

High-Throughput Production of Unlabeled, Selenomethionine and ¹⁵N-Labeled Proteins in Terrific Broth and Chemically Defined Auto-Induction Media

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Abstract

High-throughput methodologies are used to screen for protein expression and to provide large-scale *Escherichia coli* growths at the University of Wisconsin Center for Eukaryotic Structural Genomics (CESG). CESG undertakes routine production of unlabeled, Se-Met, ¹⁵N-, and ¹³C-¹⁵N-labeled proteins to support efforts in both X-ray and NMR structure determinations. The use of one cell line (*E. coli* B834-pLacIARE), a chemically defined medium that requires minimal changes in composition to produce proteins with the required labeling patterns, and the incorporation of Studier auto-induction provides a simplicity of approach that contributes to stability of pipeline operations.

Overview of the CESG Pipeline

Figure 1

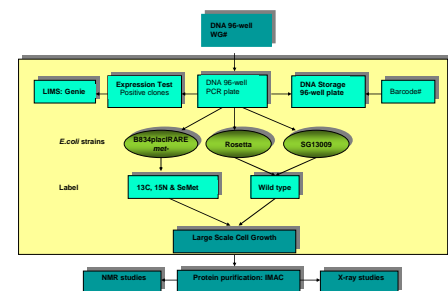


Figure 1 shows an overview of the CESG protein production pipeline. CESG uses the GENIE module of the project-developed SESAME laboratory information management system to record data generated throughout the entire pipeline. Workgroups of 96 *Arabidopsis thaliana* DNA clones (arrayed in 96-well PCR plates) are bar-coded and labeled for ease of handling, and the 96 clones are processed in parallel by a 2-step PCR protocol. Successfully cloned and sequence-verified genes are screened for positive protein expression in the *E. coli* host strain Rosetta-placIARE (Novagen). Clones that successfully express the target proteins are recorded in GENIE and then passed onto large scale cell growth for preparation of unlabeled and/or isotopically labeled protein. Individual protein targets can also be requested for re-growth using SESAME. The protein purification section purifies proteins to be used in structure determination by X-ray or NMR methods. Each part of the pipeline has been designed to be as simple as possible, provide for high-throughput operations and scalability, and flexibility to respond to new discoveries and opportunities.

Materials

Unlabeled Cell Growths

Powdered Terrific broth (TB) medium (48 g, Fisher) was mixed with 1 L of water. The solution was autoclaved at 121 °C and 15 psi for 20 min. Glycerol (90% w/w in water) was separately autoclaved. After cooling, one liter of TB medium was mixed with 10 ml of 80% glycerol, 1 ml of ampicillin (100 µg/ml) and 1 ml of chloramphenicol (34 µg/ml).

Labeled Cell Growths

PA-0.5G Starter Culture Medium: For 100 ml, this defined medium contained 92 ml of sterile water, 100 µl of 1 M MgSO₄, 10 µl of trace metal mix, 1.25 ml of 40% glucose, 5.0 ml of 20x NPS, 0.4 ml of methionine (25 mg/ml), 1 ml of 17 amino acid cocktail (each amino acid 10 mg/ml), 100 µl of ampicillin (100 mg/ml), 100 µl of chloramphenicol (34 mg/ml) and 100 µl of vitamin cocktail including vitamin B12.

P-0.5G Starter Culture Medium: This medium contained 5.0 ml of 20x ¹⁵N NPS and all other components identical to PA-0.5G starter culture medium but without amino acids.

PASM-5052 Medium: For 1 liter, this medium for large-scale growth and Se-Met labeling contained 900 ml of sterile water, 1 ml of 1 M MgSO₄, 100 µl of trace metal mix, 20 ml of 5052, 50 ml of 20x NPS, 0.4 ml of Met (25 mg/ml), 20 ml of 17 amino acid cocktail (each amino acid 10 mg/ml), 5 ml of Se-Met (25 mg/ml), 1 ml of ampicillin (100 mg/ml), 1 ml of chloramphenicol (34 mg/ml), and 1 ml of a vitamin cocktail lacking vitamin B12.

P¹⁵N-5052 Medium: For 1 liter, this medium for large scale growth and ¹⁵N labeling contained 925 ml of sterile water, 1 ml of 1 M MgSO₄, 100 µl of metal mix, 20 ml of 5052, 50 ml of 20x ¹⁵N NPS, 1 ml of ampicillin (100 mg/ml), 1 ml of chloramphenicol (34 mg/ml), and 1 ml of vitamin cocktail including vitamin B12.

Methods

Unlabeled Growths

Normally, 24 bottle growths were undertaken in parallel. Fresh transformants on LB agar were transferred into 200 ml of TB medium in a 500 ml conical flask with 100 µl of ampicillin and 100 µl of chloramphenicol and grown for 5 h at 37°C. A 50 ml aliquot of this starter culture was inoculated into 450 ml of TB medium in 2 L PETE bottles. The bottles were loosely sealed with foam plugs. The 2 L PETE bottles were incubated in temperature regulated shakers at 240 rpm, 37°C. The cell growth was continued for ~2 h to obtain an OD of 1. The TB medium culture was induced with 500 µl of 1 M IPTG (final concentration of 1 mM) and the incubation was continued at 20°C, 240 rpm up to 20 h. The cell growth OD was recorded prior to harvest. The culture was harvested by centrifugation at 5000 x g for 18 min followed by washing with 40 ml of sonication buffer with 20% ethylene glycol. Protein expression was analyzed in 150 µl samples using SDS PAGE.

Se-Met-Labeled Growths

Normally, 6-12 bottle growths were undertaken in parallel. Single colonies from fresh transformants on PA-0.5G agar plates were transferred to 3 ml of PA-0.5G starter culture medium in 10 ml test tubes and grown 5 to 6 h at 37 °C, 300 rpm. The starter culture was poured into 100 ml of PA-0.5G starter culture medium and grown for ~21 h at 25 °C. A 20 ml aliquot of this culture was inoculated to 480 ml of PASM-5052 medium in a 2 L PETE bottle and incubated in refrigerated shakers at 235 rpm, 25°C for 22 to 24 h. The cell growth OD was measured prior to harvest. Protein expression was analyzed using SDS PAGE.

¹⁵N-Labeled Growths

Single colonies were grown as for Se-Met labeling in 3 ml of PA-0.5G and poured to 100 ml of P-0.5G starter culture medium and grown for ~42 h at 25°C. A 20 ml aliquot was inoculated into 480 ml P¹⁵N-5052 medium in 2L PETE bottles and incubated at 235 rpm, 25°C for 24-26 h. The cell growth and analysis was as described above.

Results

Small-Scale Expression Screening

Figure 2 summarizes our small-scale expression screening of a workgroup of ~96 clones. The entire procedure takes 2 to 3 days to complete and is achieved in 0.5 mL of TB growth medium. We have found that ~70% of the target clones tested show positive expression (target clones are fused to the C-terminal of MBP, 45 kDa). The positive expressing clones are recorded in GENIE, which allows for tracking of individual clones or groups of proteins and coordination of requests for re-growth and protein purification. Figure 3 shows a representative small-scale expression analysis by SDS PAGE.

Figure 2

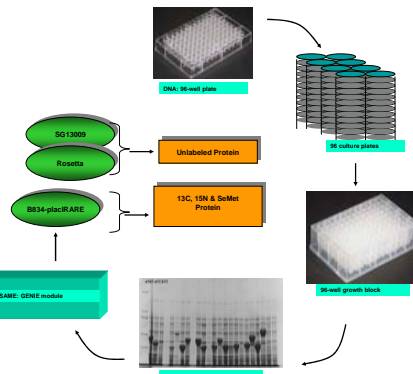
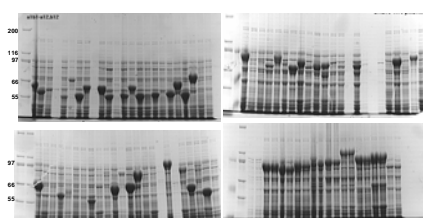


Figure 3



Clones that show positive expression are re-transformed to provide fresh cells for the large-scale cell growth section. Transformations are into Rosetta cells for growth in TB medium to produce unlabeled proteins, or *E. coli* B834-pLacIARE cells for isotopic labeling in a chemically defined medium. This approach provides simplicity of operations and flexibility. For example, B834 can be used for either Se-Met, ¹⁵N- or ¹³C-¹⁵N-labeling with only minor changes in the composition of the medium.

Large-Scale Cell Growths

Large-scale growths of a positive expressing target are carried out in an incubator shaker in PETE bottles, each containing 500 ml of culture. The Studier auto-induction medium, which uses lactose as an inducer, was found suitable for large-scale production of both unlabeled and labeled proteins. For Se-Met labeled proteins, the chemically defined auto-induction medium is supplemented with Se-Met. For ¹⁵N-labeled proteins, the medium is supplemented with ¹⁵N ammonium chloride and vitamin B12. The auto-induction medium offers the advantage of automatic induction at a targeted range of cell density without laborious monitoring of cell concentrations.

Figure 4

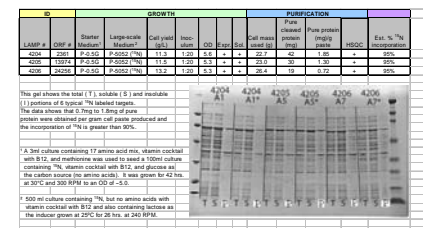
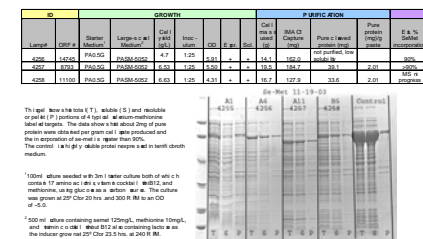


Figure 5



Figures 4 and 5 show results of large-scale expression trials to produce Se-Met and ¹⁵N-labeled proteins. Growth and expression in the Se-Met medium reliably gave greater than 90% incorporation and has been found to be suitable for X-ray studies. Similarly, the ¹⁵N-containing medium gives protein with >95% ¹⁵N incorporation, which is suitable for NMR screening by ¹⁵N-HSQC spectroscopy.

Cumulative Project Results

The cumulative project results obtained by operation of the *E. coli* expression screening and cell growth pipeline at CESG are summarized below.

Process Step	Action	Total Number of Actions in GENIE Database
ORF Selection	Selected	1440
Polymerase Chain Reaction	PCR +	1038
Entry Clone Generation	Entry Clone +	876
Destination Clone Generation	Destination Clone +	819
Small-Scale Expression Screen	Expression +	631
Large-Scale Cell Growth	Expression +	521
Large-Scale Expression: Solubility Screen	Solubility +	462