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Simplifying Progress

Performance Benchmarking of Commercial Cell-Free Expression Platforms

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Introduction

Cell-free protein synthesis (CFPS) has emerged as an interesting approach which can accelerate R&D workflows by providing rapid and simple protein screening. In comparison to cell-based expression, CFPS offers exceptional speed with weeks of cell-based protein expression timeline reduced to hours. Several CFPS platforms have been developed in the past decades and are defined by their starting cellular material or the host organism. Commercially available CFPS kits include systems derived from bacteria, mammalian HeLa cells, insect cell cultures, wheat germ extract (WGE), reticulocytes from rabbit blood, cultured tobacco BY-2 cells and Leishmania. While it is acknowledged that significant differences in performance among systems exist, no comprehensive objective comparison has been done to thoroughly test the capabilities of the systems using different classes of proteins. Here, we test the most promising commercially available CFPS kits (based on bacterial, mammalian, plant, insect and Leishmania lysates) using three distinct protein classes, namely a cytosolic fluorescence reporter, a monoclonal antibody and an enzyme for different performance parameters including protein amounts, post-translational modifications and activity.

1. Selection of Proteins for Benchmarking of the CFPS Systems

We selected three different proteins based on their different characteristics to challenge the different systems.. They are summarized below:

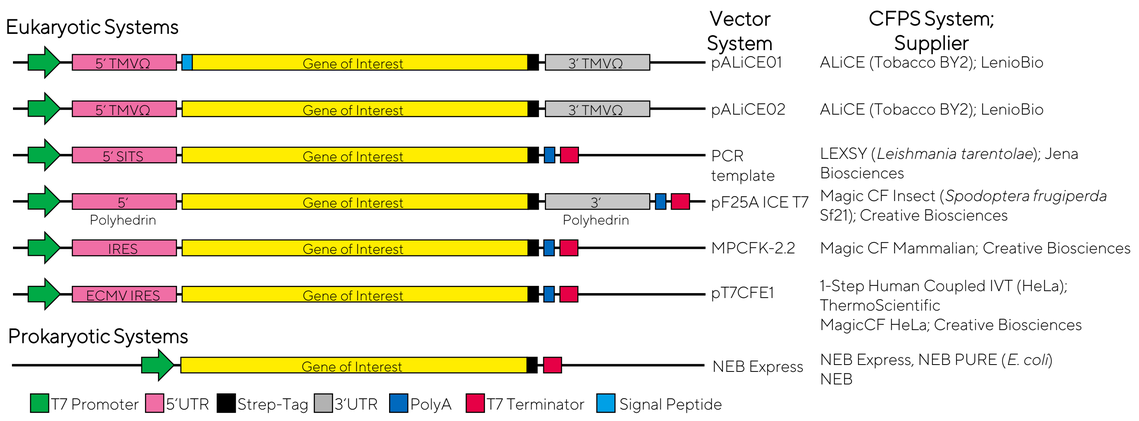
Enhanced Yellow Fluorescent Protein (eYFP)
- Simple cytosolic protein
- Standard used for CFPS development
- Easy detection and measurement

Monoclonal Antibody - Humira (Adalimumab)
- Therapeutic Protein of high interest
- Microsomal protein requiring post-translational modifications for full functionality
- Hetero-tetramer with intramolecular and intermolecular disulphide bonds
- Single N-Glycan Site
- Commercially available measurement/binding assays

Glucose Oxidase (GOx)
- Microsomal enzyme requiring post-translational modifications for full functionality
- Dimerization through disulphide bonding needed for function
- 8 N-Glycan sites and glycosylation necessary for function
- Commercially available quick functional test using colorimetric assay

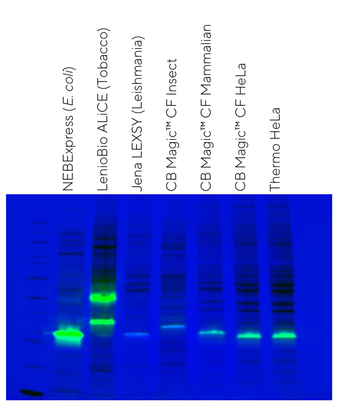
2. Commercial CFPS Systems and Cloning

Gene of Interest (GOI) gene blocks were synthesized and cloned into the respective vector systems provided with the CFPS Kits using Gibson homology cloning. For microsomal protein expression in ALiCE, genes were cloned into pALiCE02 vector to provide the nascent peptides with a signal peptide. All plasmid templates were purified using midi plasmid preparation protocol for maximum purity. Templates for the LEXSY system were generated using overlapping PCR and purified using NEB Monarch Kit.

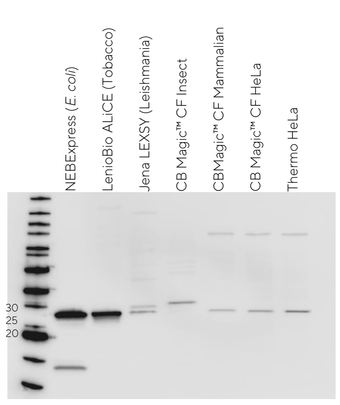


3. eYFP Expression

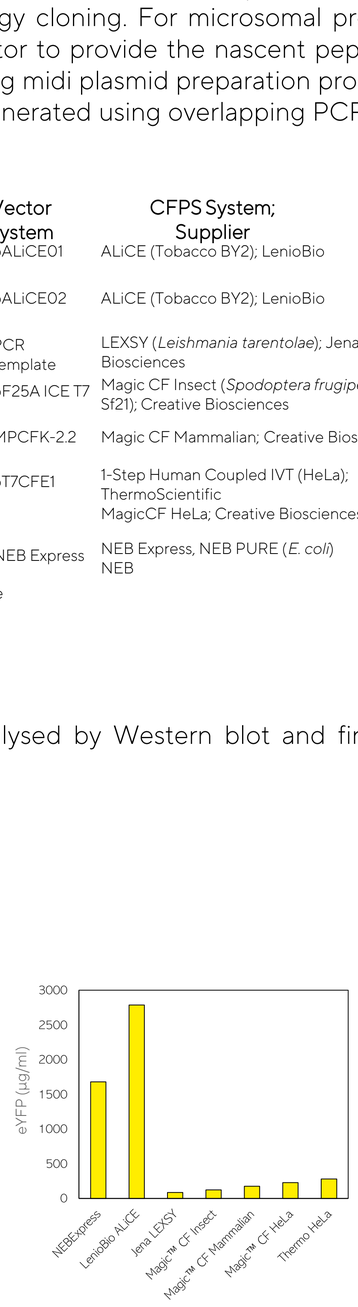
eYFP expression was monitored on SDS-gel as well as analysed by Western blot and finally measured using fluorescence spectrophotometry.



Merged image of SDS-PAGE gel fluorescent scan (Ex/Em 488/507 nm) + Stain-free gel scan. Unreduced and non heat-denatured samples were loaded onto the gel to retain fluorescence



Western Blot with anti-Strep Antibody done on heat-denatured samples

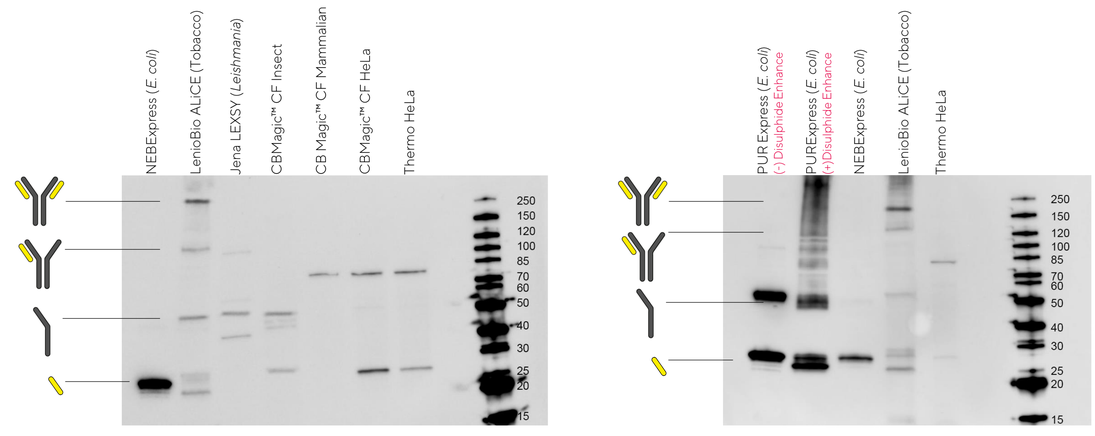


Conclusions from the eYFP expression analysis:

1. The bacterial and tobacco systems produced the highest amounts of eYFP - the bacterial system produced ~1500 µg/ml of eYFP after 3 Hours and the tobacco system produced ~2700 µg/ml of eYFP in 24 Hours.
2. The mammalian, HeLa, Leishmania and insect systems produced 10-20 times lesser eYFP compared to the tobacco system (50-250 µg/ml). Among these systems, the ThermoScientific HeLa system produced the highest amounts of eYFP (~250 µg/ml)

4. Humira (Adalimumab) Expression & Disulphide Bonding

For Humira expression, the heavy and the light chains were cloned separately, and equimolar amounts of template were added to the lysates. The NEB PURExpress system was added to this analysis as it allows disulphide bonding through the addition of disulphide enhancers to the reaction.



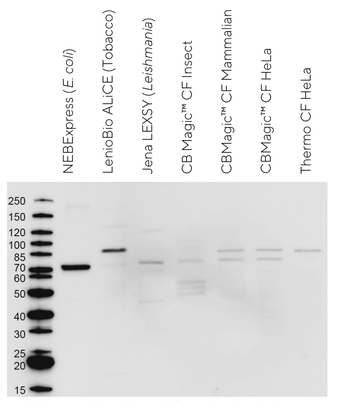
Western Blot with anti-Strep Antibody done on heat-denatured non-reduced samples

Following deductions can be made from Humira expression using the CFPS systems:

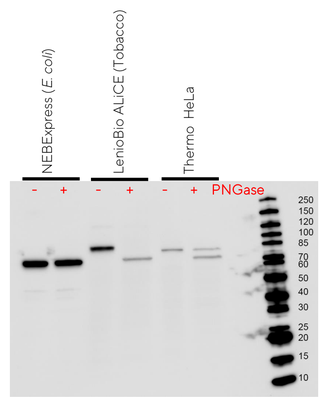
1. The mammalian, insect, *Leishmania* and HeLa systems produce very low amounts of light and heavy chains that are not disulphide bonded.
2. The mammalian and HeLa systems produced no detectable amounts of heavy chain.
3. The *E. coli*-based NEB Express is biased towards producing high amounts of the light chain molecule.
4. The NEB PURExpress produced highest amounts of both the heavy and the light chain which could be partially disulphide bonded to form heteromers using disulphide enhancer.
5. Only the tobacco-based system produced detectable amounts of fully formed heterotetrameric monoclonal antibody.

5. GOx Expression, PNGase Assay and Functional Analysis

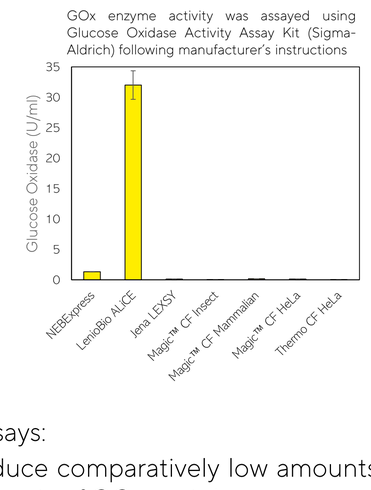
Since GOx is glycosylated on 8 N-glycan sites, the glycosylated and non-glycosylated forms of the protein can be easily separated on SDS-gels. Moreover, GOx enzyme activity can be easily assayed using commercially available chromogenic tests.



Western Blot with anti-Strep Antibody done on heat-denatured and reduced samples



Samples were treated with PNGaseF (NEB) followed by Western Blot analysis using anti-Strep Antibody



Following deductions can be made from GOx expression and assays:

1. The mammalian, insect, *Leishmania* and HeLa systems produce comparatively low amounts of GOx. The *E. coli* and Tobacco systems produced highest amounts of GOx protein.
2. The E. coli, Leishmania and insect systems produce only non-glycosylated GOx.
3. PNGase assay confirmed that the tobacco system produces fully glycosylated protein, the HeLa system produced partially glycosylated GOx whereas the *E. coli* system produces only non-glycosylated protein.
4. Chromogenic enzyme assay showed that only the tobacco based ALiCE system was able to produce functional protein indicating that the GOx produced in tobacco lysate is disulphide bonded as well as glycosylated.

6. Conclusions

- The bacterial and tobacco lysates outperform the current alternatives in terms of protein amounts and price.
- The tobacco system is the most versatile among the tested ones in terms of protein amounts, functionality and post-translational modifications.
- The mammalian lysates, although of high interest for therapeutic proteins, are true underperformers in terms of expression levels and protein functionality. Further development of mammalian CFPS would be of interest to the protein expression community in general.