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Introduction

The NEBExpress™ Cell-Free *E. coli* Protein Synthesis System has been developed for coupled *in vitro* transcription and translation reactions resulting in high yields, including proteins with various sizes and origins.

Attributes which promote enhanced performance:

- A genetically engineered *E. coli* strain ensures stability of template DNA, RNA, and protein product.
- Fully accommodates varying reaction conditions, i.e., temperature and time, resulting in optimal synthesis of large size proteins up to 230 kDa
- Compatible with PURExpress Disulfide Bond Enhancer for better folding, and NEBExpress™ GamS Nuclease Inhibitor for enhanced yield from linear templates
- Reaction buffer formulation is compatible with SDS-PAGE (no acetone or TCA precipitation needed)
- Sustained reaction: protein synthesis can be sustained for 10 hours at 37 °C or up to 24 hours at lower temperatures
- Reproducible batches of lysate, using highly stringent biomanufacturing processes and quality standards

Methods

- **Materials:** NEBExpress™ Cell-Free *E. coli* Protein Synthesis System (NEB# E5360), GamS REcBCD Inhibitor (NEB# P0774), PURExpress® Disulfide Bond Enhancer (NEB# E6820), NEBExpress™ Ni-NTA Magnetic Beads (NEB# S1423).
- **Cell-free Protein Synthesis (CFPS):** reagents were combined in 1.5 mL micro-centrifuge tubes: 25 µL Protein Synthesis Buffer (2X), 1 µL T7 RNA Polymerase, 1 µL RNase Inhibitor, 12 µL NEBExpress S30 Synthesis Extract, 2 µL template (125 ng/µL), and water to 50 µL. Reactions were incubated with shaking for 3h at 37C (unless indicated).
- **Target list:** βGalactosidase from *Streptococcus* (β GalS), *E. coli* βGalactosidase (βGal), SP6 RNA polymerase (SP6), Firefly luciferase (Fluc), 6-phospho-beta-glucosidase (BglA), citrate synthase (Cit), venus green fluorescent protein (vGFP), guanylate kinase (Gmk), di-hydrofolate reductase (DHFR), calmodulin (CALML).
- **vGFP assay:** reagents were combined in 96 well plates (black, clear bottom) as follows: 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), and water to 50 µL. Reactions were incubated in a fluorescent reader with intermittent shaking at 37C for 5h (unless indicated). Detection: em 513 - ex 532, 6 flashes/read.
- **βGalactosidase assay:** CFPS samples were diluted 1:10 in water, 5 µL of each dilution was combined in with 200 µL of 5mM ortho-Nitrophenyl-β-galactoside (*onp*-βGal) in 50mM NaPO₄ pH 7 (Sigma). Samples were incubated at 37C for 30min, free o-Nitrophenol was measured by Abs 420nm.
- **Chitinase assay:** CFPS samples (1-10 µL) were mixed with 200 µL of 40µM 4-Methylumbelliferyl β-D-N,N',N"-triacetylchitotrioside (4-MU-chitotrioside) in 20 mM NaPO₄-Aco ~pH6, 200 mM NaCl (Sigma). Reactions were incubated at 37C for 30min, free 4-Methylumbelliferone was measured at em 513 - ex 532.
- **Linear DNA:** template was prepared by PCR using Q5® High-Fidelity 2X Master Mix (NEB #: M0492) as indicated (T7 promoter and terminator regions included). PCR product was purified using Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB#: T1030), concentration was adjusted to 250ng/µL.
- **RNA prep:** mRNA of vGFP was prepared using NEB HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB #: E2050) followed by purification using NEB Monarch® RNA Cleanup Kit (NEB #: T2030).
- **SDS-PAGE:** 2 µL of CFPS reaction were mixed with water and Blue Loading Buffer Pack (NEB# B7703), and loaded into 10/20% Tris-Glycine gels (Invitrogen), MW marker: Unstained Protein Standard, Broad Range (10-200 kDa) (NEB# P7717). Gels were stained with Coomassie Blue.
- **Protein purification:** His tagged proteins were purified using NEBExpress™ Ni Spin Columns (NEB# S1427) as directed. Eluted fractions were cleaned (to remove imidazole) using Zeba™ Spin Desalting Columns (Thermo), and protein concentration was measured using a nanodrop spectrophotometer (Thermo).

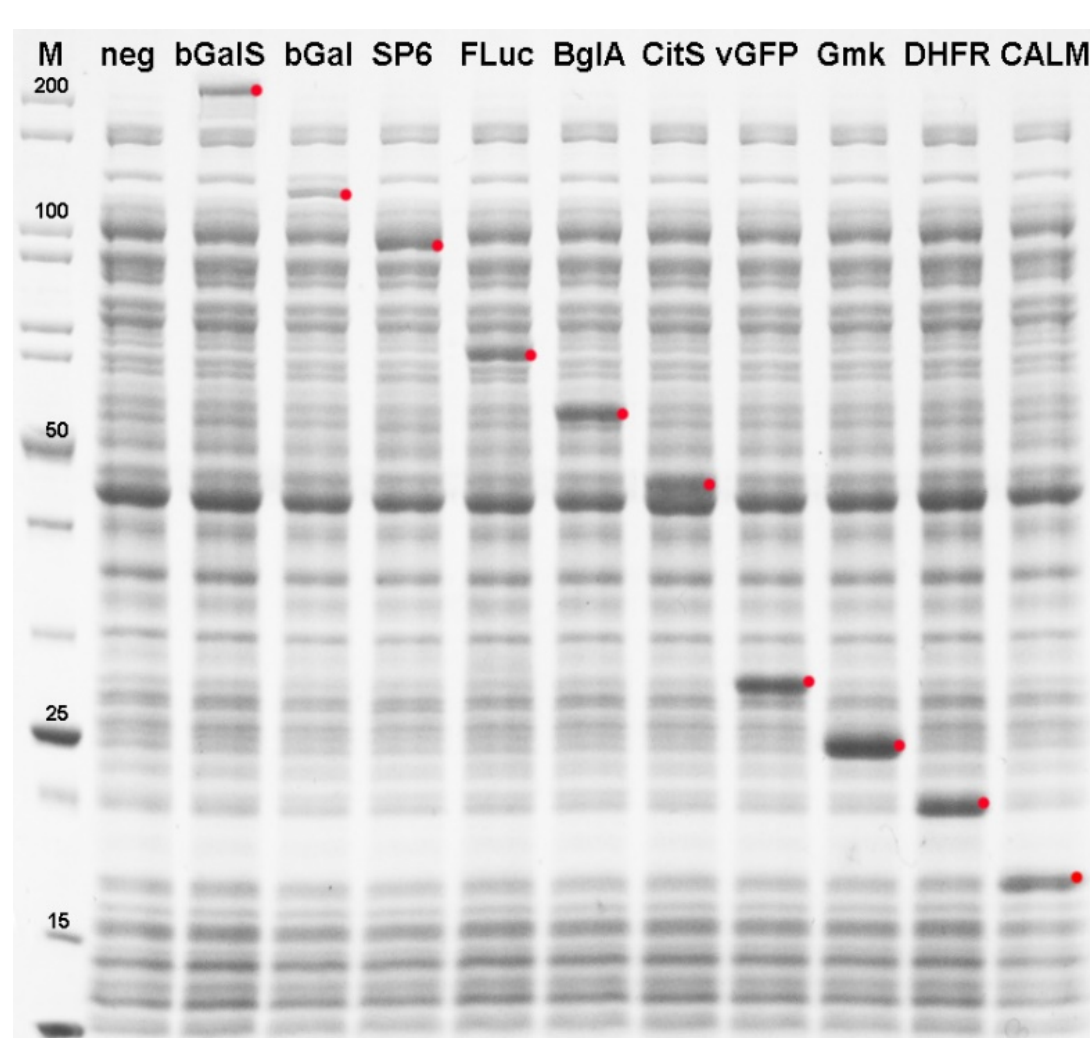
Results

Size of Proteins synthesized

- 17 to 230 kDa MW range

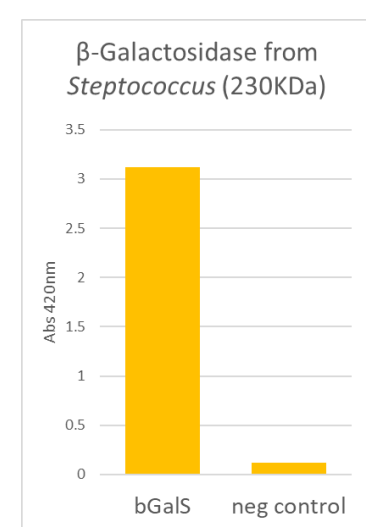
Protein analysis

- Load gel without TCA/acetone precipitation



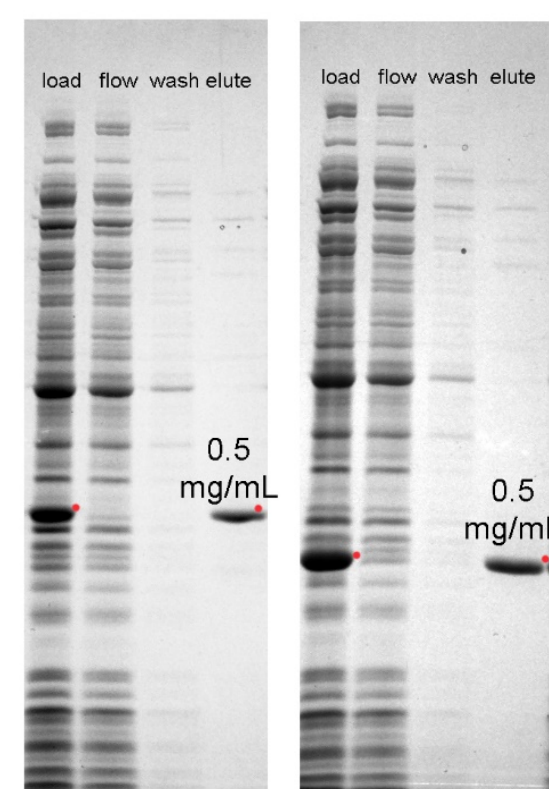
Active Protein

- Functional assay



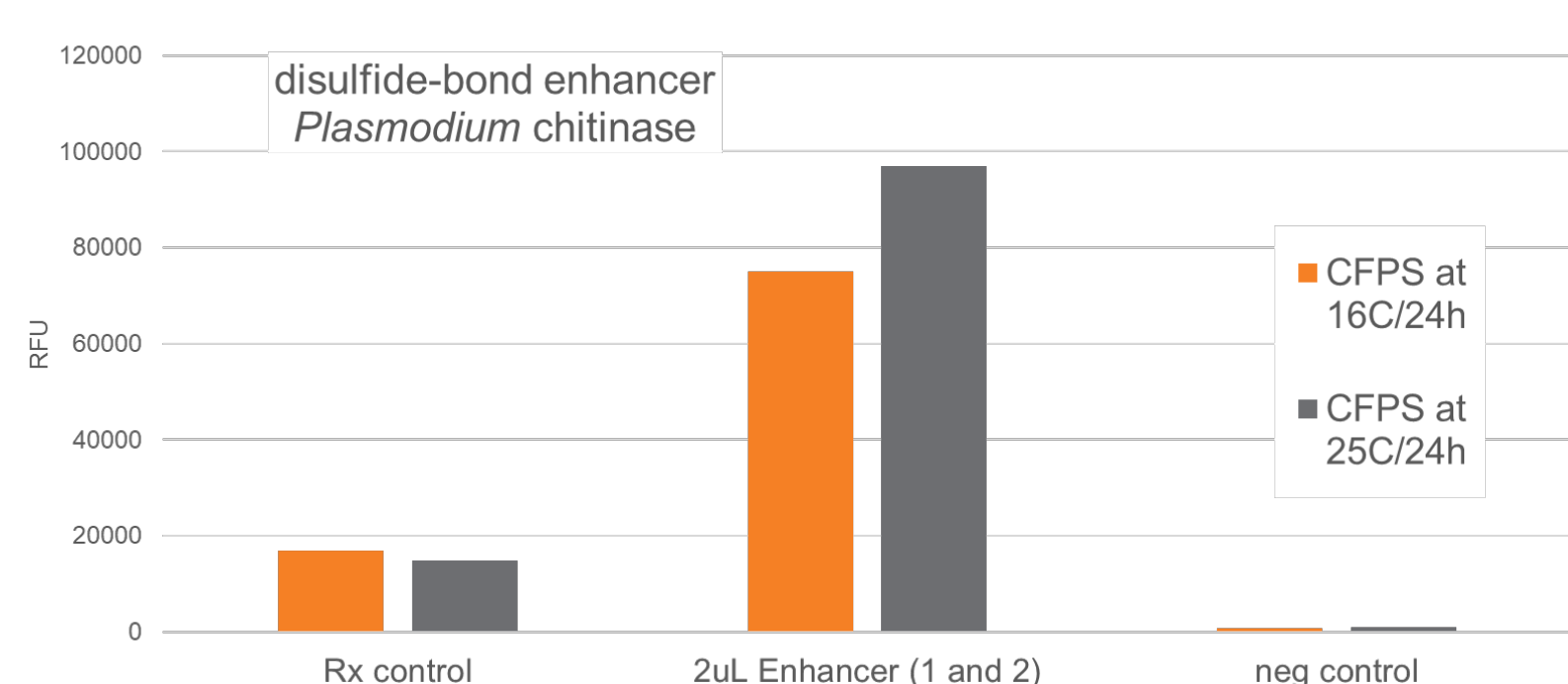
Yields, after Ni-NTA purification

- 0.5 mg/mL



Protein Folding

- PURExpress® Disulfide Bond Enhancer increases yield of active chitinase

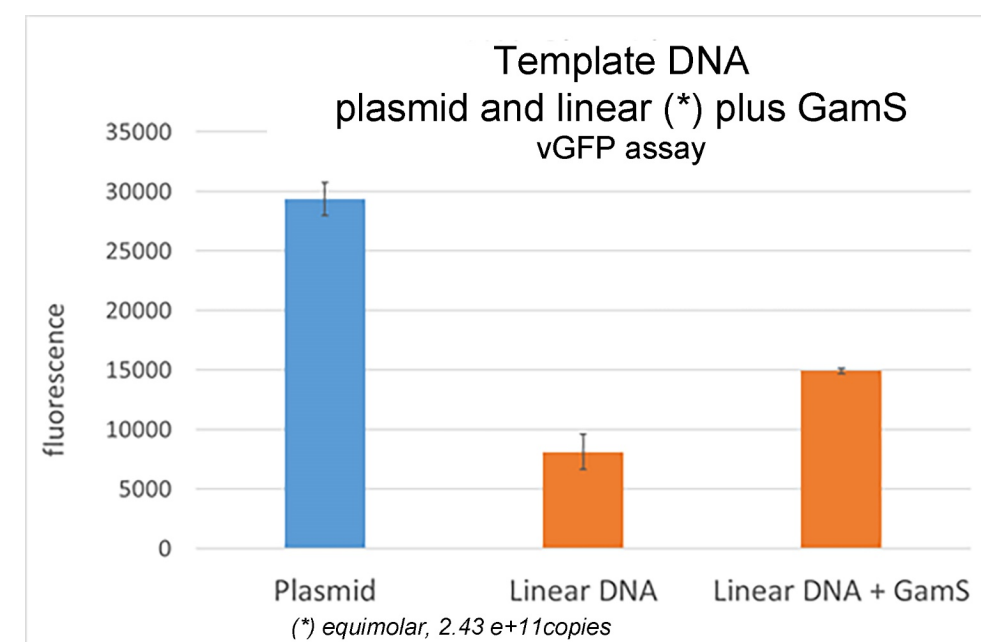


Results (continued)

DNA Templates

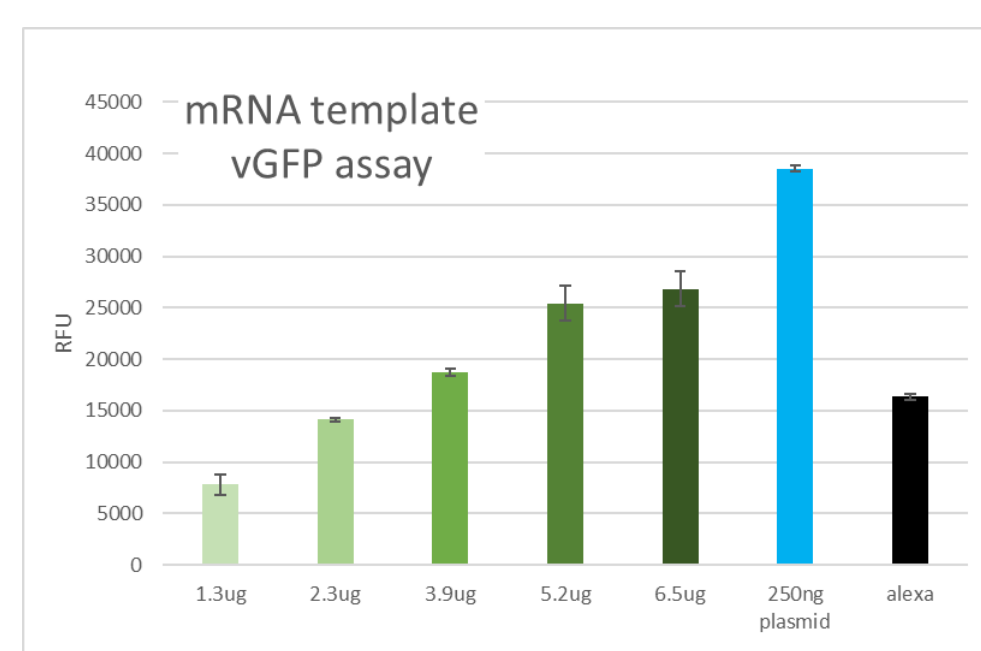
Circular and linear DNA

- PCR amplification: from sequence to functional protein in only a few hours.
- If time is not a constraint, highest yields can be achieved with plasmid DNA



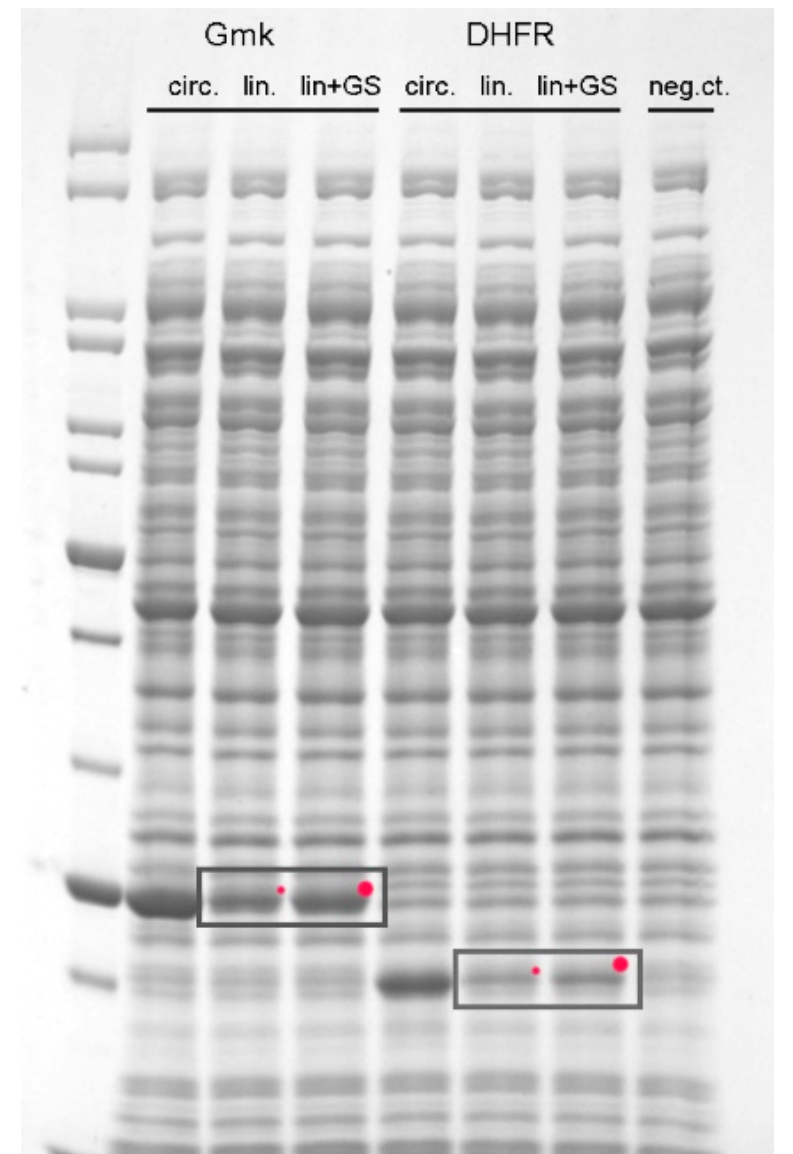
mRNA template

- RNA stability, sustained protein synthesis



GamS Nuclease Inhibitor

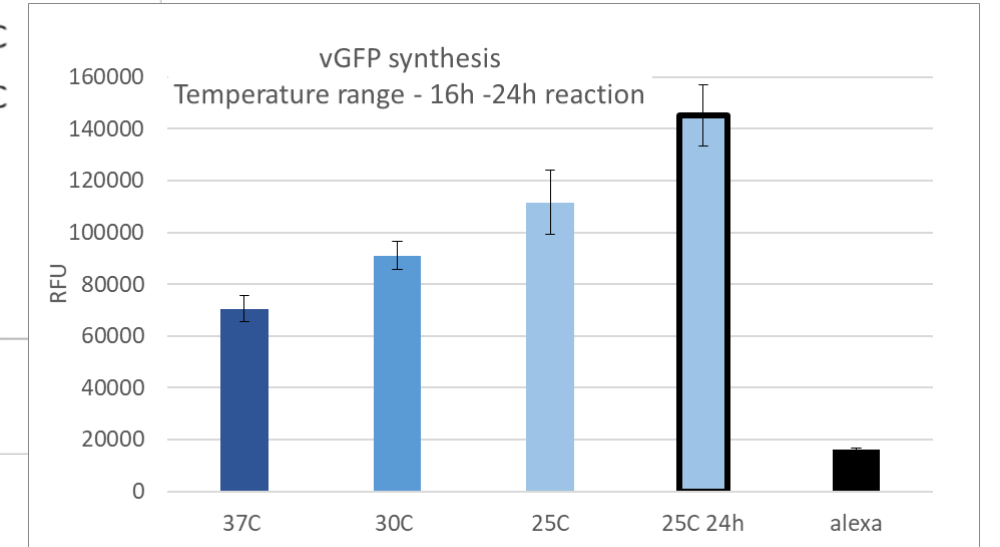
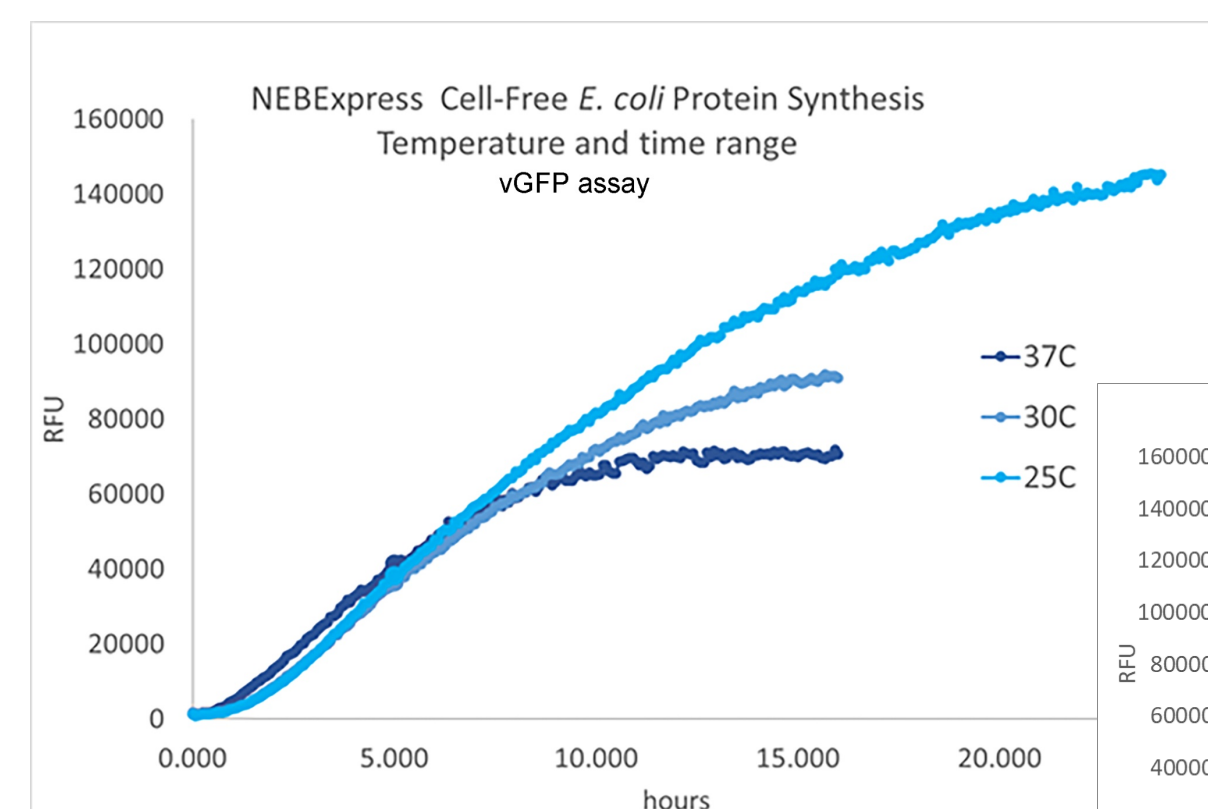
- Enhances yields with linear DNA



Reaction Conditions

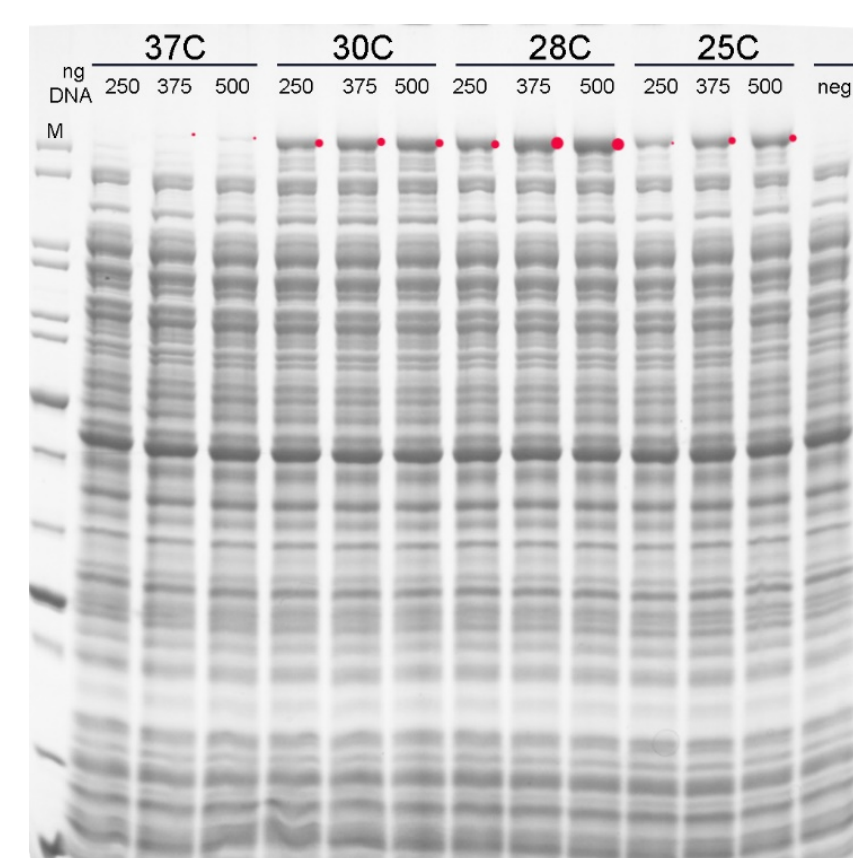
Incubation time

- Sustained synthesis and increased yields at lower temperatures for up to 24 hours



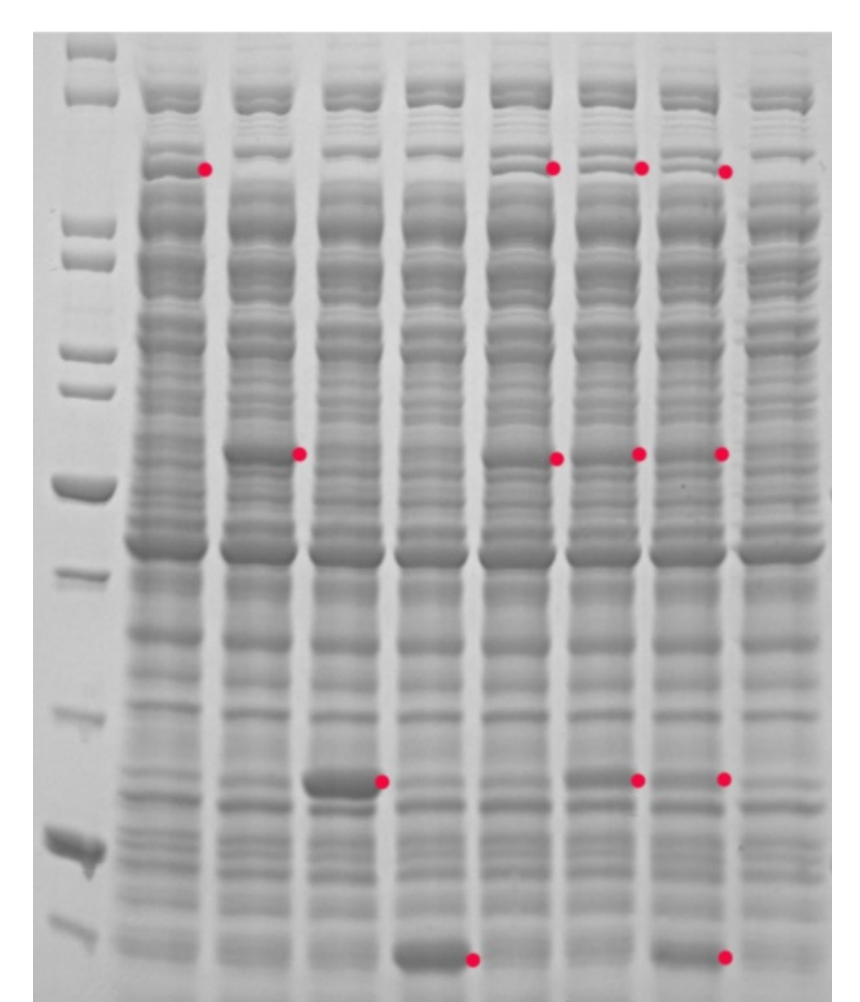
Time and Temperature

- Varying temperature can increase yields of high MW targets



Co-expression

- Simultaneous expression of four different targets



Conclusions

A high-performing, versatile, and robust cell-free protein synthesis system was developed by genetic engineering of *E. coli*, by optimizing a reaction buffer, and by employing stringent manufacturing practices. This system can be used for a variety of applications such as high throughput protein screening and engineering, as well as synthetic biology.

Acknowledgement

We thank Donald Comb, the founder of New England Biolabs, for his continued support to NEB's basic and applied research.