

## Small-Scale Expression Testing

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The SMALL-SCALE EXPRESSION TESTING (SSET) Team, headed by Brian Fox, PhD, is responsible for small-scale protein expression trials. Trials are used by staff to evaluate the use of lowered temperature, different medium compositions, and different host strains on protein expression and stability.

### Goals for CESH Small-Scale Expression Testing

- Develop small-scale screening methodologies that are rapid, simple, and efficient.
- Identify protein targets that are statistically likely to proceed through all downstream processing stages.
- Yield sufficient sample for functional studies and preliminary structural analysis.
- Minimize costs while maximizing the output of soluble proteins suitable for purification.

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### Overview of Small-Scale Expression Testing at CESH

One of the main goals of the NIH Protein Structure Initiative is to develop high-throughput technologies to assist protein structure determination. CESH has developed a rapid, small-scale, high-throughput screening method for identifying positively expressed cloned eukaryotic genes (from *Arabidopsis thaliana*, rice, human, yeast, zebrafish, mouse, and others) suitable for downstream large-scale protein production and subsequent protein purification efforts. The genes are produced with maltose-binding protein (MBP) as an N-terminal fusion to enhance solubility and folding.

CESH small-scale methodologies include expression vector engineering, optimization and improvement of auto-induction medium using factorial evolution techniques, and automated protein production analysis. These approaches have been used to identify the best eukaryotic proteins (*Arabidopsis*, rice, human, yeast, zebrafish, mouse, and algae) for large-scale cell growths (2-liter), and also yield sufficient sample for functional studies and preliminary structural analysis. High-throughput processing of workgroups of 96 eukaryotic cloned genes are processed and screened in parallel, on the small-scale, for positive expression, solubility, and TEV protease cleavage using the *E. coli* host strain B834-pRARE2. Large-scale growths are used to produce isotopically enriched proteins for IMAC purification leading to samples for structural analysis by NMR spectroscopy and X-ray crystallography.

The CESH cell-based small-scale methodologies have proven to be rapid, simple, efficient, and cost-effective. The approaches developed provide information on the level of expression, the potential for *in vivo* degradation, the solubility of the MBP fusion, the ability of TEV protease to cleave the fusion, and the yield of cleaved and purified target protein. The small-scale screening method is flexible and assessments of the above mentioned properties are made prior to committing valuable resources for downstream processes. Over a two-year period, this approach has successfully predicted proteins that are suitable for large-scale production and purification with ~80% accuracy. In summary, the CESH small-scale purification screening approach saves considerable resources and labor, and reduces expenditure of resources on proteins targets that will ultimately fail in protein purification.

CESH's strategy is to minimize costs and maximize the output of soluble proteins suitable for purification uses small-scale screening methodologies to identify protein targets that are statistically

likely to proceed through all downstream processing stages (large-scale protein production and generic IMAC purification). Passage through the entire cell-based protein production process, beginning with gene cloning, requires ~2-3 months to deliver ~10 mg of purified, isotopically enriched protein for either NMR spectroscopy screens or X-ray crystallization trials. Small-scale screening identifies several important features of cloned eukaryotic proteins that together indicate whether the proteins will be "suitable" for structural studies.

## Methods Being Investigated and Publications

### + Expression Plasmid Design

All protein targets are first produced as MBP-fusion protein in a single vector (pVP16) in the methionine auxotroph *E. coli* strain, B834-pRARE2 (metE-) (Studier, 2005 and Frederick et al., 2007), using seleno-methionine medium (5SM), and then purified using a generic immobilized metal-chelating chromatography (IMAC) scheme. In small-scale screening, the production of MBP-target protein fusions are analyzed by SDS-PAGE to evaluate their levels of expression, solubility, and TEV protease cleavage. The cloned eukaryotic genes are expressed under control of either a T5 or T7 phage based promoter that is lactose inducible. The expressed proteins are fused to an N-terminal (His)<sub>n</sub>-tagged (n=6 or 8) maltose-binding protein (MBP), which enhances solubility and expression. The construct includes a TEV protease cleavage site (located between the MBP and target protein). The modular architecture of the expression plasmid allows for flexibility for future design modifications. For example, the MBP segment can now be readily replaced with many other solubility enhancers such as glutathione S-transferase (GST), thioredoxin or NusA. The *E. coli* host strain, B834-pRARE2 (supplemented with seven rare aminoacyl tRNAs) is used for both small scale expression screening and for large-scale protein production (2-L cell growths).

### + Small-Scale Semi-Automated Purification of Eukaryotic Proteins for Structure Determination

A simple approach that allows cost-effective automated purification of recombinant proteins in levels sufficient for functional characterization or structural studies is described. Studies with four human stem cell proteins, an engineered version of green fluorescent protein, and other proteins are included. The method combines an expression vector (pVP62K) that provides *in vivo* cleavage of an initial fusion protein, a factorial designed auto-induction medium that improves the performance of small-scale expression, and rapid, automated metal affinity purification of His<sub>8</sub>-tagged proteins. For initial small-scale expression screening, single colony transformants were grown overnight in 0.4 mL of auto-induction medium, expressed proteins were purified using the Promega Maxwell 16, and purification results were analyzed by Caliper LC90 capillary electrophoresis. The yield of purified [U-<sup>15</sup>N]- His<sub>8</sub>-Tcl-1 was 7.5 ug per mL of culture medium, of purified [U-<sup>15</sup>N]-His<sub>8</sub>-GFP was 68 ug per mL, and of purified selenomethione- labeled AIA-GFP (His<sub>8</sub> removed by treatment with TEV protease) was 345 ug per mL. The yield information obtained from a successful automated purification from 0.4 mL was used to inform the decision to scale-up for a second meso-scale (10-50 mL) cell growth and automated purification. <sup>1</sup>H-<sup>15</sup>N NMR HSQC spectra of His<sub>8</sub>-Tcl-1 and of His<sub>8</sub>-GFP prepared from 50 mL cultures showed excellent chemical shift dispersion, consistent with well-folded states in solution suitable for structure determination. Moreover, AIA-GFP obtained by proteolytic removal of the His<sub>8</sub> tag was subjected crystallization screening, and yielded crystals under several conditions. Single crystals were subsequently produced and optimized by the hanging drop method. The structure was solved by molecular replacement at a resolution of 1.7 Å. This approach provides an efficient way to carry out several key target screening steps that are essential for successful operation of proteomics pipelines with eukaryotic proteins: examination of total expression, determination of proteolysis of fusion tags, quantification of the yield of purified protein, and suitability for structure determination.

Blommel, P.G., Martin, P.A., Wrobel, R.L., Steffen, E., Fox, B.G. (2006) **High efficiency single step production of expression plasmids from cDNA clones using the Flexi Vector cloning system.** *Protein Expr Purif* 47(2):562-70, 562-570. |[16377204](#)|

Blommel, P.G., Becker, K.J., Duvnjak, P., Fox, B.G. (2007) **Enhanced bacterial protein expression during auto-induction obtained by alteration of lac repressor dosage and medium composition.** *Biotechnol Prog* 23(3):585-98. |[17506520](#)|

Frederick, R.O., Bergeman, L., Blommel, P.G., Bailey, L.J., Song, J., Meske, L., Bingman, C.A., Ritters,

M., Dillon, N., Kunert, J., Yoon, J., Lim, A.-Y., Cassidy, M., Bunge, J., Aceti, D.J., Primm, J.P., Markley, J.L., Phillips, G.N., Jr., Fox, B.G. (2007) **Small-scale, semi-automated purification of eukaryotic proteins for structure determination.** *JSFG* 8(4):153-66. | [17985212](#) |

**+ Improved Methodologies Presently Being Investigated**

Several improved methodologies are being researched by CESG to improve protein production efficiency and increase the output of protein structures by the pipeline. Some of these methodologies aim to improve the ability of the small-scale screen to accurately predict events further down pipeline into the protein purification section. To achieve these goals, CESG has developed new technologies for expressing and purifying sufficient target protein directly from the small-scale screens for initial biophysical analysis, and meso-scale protein production for NMR and X-ray analysis.

We have combined two basic improvements at several steps of our screening pipeline whose two relevant underlying technologies: (1) factorial evolution of auto-induction medium to improve growth and expression; and (2) expression vector engineering to better match performance in small- and large-scale cell growths. Factorial evolution of the auto-induction medium revealed new combinations of carbon sources that were better matched to CESG pipeline activities. Furthermore, by altering the promoter for Lac repressor (provided by LacIQ-type plasmids, such as the pQE80 (Qiagen) derivatives used in the CESG), we obtained close correlation between small- and large-scale expression trials, while also increasing total protein expression.

## Small-Scale Expression Testing Team

- Brian Fox, PhD (PI)
- Ronnie Frederick, PhD
- Katarzyna Gromek, PhD
- Lai Bergeman

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Center for Eukaryotic Structural Genomics (CESG), Department of Biochemistry  
University of Wisconsin-Madison, 445 Henry Mall, Madison, WI 53706 | [Map](#) |  
Telephone: 608.263.2183 Fax: 608.890.1942 Email: [cesginfo@biochem.wisc.edu](mailto:cesginfo@biochem.wisc.edu)

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