

Use of the PURExpress[®] *In Vitro* Protein Synthesis Kit, Disulfide Bond Enhancer and SHuffle[®] Competent *E. coli* for heterologous *in vitro* and *in vivo* cellulase expression

Richard Cohen, Charles Emrich, Nitzan Koppel, Nicholas Toriello and Robert Blazej. Allopatis Biotechnologies, Inc.

Introduction

Advances in industry and medicine have led to the engineering of complex “designer” proteins, such as antibodies in targeted therapeutics and enzymes in process development. The ability to easily generate an almost infinite number of variants at the DNA level has increased the demand for improved protein expression methodologies to fully capture what can be produced genetically. Often, the protein of interest is eukaryotic in origin and may require posttranslational modifications specific to its native host or may be toxic to the host cells expressing them. Cell-free protein expression systems have allowed us to step beyond the limits of traditional *in vivo* expression methodologies by decoupling protein expression from host cell viability (1,2,3). Furthermore, the ability to produce complex proteins using cell-free transcription/translation systems uniquely enables high-throughput directed evolution and protein engineering efforts (4,5). Several cell-free protein expression systems have been developed in the last decade with recent advances focusing on special folding or assembly environments (6,7,8). Equally as important is the capability to transition from the *in vitro* system to larger-scale *in vivo* expression, while maintaining activity of the target protein (9,10).

The PURExpress *In Vitro* Protein Synthesis Kit, supplemental PURExpress Disulfide Bond Enhancer, and SHuffle Competent *E. coli* from New England Biolabs provide a seamless system for *in vitro* to *in vivo* protein expression. PURExpress is a novel cell-free transcription/translation system reconstituted from the purified components necessary for *E. coli* translation. The nuclease-free and protease-free nature of the PURExpress platform preserves the integrity of DNA and RNA templates and results in proteins that are free of modification and degradation. The PURExpress Disulfide Bond Enhancer (PDBE) is a blend of proteins and buffer components designed to correctly fold target proteins with multiple disulfide bonds produced in PURExpress reactions. These enhancements can increase the yield of soluble and functionally active complex proteins containing disulfide bonds. Target proteins expressed in the PDBE/PURExpress environment can then be transitioned to *in vivo* expression using SHuffle *E. coli* strains. These engineered strains are capable of expressing proteins with increasing disulfide bond complexity in the cytoplasm. SHuffle strains express a disulfide bond isomerase that isomerizes mis-oxidized substrates into their correctly folded state, greatly enhancing the fidelity of disulfide bond formation. When used in conjunction, these three products increase the likelihood of generating fully active, complex proteins with multiple disulfide bonds.

Cellulases are glycoside hydrolases that catalyze the cleavage of β -1,4-D-glycosidic linkages in cellulose, a linear polymer of glucose units. Cellulases are often multidomain proteins consisting of a catalytic core, a flexible linker and a carbohydrate-binding module. Bacterial and eukaryotic cellulase mixtures perform key functions in the conversion of lignocellulosic biomass into fermentable sugars for renewable chemical and biofuel production, textile processing, and detergent formulations. EG3 from the fungi *Humicola insolens*, an endoglucanase that creates internal glucan chain scissions, is a multi-domain 42 kDa protein containing a carbohydrate-binding module (CBM1) having two non-consecutive disulfide bonds connected by a flexible linker to a catalytic core (GH5) with one disulfide bond (based on the predicted crystal structure; See Figures 1 and 2). This enzyme is used as a detergent cellulase for color brightening, softening and soil removal. The capabilities of the PDBE/PURExpress system with downstream expression in SHuffle *E. coli* strains are well-matched to the expression of this complex fungal protein in a flexible *E. coli*-based system.

(see other side)

DNA CLONING

DNA AMPLIFICATION & PCR

EPIGENETICS

RNA ANALYSIS

LIBRARY PREP FOR NEXT GEN SEQUENCING

PROTEIN EXPRESSION & ANALYSIS

CELLULAR ANALYSIS

Materials

- PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800)
- PURExpress Disulfide Bond Enhancer (NEB #E6820)
- SHuffle T7 Competent *E. coli* (NEB #C3026)
- Mature chain *Humicola insolens* EG3 (UniProt #Q12624, amino acids 17 – 388) codon optimized for *E. coli* cloned into a suitable expression vector.
- 3,5-dinitrosalicylic acid (Sigma Aldrich #D0550)
- CMC solution: 1.1% w/v Carboxymethylcellulose Sodium Salt (Sigma Aldrich #21902) in 50 mM HEPES, pH 7.4
- Potassium Sodium Tartrate Tetrahydrate (Acros Organics #20286)
- MagicMedia™ (Life Technologies #K6801)
- BugBuster Plus (Novagen) with protease inhibitors; 1 μ g/ml pepstatin, leupeptin, 1 mg/ml lysozyme (Sigma P4265, L2884, L6876)
- 96-well spectrophotometer and plates (Guenier #67518)

Figure 1: *H. insolens* EG3 carbohydrate-binding module showing two non-consecutive disulfide bonds (cysteines highlighted in yellow) (Residues 19 to 52, SWISS-MODEL prediction based on template: 1az6).

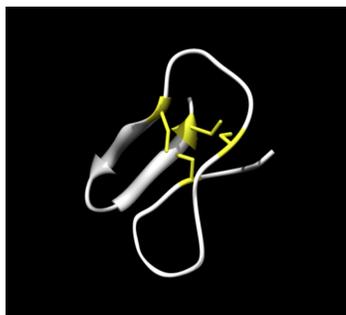
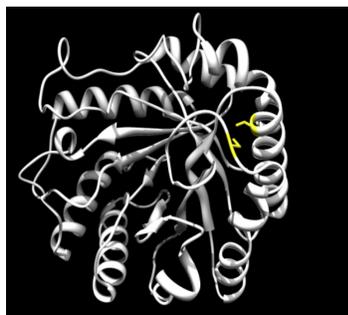


Figure 2: *H. insolens* EG3 catalytic core, a family 5 glycoside hydrolase, contains one disulfide bond (cysteines highlighted in yellow) (Residues 86 to 387, SWISS-MODEL prediction based on template: 1gzj).



General Protocol

in vitro Expression:

Thaw PURExpress tubes A, B, and Disulfide Bond Enhancer (PDBE 1 and 2) on ice. Adjust template DNA to 100 ng/μl.

Assemble reactions on ice in nuclease-free, 0.5 ml microcentrifuge tubes as indicated*:

TUBE	1	2	3	4	5
H ₂ O	7.5 μl	6.5 μl	4.5 μl	1.5 μl	3.5 μl
PURExpress Solution A	12.5 μl				
PURExpress Solution B	5.0 μl				
PDBE 1**	0.0 μl	0.0 μl	1.0 μl	1.0 μl	2.0 μl
PDBE 2**	0.0 μl	0.0 μl	1.0 μl	4.0 μl	1.0 μl
Template DNA	0.0 μl	1.0 μl	1.0 μl	1.0 μl	1.0 μl

* Refer to current PURExpress (<http://www.neb.com/nebecomm/products/productE6800.asp>) and PDBE (<http://www.neb.com/nebecomm/products/productE6820.asp>) manuals for latest protocols, as recommended volumes and incubation times have been updated.

** Typical starting amounts are 1 μl for both PDBE 1 and 2. Titration of reagent may be necessary.

Incubate the reactions at 37°C for 1 hour to express and fold the protein. Cool and store at 4°C to stop reaction.

in vivo Expression:

To produce larger quantities of purified enzyme, clone the *E3* gene into a suitable expression plasmid and transform into SHuffle using the protocol found on the NEB website (<http://www.neb.com/nebecomm/products/protocol390.asp>). Plate onto antibiotic selection plates and incubate for 24 hours at 37°C. Resuspend a single colony in 3 ml LB containing antibiotic and grow the starter culture overnight at 37°C. Inoculate 50 ml MagicMedia (Life Technologies) with 50 μl of the starter culture and grow in 250 ml baffled flasks at 37°C until reaching 1 OD, upon which transfer to growth at 25°C for a total of 24 hours.

Harvest cells by centrifugation at 3,000 rpm for 30 minutes at 4°C. Resuspend cell pellets in BugBuster Plus solution containing protease inhibitors and lysozyme [see note 1, page 3] at 4 ml/g wet cell mass. Lyse for 30 minutes according to the manufacturers protocol. Affinity purify EG3 from the lysate (e.g. immobilized metal affinity purification), quantitate by using absorbance at 280 nm, and assess for purity by SDS-PAGE.



Assay:

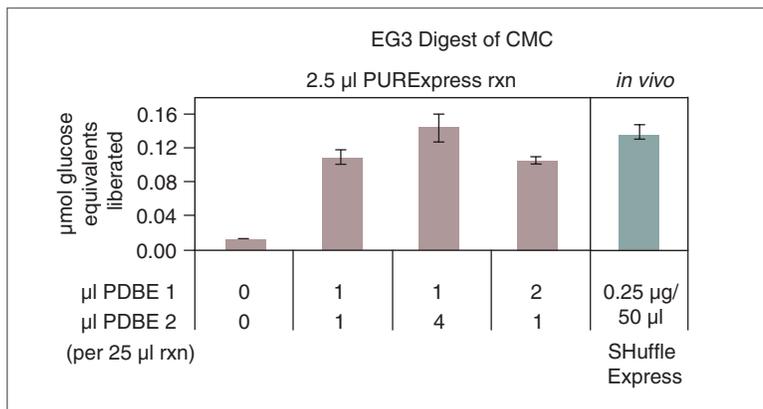
For each *in vitro* expression reaction, prepare triplicate CMC digestions in 200 µl PCR tubes as follows: Combine 45 µl 1.1% w/v CMC solution with 2.5 µl of the *in vitro* expression reaction and 2.5 µl water (1% CMC final). Similarly, combine 0.25 µg purified *in vivo*-expressed enzyme with 45 µl 1.1% w/v CMC solution and water to a final 50 µl volume. Place the tubes in a thermal cycler with a heated lid and incubate at 50°C for 1 hour. Cool and hold at 4°C.

Prepare DNS solution [see note 2]. Transfer 120 µl DNS solution to each CMC reaction and mix gently by pipetting. Cap the PCR tubes and heat the reactions to 95°C for 5 minutes with the lid set to 105°C. Cool the reaction to room temperature. Transfer 100 µl from each reaction to a half-area spectrophotometer plate and read the absorbance at 540 nm. Similarly processed glucose standards may be used to calculate liberated glucose-equivalents in the CMC digestion reactions. Dispose of residual DNS solution and used pipette tips in appropriate chemical waste.

Results

Active *Humicola insolens* EG3 was successfully expressed in *in vitro* transcription and translation (IVTT) reactions by using New England Biolabs' PURExpress *In Vitro* Synthesis Kit. The addition of NEB's PURExpress Disulfide Bond Enhancer (PDBE) increased enzyme activity 13-fold. SHuffle Competent *E. coli* provide a quick path to milligram quantities of difficult-to-express eukaryotic proteins in *E. coli*. Several growth conditions, including temperature, media, and inoculum volume were investigated for optimal EG3 expression. Using the protocol described above, soluble, active yields of 46 mg/L were achieved from SHuffle cells, significantly greater than titers from BL-21 lines (≥ 0.6 mg/L). Figure 3 presents the relative activities of EG3 expressed *in vitro* with varying amounts of PDBE supplementation and *in vivo* by using SHuffle cells.

Figure 3. Liberated glucose-equivalents assay showing the effect of PDBE supplementation in the *in vitro* expression and the activity relative to 0.25 µg *in vivo*-expressed EG3.



Notes

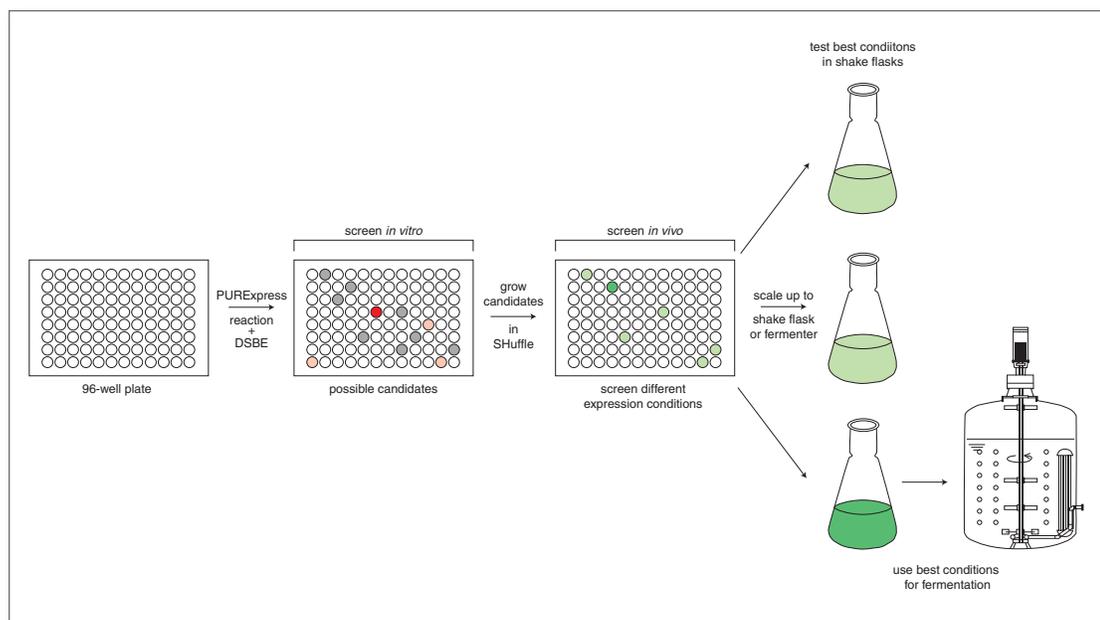
1. Stock BugBuster solution. Add the following to 40 ml BugBuster: 400 µl 100 mM PMSF, 40 µl 1mg/ml pepstatin, 40 µl 1 mg/ml leupeptin and 40 mg lysozyme.
2. Preparation of stock 3,5-dinitrosalicylic acid (DNS) solution: combine 5 g 3,5-dinitrosalicylic acid and 250 ml H₂O. Heat to 40°C, while stirring, add 150 g potassium sodium tartrate tetrahydrate. Add 50 ml 4N NaOH. Bring up to 500 ml with H₂O. Store at room temperature in brown, light-proof bottles.

Summary

The PURExpress *In Vitro* Protein Synthesis Kit supplemented with PURExpress Disulfide Bond Enhancer was used to express active *Humicola insolens* EG3, a multi-domain fungal cellulase containing three disulfide bonds, from a synthetic gene construct. Useful quantities of EG3 were produced directly from the *in vitro* expression reaction. Direct scale-up to milligram quantities was made possible by transferring the gene construct into SHuffle Competent *E. coli*.

These tools can be utilized in a general workflow where the high throughput screening and selection of variants of a complex protein of interest can be done in a cell-free environment (Figure 5). The successful variant can then be expressed *in vivo* by using a specialized *E. coli* strain that also facilitates the required protein folding needs. In this case, the formation of complex disulfide bonds with the correct pairing was crucial to successful expression of active EG3 in both *in vitro* and *in vivo* expression environments.

Figure 5. Workflow illustration of *in vitro* protein expression and screening followed by *in vivo* expression and scale up.



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