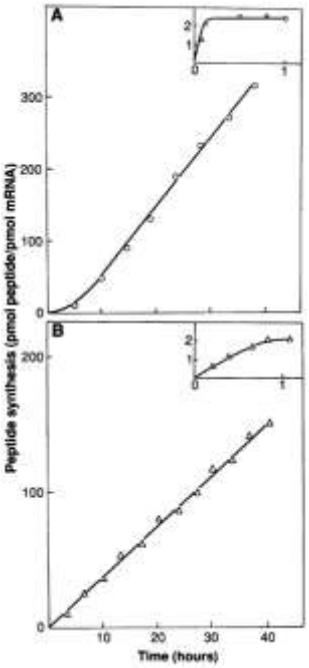


Fig. 2. Kinetics of the Val⁵-calcitonin synthesis in the continuous cell-free translation systems (40 hours). Inset (same axes units): Kinetics of the polypeptide synthesis in the standard cell-free systems of the same composition and volume. (A) Synthesis of calcitonin in the *E. coli* system. All reaction conditions were the same as in Fig. 1A, except that 0.06 nmol of synthetic calcitonin mRNA was added instead of viral RNA, and the PM-10 membrane was used instead of the PM-30 for removing the product from the ultrafiltration cell. (B) Synthesis of calcitonin in the wheat system. All reaction conditions were the same as in Fig. 1B, except that 0.06 nmol of synthetic calcitonin mRNA was added instead of viral RNA, 75 mM potassium acetate was found to be optimal for this synthesis, and the PM-10 membrane was used instead of the XM-50 for removing the product from the ultrafiltration cell.

Spirin, Alexander S., et al. "A continuous cell-free translation system capable of producing polypeptides in high yield." Science 242.4882 (1988): 1162-1164.

6

ENGINEERING THE SYSTEMS

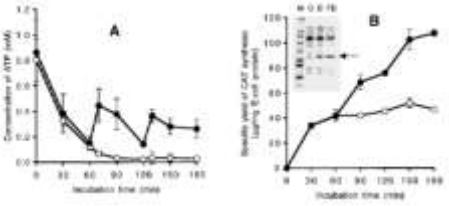
DEVCOM

Regeneration of Adenosine Triphosphate from Glycolytic Intermediates for Cell-Free Protein Synthesis

Dong-Myung Kim, James R. Swartz

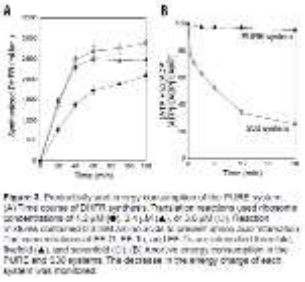
Department of Chemical Engineering, Stanford University, Stanford, California 94305-5025, USA; telephone: 650-723-5388; fax: 650-725-0555; e-mail: swartz@cheme.stanford.edu

Received 14 September 2000; accepted 29 January 2001



Kim, Dong-Myung, and James R. Swartz. "Regeneration of adenosine triphosphate from glycolytic intermediates for cell-free protein synthesis." Biotechnology and bioengineering 74.4 (2001): 309-316.

Shimizu, Y., Inoue, A., Tomari, Y. et al. Cell-free translation reconstituted with purified components. Nat Biotechnol 19, 751–755 (2001). https://doi.org/10.1038/90802



RESEARCH ARTICLE

Cell-free translation reconstituted with purified components

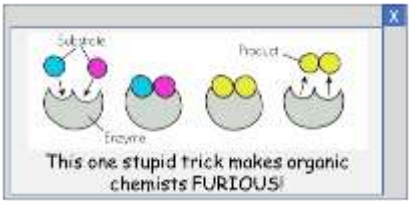
Yoshihiro Shimizu[1], Akio Inoue[2], Yukihide Tomari[1], Tsuzumi Suzuki[2], Takashi Yokogawa[2], Kazuya Nishikawa[2], and Takuya Ueda[1]*

We have developed a protein-synthesizing system reconstituted from recombinant tagged protein factors purified to homogeneity. The system was able to produce protein at a rate of about 160 μg/ml/h in a batch mode without the need for any supplementary apparatus. The protein products were easily purified within 1 h using affinity chromatography to remove the tagged protein factors. Moreover, omission of a release factor allowed efficient incorporation of an unnatural amino acid using suppressor transfer RNA (tRNA).

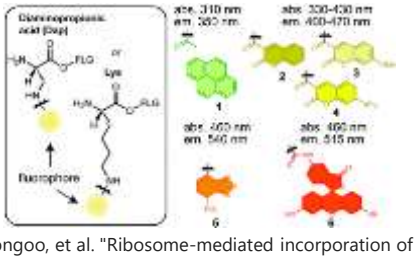
CURRENT PROMINENT APPLICATIONS

DEVCOM

Metabolic Engineering

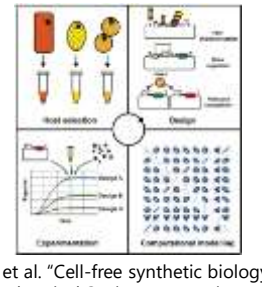


Continued Basic Research (Engineering Translation!)



Lee, Joongoo, et al. "Ribosome-mediated incorporation of fluorescent amino acids into peptides in vitro." *Chemical Communications* 57.21 (2021): 2661-2664.

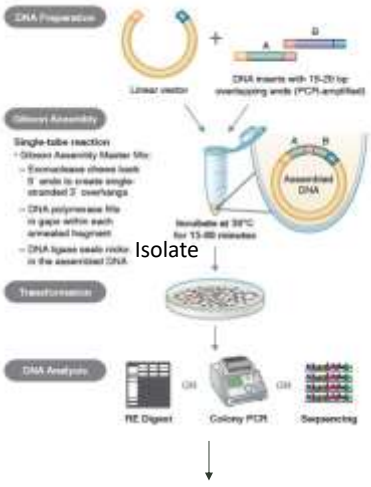
"Breadboarding" / Screening



Fielding

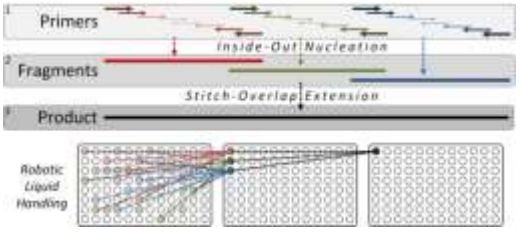
Moore, Simon J et al. "Cell-free synthetic biology for in vitro prototype engineering." Biochemical Society transactions vol. 45,3 (2017): 785-791. doi:10.1042/BST20170011

Testing an Engineered Protein



Overnight culture, grow to density, induce, lyse, purify, label, test.

Stitch together a linear ORF from primers on a robot,



Express *in vitro*

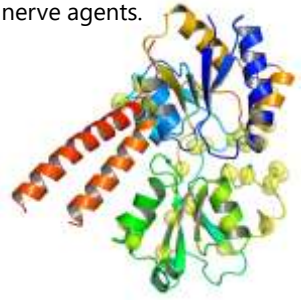
Throw that PCR product into a tube with lysate and reaction buffer, purify, label, test.

Miklos, A. E., R. A. Hughes, and A. D. Ellington. "Design and assembly of large synthetic DNA constructs." *Current Protocols in Molecular Biology* (2012): Unit3-23.

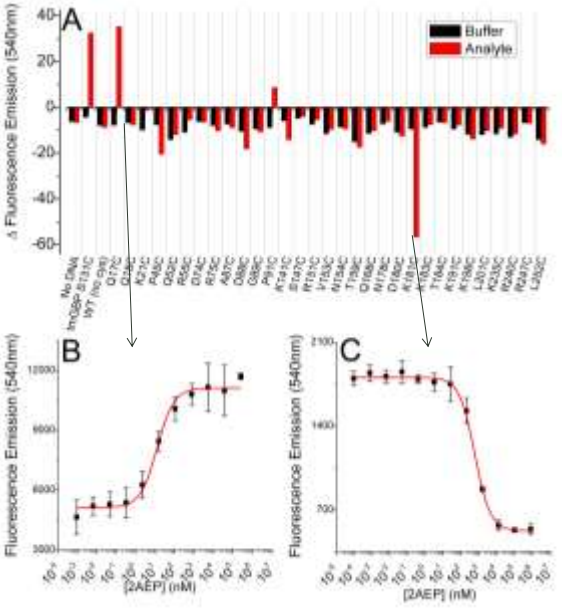
9

Screening for Biosensors

Phosphonate-binding protein
Starting point for sensing pesticides and nerve agents.



This type of screening can take months with conventional methods, days with *in vitro* expression

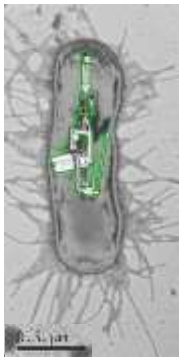


Alicea, I., et. al. (2011). Structure of the Escherichia coli phosphonate binding protein PhnD and rationally optimized phosphonate biosensors. *Journal of molecular biology*, *414*(3), 356-369.

10

Using Syn Bio in the Field - The Question:

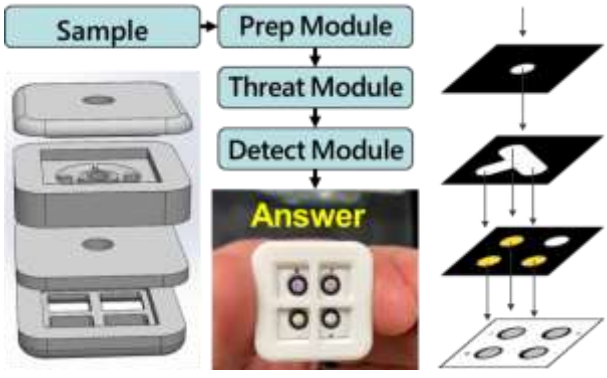
If I had an engineered organism which could turn latrine contents into freshly-crimped 5.56 rounds, would someone let me USE it?



11

Cell-Free Syn Bio Sensing

Lysates and energy mixes can be stable when freeze-dried, and you can even embed these reagents in paper before freeze-drying, so we can make shelf-stable devices that run various sensing schemes.



12

ACKNOWLEDGEMENTS

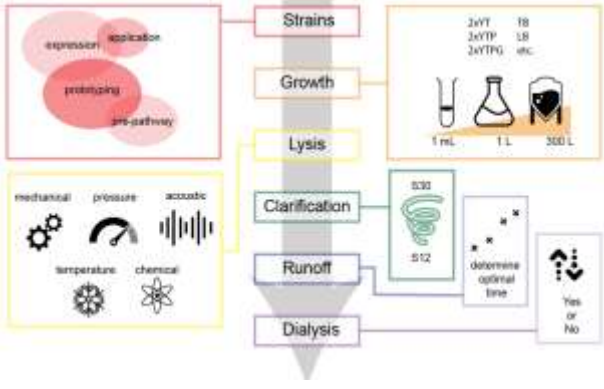
Lysate Team
Dr. Nathan McDonald
Dr. Patricia Buckley
Katherine Rhea
Dr. Stephanie Cole

Funding:
Defense Threat Reduction Agency

13

In-house experience making cell-free systems

- CBC established protocol for generating *E.coli* cell-free lysate
- CBC experience in creating novel cell-free lysate for *V. natriegens*
- Known key considerations:
  - Tuning mRNA and protein degradation rates
  - DNA stabilization
  - Regeneration of energy (ATP)



Cole et al. 2020

14

Non-traditional cell free lysate systems

Organism	Type	Proteins Made
Bacillus megaterium	Gram positive	GFP, mCherry
Bacillus subtilis	Gram positive	GFPmut3b, renilla luciferase
Clostridium autoethanogenum	Gram positive	Luciferase, metabolic enzymes
Corynebacterium glutamicum	Gram positive	eGFP
Escherichia fergusonii	Gram negative	eGFP
Klebsiella oxytoca	Gram negative	eGFP
Lactococcus lactis	Gram positive	None (only transcription active)
Pantoea agglomerans	Gram negative	eGFP
Pseudomonas fluorescens	Gram negative	GFP, apolipoprotein, pancreatic RNase, p37a, glucokinase, peptidases
Pseudomonas putida	Gram negative	sfGFP
Salmonella enterica	Gram negative	eGFP
Streptomyces species	Gram positive	eGFP, sfGFP, metabolic proteins
Sulfolobus solfataricus	Archaeal thermophile	ORF 104, ORF 143
Sulfolobus tokodaii	Archaeal thermophile	Polyphenylalanine
Thermococcus kodakaraensis	Archaeal thermophile	Chitinase
Thermus thermophilus	Gram negative	Polyphenylalanine
Vibrio natriegens	Gram negative	sfGFP, eGFP

Cole et al. 2020

15

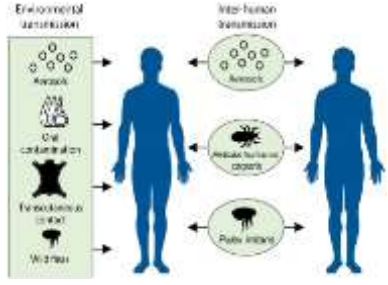
New Reason to go cell-free – Risk Reduction

Yersinia pestis

Gram-negative enterobacteria,
Causative agent of the plague
- Route of transmission proceeds typically through flea bites

Encodes a variety of virulence factors including multiple virulence plasmids
- pCD1/pMT1/pPCP1
- Pigmentation locus (pgm)
- F1 proteinaceus capsule
- Type III secretion system
- Lipopolysaccharide

Experiences dramatic environmental shifts during lifecycle from flea hindgut through human infection
- Temperature transitions range from 21^{0}C-37 ^{0} C



Barbieri et al. 2020

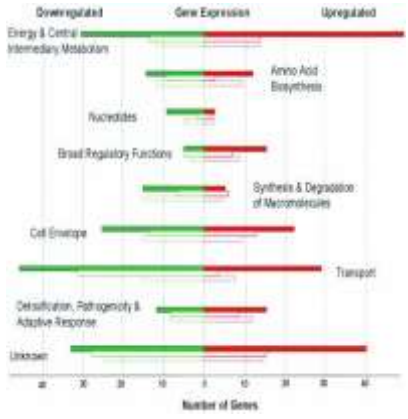
16

BROAD EXPRESSION CHANGES ACROSS TEMPERATURES

Y. pestis transitions from ~21^{0}C to 26 ^{0} C to 37 ^{0} C during the infection of a human host from a carrier flea.

The shift in temperature results in dynamic gene expression changes associated with pathogenesis

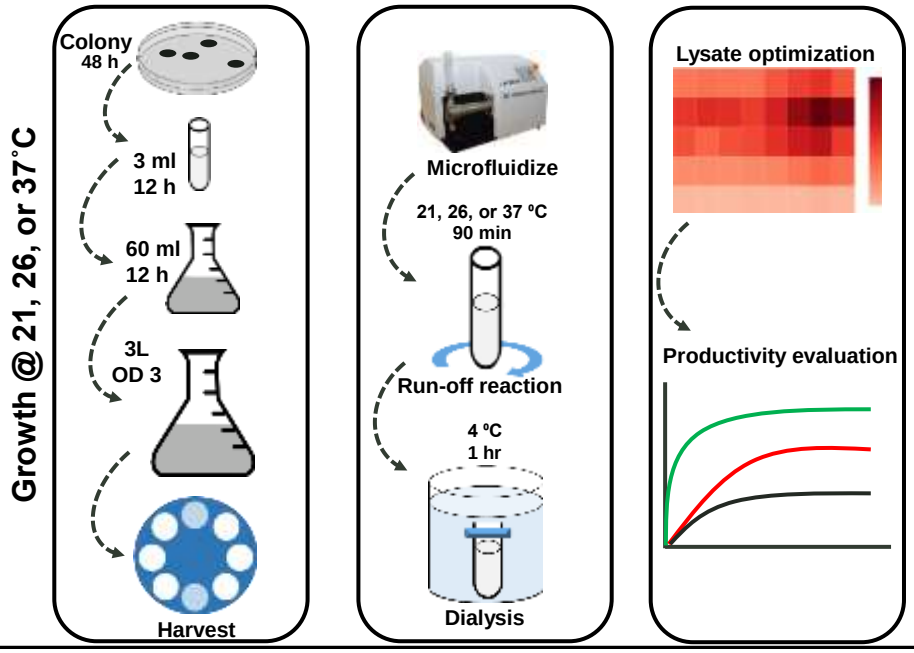
- Stimulates expression of F1 capsule antigen which is essential for evading phagocytosis
- Upregulation of T3SS effector proteins
- Modifications to the lipopolysaccharide to a low immunostimulatory form



Motin et al. 2014

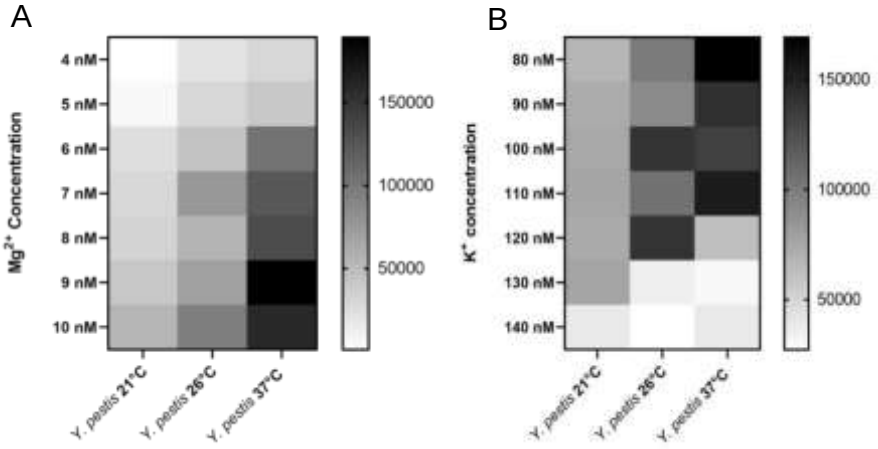
17

Generating a *Yersinia pestis* CO92- extract



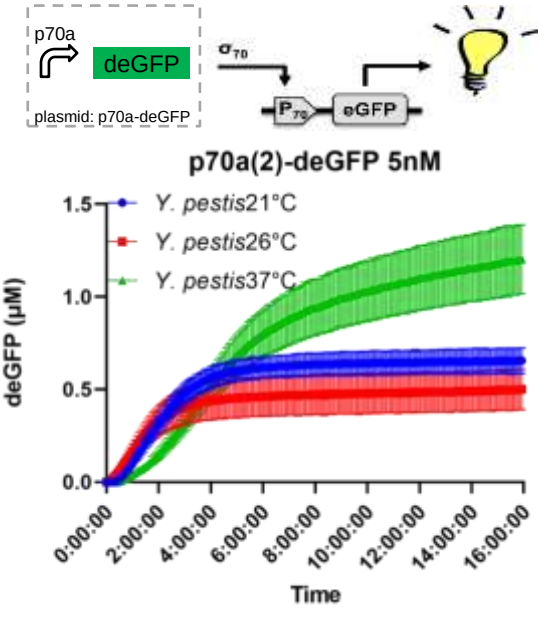
18

OPTIMIZATION OF EXTRACTS



19

Y. Pestis CO92$^-$ 37ºC lysate is most productive



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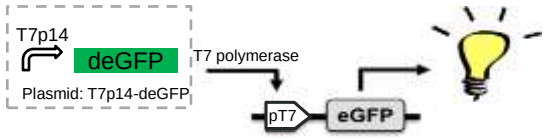
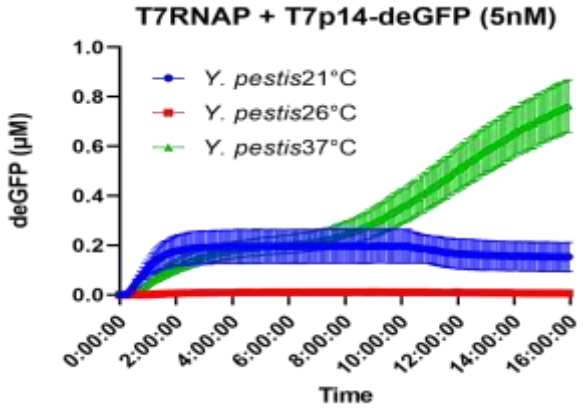
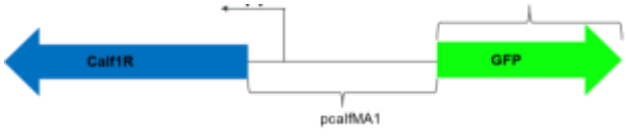
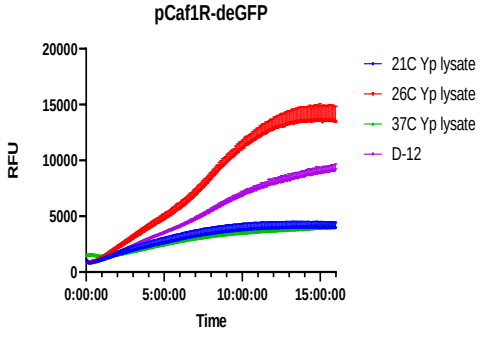
T7 promoters less active in *Y. pestis* CO92- lysates



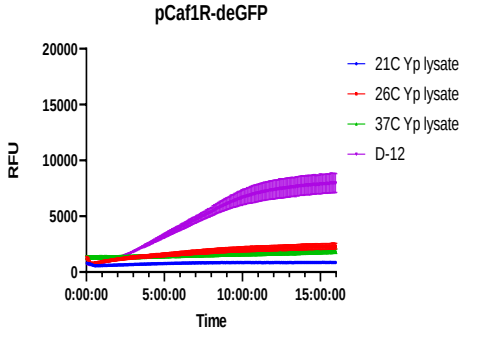


Yp Lysates work with Native Promoters





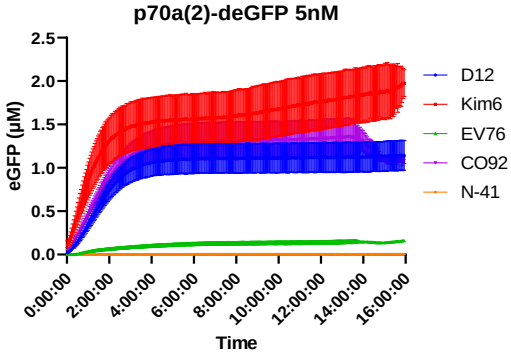
Cell-free reaction run at 29℃



Cell-free reaction run at 37˚C

Extract Productivity is Strain Specific

Strain	Plasmids	Attenuation
D-12	pCD1/pMT1	pCD1-
Kim6	pCD1/pMT1	Lcr-
EV76	pCD1/pMT1	Pgm-
CO92	pCD1/pMT1	Pgm-
N-41	pPCP/pCD1/pMT1	Pgm-



All extracts grown at 21°C

23

pubs.acs.org/synthbio Technical Note

Cell-Free Protein Systems from *Yersinia pestis* are Functional and Growth-Temperature Dependent

Nathan D. McDonald,[#] Katherine A. Rhea,[#] Kimberly L. Berk, Julie L. Zacharko, and Aleksandr E. Miklos*

Cite This: https://doi.org/10.1021/acssynbio.1c00505 Read Online

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ABSTRACT: Cellular lysates capable of transcription and translation have become valuable tools for prototyping genetic circuits, screening engineered functional parts, and producing biological components. Here we report that lysates derived from *Yersinia pestis* CO92[−] are functional and can utilize both the *E. coli* σ70 and the bacteriophage T7 promoter systems to produce green fluorescent protein (GFP). Because of the natural lifestyle of *Y. pestis*, lysates were produced from cultures grown at 21 °C, 26 °C, and 37 °C to mimic the infection cycle. Regardless of the promoter system the GFP production from 37 °C was the most productive and the 26 °C lysate was the least. When reactions are initiated with 5 nM of DNA, the GFP output of the 37 °C lysate is comparable with the productivity of other non-*E. coli* systems. The data we present demonstrate that, without genetic modification to enhance productivity, cell-free extracts from *Y. pestis* are functional and dependent on the temperature at which the bacterium was grown.

KEYWORDS: *cell free lysates, in vitro transcription and translation, alternative chassis organisms*

24

CONCLUSIONS AND A QUESTION

- Cell-free production systems can be generated from *Yersinia pestis* extracts
- *Yersinia pestis* extracts are able to express deGFP from native *E. coli* σ_{70} and bacteriophage T7 promoters,
- Growth temperature impacts productivity of the extracts
- *Yersinia pestis* strains have variable production in cell-free expression tests
- How much "live organism" work could be replaced with cell-free?

25