NEBExpress® Cell-free *E. coli* Protein Synthesis System

A High Performance *E. coli* Cell Lysate-Based System for *in vitro* Protein Synthesis

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Introduction

Cell-free protein synthesis (CFPS) systems based on bacterial cell lysates have been widely used for an array of applications. These systems offer a number of advantages; for example, the tight coupling of translation and transcription from prokaryotic cells is preserved in the lysate, rendering the protein synthesis process exceptionally efficient. Additionally, genetic manipulation can be performed on the strain in which the lysates are made to enhance its ability to generate high yields of protein. Finally, the lysates can be manufactured at a larger scale, compared to reconstituted systems. Lysate-based protein synthesis systems are conceptually simple and relatively less expensive, and have therefore been home-brewed in many laboratories over the past decades, as well as supplied by several commercial sources. These systems, however, exhibit varying levels of performance, ease of use, and often do no produce consistent results across broad size ranges and types of proteins.

The NEBExpress Cell-free *E. coli* Protein Synthesis system was developed using several strategies to enhance performance, ease of use, and ensure robustness. These include the use of an *E. coli* strain genetically engineered to maximize the stability of template DNA and RNA and the protein products, a highly optimized reaction buffer, and a stringent biomanufacturing process.

In this technical note, we examine how the NEBExpress Cell-free *E. coli* Protein Synthesis System performs in several frequently encountered applications, and demonstrate this system's high performance and versatility.

Methods

Cell-free Protein Synthesis (CFPS) reaction

The following reagents were combined in 1.5 ml microcentrifuge tubes:

- 25 µl Protein Synthesis Buffer (2X)
- 1 µl T7 RNA Polymerase
- 1 µl RNase Inhibitor
- 12 µl NEBExpress S30 Synthesis Extract
- 2 µl DNA template (125 ng/µl)
- Water to 50 µl

Reactions were incubated with shaking for 3 hours at 37°C (unless otherwise indicated).

Green fluorescent protein (vGFP) assay

The following reagents were combined in a black 96-well plate (Corning #3165):

- 25 µl Protein Synthesis Buffer (2X)
- 1 µl T7 RNA Polymerase
- 1 µl RNase Inhibitor
- 12 µl NEBExpress S30 Synthesis Extract
- 2 µl vGFP template (125 ng/µl)
- Water to 50 µl

Plates were covered with a breathable seal, and reactions were incubated with intermittent shaking for 5 hours at 37°C (unless otherwise indicated). Fluorescence detection: emission at 513 nm, excitation at 532 nm, 6 flashes/read.

β-Galactosidase assay

CFPS samples were diluted 1:10 in water. 5 µl of solution was combined with 200 µl of 5 mM 2-nitrophenyl β-D-galactopyranoside (Sigma) in 50 mM sodium phosphate buffer pH7.3. Samples were incubated at 37°C for 30 min. Free 2-nitrophenol was measured by absorption at 420 nm.

Chitinase assay

CFPS samples (1-10 µl) were mixed with 200 µl of 40 µM 4-Methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside (4-MU-chitotrioside) in 20 mM Na Acetate pH6, 200 mM NaCl. Reactions were incubated at 37°C for 30 minutes, free 4-Methylumbelliferone was measured at emission at 513 nm and excitation at 532 nm.

Linear DNA

Template DNA was prepared by PCR using the Q5 High-Fidelity 2X Master Mix (NEB #M0492) (T7 promoter and terminator regions included). PCR product was purified using the Monarch PCR & DNA Cleanup Kit (5 µg) (NEB #T1030). Final concentration was adjusted to 250 ng/µl.

RNA preparation

vGFP mRNA was prepared using NEB's HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050), according to the recommended protocols, followed by purification using the Monarch RNA Cleanup Kit.

SDS-PAGE

2 µl of CFPS reaction was mixed with water and Blue Loading Buffer Pack (NEB #B7703) and loaded onto 10/20% Tris-Glycine gels (Invitrogen). The molecular weight marker used was the Unstained Protein Standard, Broad Range (10-200 kDa) (NEB #P7717).

Protein purification

His-tagged proteins were purified using the NEBExpress Ni Spin Columns (NEB #S1427) as directed. Eluted fractions were cleaned (to remove imidazole) using Zeba Spin Desalting Columns, and protein concentration was measured using a Nanodrop™ spectrophotometer (Thermo Fisher).

Materials

- NEBExpress Cell-free *E. coli* Protein Synthesis System (NEB #E5360)
- NEBExpress GamS Nuclease Inhibitor (NEB #P0774)
- PURExpress® Disulfide Bond Enhancer (NEB #E6820)
- NEBExpress Ni-NTA Magnetic Beads (NEB #S1423)
- NEBuilder® HiFi DNA Assembly Master Mix (NEB #E2621)
- Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB #T1030)
- HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB #E2050)
- Monarch RNA Cleanup Kit (NEB #T2030)
- Q5® High-Fidelity 2X Master Mix (NEB #M0492)
- Blue Protein Loading Dye (NEB #B7703)
- 2-nitrophenyl β-D-galactopyranoside (Sigma), 4-Methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside (Sigma)
- Zeba™ Spin Desalting Columns (Thermo Fisher)

Results

Efficiency on different templates

Traditionally, cell-free protein synthesis systems use DNA plasmids as templates because they are readily available and easily prepared. Recently, there has been an increasing need for linear DNA templates in applications such as high throughput screening, where linear DNA can be used directly from amplification or site-directed mutagenesis. Additionally, there are situations where protein synthesis from mRNA templates is desired.

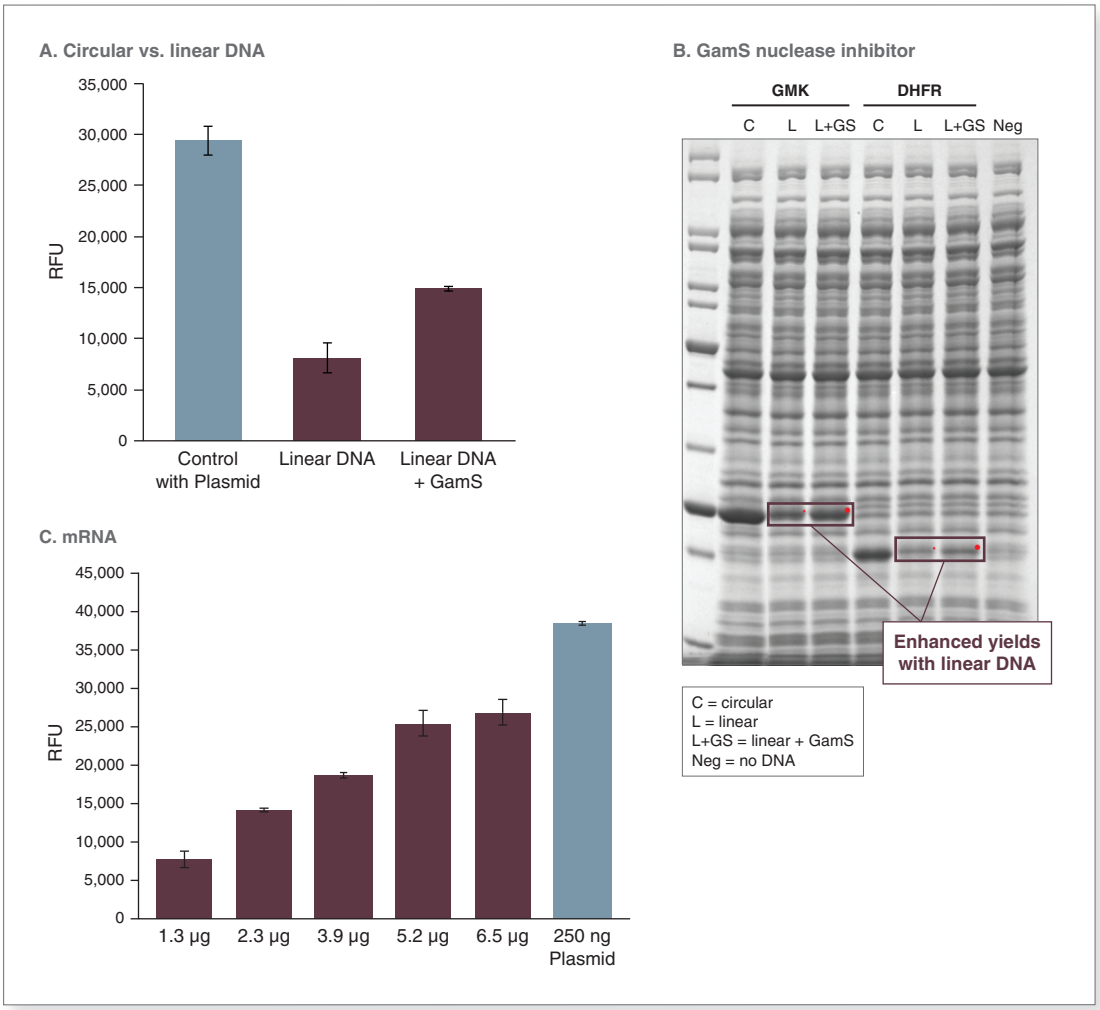
For these reasons, a lysate system that can utilize plasmid DNA, linear DNA or mRNA as a template is desirable. As shown in Figure 1A, when equimolar linear DNA templates were introduced in the NEBExpress Cell-free *E. coli* Protein Synthesis System, the target vGFP protein was produced, albeit at a reduced yield. Yield was almost doubled by adding 1.5 µg of NEBExpress GamS Nuclease Inhibitor, which is known to stabilize linear DNA templates in *in vitro* protein synthesis reactions (Figure 1A and 1B). Protein yield was also enhanced by the addition of more linear DNA. With a combination of GamS and an increased amount of linear DNA, protein synthesis can be achieved at almost the same level as the plasmid DNA templates (data not shown). Figure 1C shows vGFP synthesis from increasing amounts of mRNA, in comparison with a standard reaction with plasmid DNA.

FIGURE 1: **The NEBExpress Cell-free *E. coli* Protein Synthesis System can efficiently synthesize proteins from plasmid DNA, linear DNA or mRNA templates**

A. Equimolar amounts of plasmid DNA, linear DNA, and linear DNA supplemented with GamS were used in CFPS reactions. Addition of GamS to linear DNA almost doubled the vGFP protein yield.

B. CFPS of guanylate kinase (GMK) and dihydrofolate reductase (DHFR) using plasmid DNA (C), linear DNA (L) and linear DNA supplemented with GamS (L+GS). Boxed region shows an increase in yield from linear DNA with the addition of GamS.

C. Increasing amounts of input mRNA led to increasing yields of target vGFP. Yields were compared to the yield from a CFPS reaction using 250 ng plasmid DNA.



Effects of target size and temperature

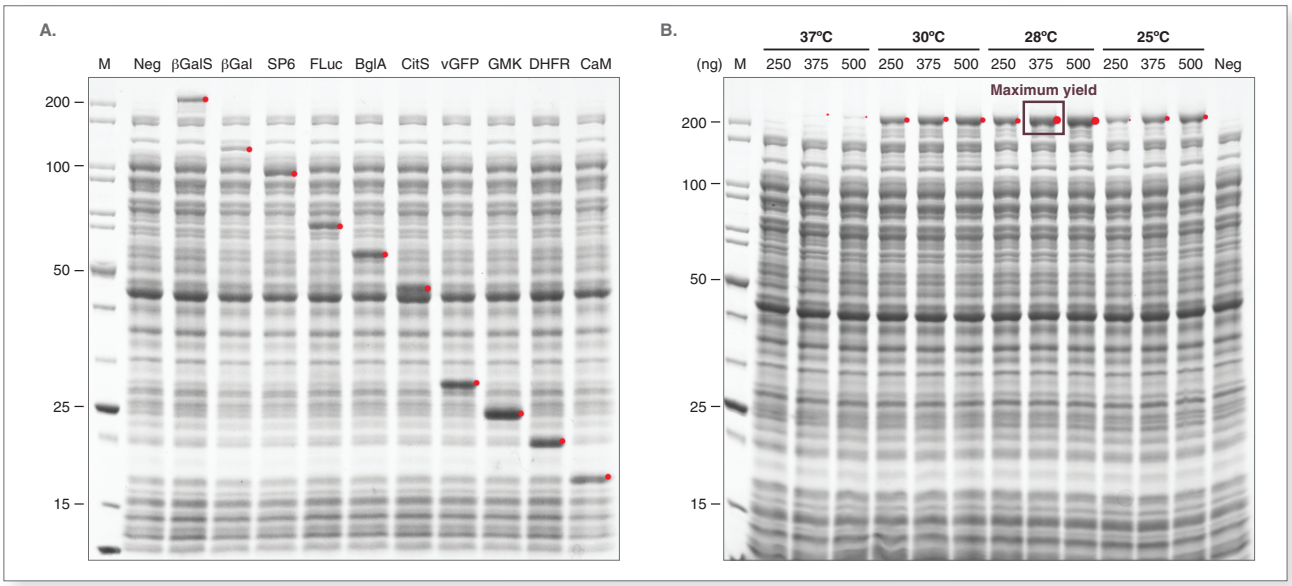
The ability to use one kit for synthesis of a wide range of proteins can have a big impact on experimental efficiency. The NEBExpress Cell-free *E. coli* Protein Synthesis System has been shown to generate high yield of proteins from a wide range of targets (16.7–230 kDa) (Figure 2A). Further, the enzymatic activity of β-galactosidase S (230 kDa) was confirmed via colorimetric assay using 2-nitrophenyl β-D-galactopyranoside as a substrate. To our knowledge, this is the largest active protein synthesized using a cell-free system.

Moreover, protein synthesis using the NEBExpress Cell-free *E. coli* Protein Synthesis System can be carried out at different temperatures. As shown in Figure 2B, the β-galactosidase S showed highest yield at the optimal synthesis temperature of 28°C. Similar to *in vivo* protein expression, the relative rate of protein translation and folding can determine how much soluble form of the protein can be obtained. These variables can be easily tuned by incubation at different temperatures in *in vitro* reactions.

FIGURE 2: **The NEBExpress Cell-free *E. coli* Protein Synthesis System can generate high yields of proteins from a wide range of targets, and can be used to optimize synthesis temperature**

A. CFPS of ten different targets, indicated by red dots, and a no DNA control (neg; Lane 2). Target proteins varied in size between 16.7 and 230 kDa. Synthesized proteins include (Lanes 3 to 12): β-Galactosidase from Streptococcus (βGalS), *E. coli* β-Galactosidase (βGal), SP6 RNA polymerase (SP6), Firefly luciferase (FLuc), 6-phospho-beta-glucosidase (BglA), citrate synthase (CitS), venus green fluorescent protein (vGFP), guanylate kinase (Gmk), dihydrofolate reductase (DHFR), calmodulin (CaM).

B. CFPS of β-Galactosidase S from *Streptococcus* (βGalS) using different temperatures and amounts of input plasmid DNA template. βGalS showed the highest yield using 500 ng of DNA when synthesized at 28°C.

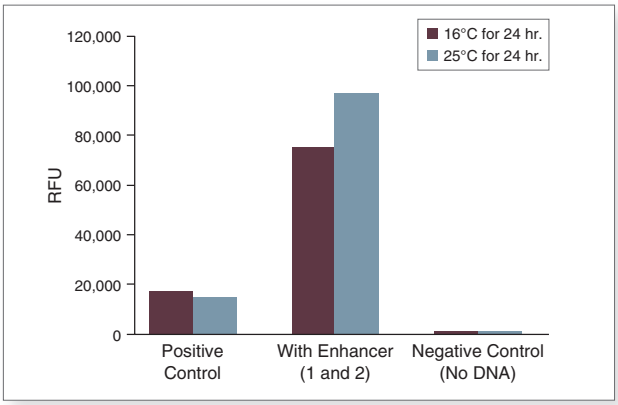


Disulfide bond formation

Disulfide bond formation is a challenging issue for protein expression in bacteria. This can be addressed using specialized *E.coli* strains that have been optimized for expression of proteins with multiple disulfide bonds, such as SHuffle®. Additionally, disulfide bond enhancers can be added to *in vitro* protein synthesis reactions to help correct disulfide bond formation in the target proteins. Figure 3 shows that, in the absence of such enhancers, chitinase from *Plasmodium* that was synthesized using the NEBExpress Cell-free *E. coli* Protein Synthesis System displayed minimal activity. However, in the presence of PURExpress® Disulfide Bond Enhancer, there was a significant increase in the chitinase activity.

FIGURE 3: **PURExpress® Disulfide Bond Enhancer increases yield of active protein in NEBExpress Cell-free *E. coli* Protein Synthesis reactions**

CFPS of chitinase assay under standard conditions (positive control) or following addition of PURExpress Disulfide Bond Enhancer. Chitinase CFPS with enhancer produced a higher yield of active protein at both 16°C or 25°C compared with synthesis under standard conditions.



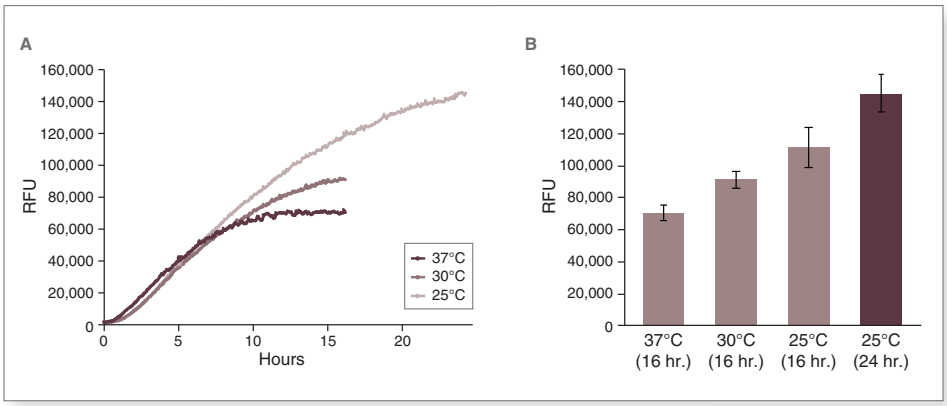
Sustained protein synthesis

With the NEBExpress Cell-free *E. coli* Protein Synthesis System it is possible to detect high yields of target protein in approximately three hours. However, given adequate aeration and agitation, the reaction can continue for more than 10 hours, producing greater than 1 mg protein/ml (Fig 4A). The longer incubation time is particularly useful when it is necessary to carry out the protein synthesis reaction at milder temperatures. As shown in Figure 4B, the synthesis of vGFP sustained over 24 hours at 25°C produced the highest yield, estimated at 3 mg/ml.

FIGURE 4: **The flexibility NEBExpress Cell-free *E. coli* Protein Synthesis System enables extended incubations**

A. CFPS of vGFP reaction at 25, 30 and 37°C. vGFP fluorescence intensity was greatest at 25°C after 24 hours.

B. End-point fluorescent values illustrate significantly higher yields with longer incubation times at low temperatures (25°C for 24 hours) than reactions at 37°C (for 16 hours).

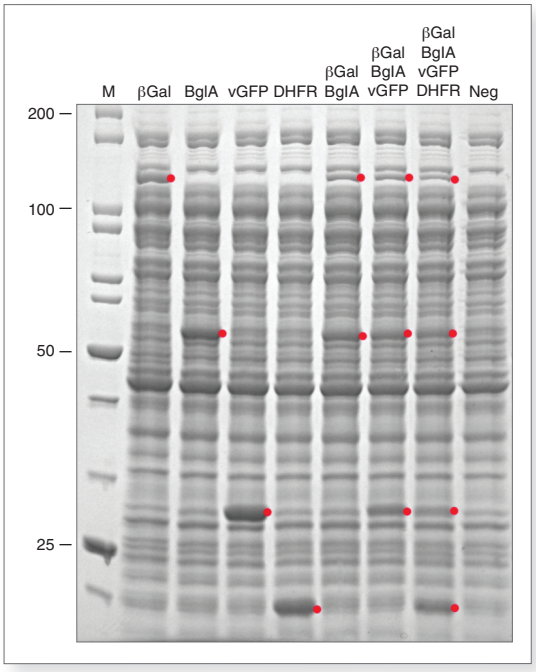


Co-expression of proteins

It is possible to co-express multiple protein targets in a single reaction, without consideration of co-transformation and viability of the cells, which becomes limiting with *in vivo* expression. As shown in Figure 5, four DNA targets were added to a single reaction using the NEBExpress Cell-free *E. coli* Protein Synthesis System. All four targets were produced, in a clear band, although with a slightly reduced yield compared to experiments where only one plasmid is introduced. This demonstrates the potential for the NEBExpress system to make multiple proteins for the purpose of assembling a protein complex, or to introduce multiple enzymes to engineer a metabolic pathway.

FIGURE 5: **The NEBExpress Cell-free *E. coli* Protein Synthesis System can be used for simultaneous expression of multiple targets**

CFPS reactions of four different targets, expressed individually or in combination of two, three or four targets simultaneously. Specific bands are indicated with red dots. Synthesized proteins include: *E. coli* β-Galactosidase (βGal), 6-phospho-beta-glucosidase (BglA), venus green fluorescent protein (vGFP), dihydrofolate reductase (DHFR); negative control (neg; Lane 9). Reactions containing four simultaneously expressed targets produced four distinct bands at a slightly less yield.

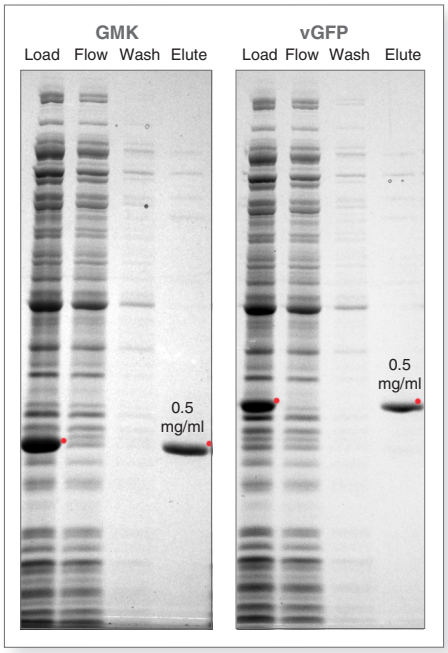


Purification of the protein product

Once protein is synthesized, it is beneficial to have a rapid method for purification, in order to be able to perform further characterization. Figure 6 shows two 50 µl NEBExpress reactions, synthesizing His-tagged green fluorescent protein (vGFP) and His-tagged guanylate kinase (GMK) that were purified using NEBExpress Nickel Spin Columns following the recommended standard protocol. The final 50 µl elution had a concentration of 0.5 mg/ml, demonstrating that cell-free protein synthesis can produce enough protein for functional and structural characterization.

FIGURE 6: **Purification of protein synthesized by the NEBExpress Cell-free *E.coli* Protein Synthesis System yields sufficient protein for downstream analysis**

SDS-PAGE gel showing two 50 µl CFPS experiments, synthesizing His-tagged guanylate kinase (GMK) and His-tagged green fluorescent protein (vGFP), purified using NEBExpress Nickel Spin Columns. Lanes show samples before purification (load), unbound material (flow), unspecific binding (wash) and bound (elute) purified sample.



Conclusion

A high-performing, versatile, and robust cell-free protein synthesis system offers the ability to rapidly produce a large number of proteins for further characterization. The NEBExpress Cell-free *E. coli* Protein Synthesis System was developed using genetically engineered *E. coli* strains, an optimized reaction buffer, and stringent manufacturing practices, and is capable of synthesizing proteins as large as 230 kDa. The yield, under optimal conditions, can reach milligrams per milliliter, with protein synthesis continuing for up to 24 hours. The versatility of this system makes it ideal for a variety of applications, including high throughput protein screening and engineering as well as synthetic biology.

Ordering Information

PRODUCT	NEB #	SIZE
NEBExpress Cell-free *E. coli* Protein Synthesis System	E5360S/L	10/100 reactions
PURExpress Disulfide Bond Enhancer	E6820S	50 reactions
NEBExpress GamS Nuclease Inhibitor	P0774S	75 µg

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