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Autoinduction of Protein Expression

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Abstract

This unit contains protocols for the use of lactose-derived autoinduction in *Escherichia coli*. The protocols allow for reproducible expression trials to be undertaken with minimal user intervention. A basic protocol covers production of unlabeled proteins for functional studies. Alternate protocols for selenomethionine labeling for X-ray structural studies, and multi-well plate growth for screening and optimization are also included.

Keywords

recombinant protein; expression; *Escherichia coli*; high-throughput methods; X-ray crystallography

INTRODUCTION

Autoinduction is a simple approach for protein expression that needs little user intervention after inoculation of the culture (Studier, 2005). This unit describes host strains, expression vectors, factorial-designed medium preparations, and growth conditions for lactose-driven autoinduction of recombinant protein expression in *Escherichia coli* (Blommel et al., 2007). In the factorial design process, the concentrations of the three carbon sources (glucose, glycerol, lactose) in the autoinduction medium were systematically varied as part of a five level, three-parameter design based on two full, three-level cubic factorials, with one nested within the other (Myers and Montgomery, 2002). Each level of the factorial constitutes a unique combination of carbon source concentrations. The Basic Protocol outlines the preparation and use of the best-performing unlabeled autoinduction medium obtained from the factorial design, which can be a cost-effective and efficient way to produce recombinant proteins for functional characterizations. Alternate Protocol 1 takes advantage of the modular assembly of the autoinduction medium to provide selenomethionine labeling for X-ray structural studies. Alternate Protocol 2 outlines assembly of a multi-well format of the autoinduction medium that can be used for finer assessment of induction conditions such as intensity of induction, expression at different cell densities, or investigation of induction in early-, mid-, or late-log conditions for study of proteins of focused interest.

STRATEGIC PLANNING

The expression strain selected for lactose-based autoinduction must be capable of lactose uptake controlled by natural operation of the *lac* operon and must also be capable of growth on this carbon source. This requires the presence of functional LacY permease and LacZ β -

galactosidase. Many strains used for DNA cloning purposes have a deletion in the *lacZ* gene to permit blue/white screening in the presence of the LacZ alpha-complementing peptide, and so will not be suitable for use in lactose autoinduction protocols.

These protocols have been developed for use with *Escherichia coli* B834 [Novagen; genotype F⁻ ompT hsdS_B(r_B⁻m_B⁻) gal dcm met]. This strain is a methionine-conditional auxotroph because of an insertion in the metE gene (Frederick et al., 2007), which allows efficient incorporation of selenomethionine into expressed proteins for determination of phases in X-ray crystallography studies by multi-wavelength anomalous diffraction. *E. coli* B834 is suitable for use with T5 promoter plasmids. A variant, *Escherichia coli* B834(DE3) (Novagen), is suitable for use with T7 promoter plasmids.

For rare codon adaptation, the expression host is transformed with the pRARE2 plasmid (Novagen). Expression of certain classes of proteins is apparently improved by this approach (Sharp and Li, 1987). This plasmid can be isolated from commercial strains using standard plasmid isolation procedures and transformed into other desired expression strains. The pRARE2 plasmid is maintained by including chloramphenicol in the culture medium.

The autoinduction protocols given here assume the availability of a sequence-verified bacterial expression plasmid whose promoter is controlled by the *lac* operator. Protein expression vectors that have tight control of basal expression are most desirable for use in autoinduction (Blommel et al., 2007). The University of Wisconsin Center for Eukaryotic Structural Genomics has deposited vectors of this type in the NIH Protein Structure Initiative Material Repository (<http://www.hip.harvard.edu/PSIMR/index.htm>). These are derived from pQE80-type plasmids (Qiagen), where expression is controlled through a hybrid *lac* operator placed upstream from the viral T5 promoter. This configuration allows tight control of basal expression. Expression plasmids whose promoters are indirectly controlled by the *lac* operon regulatory elements may also be acceptable; however, attention must be paid to the level of uncontrolled basal expression (e.g., pET32 and comparable). Furthermore, although tight control of the *lac* operator can be obtained by increased expression of the LacI repressor, detailed analysis has shown this approach can introduce other significant complications to the use of autoinduction (Blommel et al., 2007).

The use of chemically defined medium formulations lacking constituents prepared from casein-based products minimizes possible contamination from lactose and inadvertent induction of expression during culture passage and scale-up (Grossman et al., 1998). Inadvertent induction during scale-up can adversely influence the outcome of expression experiments, especially when toxic, poorly expressed, or unstable proteins are being studied.

It is necessary to decide whether the expressed protein is to be made unlabeled, or labeled with selenomethionine, and choose protocols for assembly of the medium accordingly. The preparation of the medium stock solutions requires attention to detail and is time-consuming. However, when stored as indicated, the stock formulations are stable for many months, so they can be made on a periodic basis in large batches.

BASIC PROTOCOL. AUTOINDUCTION OF UNLABELED PROTEIN EXPRESSION

This protocol describes use of autoinduction medium to produce unlabeled protein. It includes improvements based on analysis of carbon source consumption patterns and completion of a three-factor, five-level factorial evolution of the medium composition with a pQE80-derived vector and *E. coli* B834 pRARE2 (Blommel et al., 2007). The medium composition described here allows high expression of an enhanced green fluorescent protein variant and *Photinus* luciferase. The convenient assays for these two proteins provide many orders of magnitude in sensitivity for detection of basal and induced expression levels, and so they have been used for methods development. However, many other proteins and enzymes have been successfully produced at high levels in this medium.

Materials

- Sequence-verified expression plasmid
 - Expression host
 - Agar plates of non-inducing medium (see recipe)
 - Non-inducing medium (see recipe)
 - Autoinduction medium (see recipe)
 - Cell suspension buffer (see recipe)
 - 37°C incubator (e.g., New Brunswick C24KC refrigerated shaker or equivalent)
 - 16-ml snap-top growth tubes used for culture scale-up (BD Biosciences) or equivalent
 - 500-ml Erlenmeyer flasks
 - 25°C shaking incubator
 - 2-liter polyethyleneterephthalate beverage bottles used for bacterial cell growth (Ball Corporation; standard 2-liter Erlenmeyer or Fernbock flasks can be substituted for the polyethyleneterephthalate beverage bottles)
 - Allegra 6R centrifuge with a GH3.8 rotor (Beckman Coulter) or equivalent
 - 50-ml centrifuge tubes
1. Transform a sequence-verified expression plasmid into the expression host using heat shock, and plate the transformed cells onto agar plates of non-inducing medium. Place the freshly transformed cells in a 37°C incubator.
 2. After 24 to 36 hr, pick a single colony from the agar plate and inoculate into 3 ml of non-inducing medium prewarmed to 37°C in a sterile 16-ml snap-top growth tube. Loosely cap the tube, place it in a 37°C incubator, and shake it at 300 rpm.
 3. After 7 hr, add the 3-ml culture to 100 ml of non-inducing medium prewarmed to 37°C in a 500-ml Erlenmeyer flask. Loosely cap the flask, place it in a 25°C incubator, and shake it at 250 rpm.

4. After 24 hr, transfer 25 ml of the 100 ml culture to each of four 2-liter polyethyleneterephthalate beverage bottles containing 475 ml of autoinduction medium prewarmed to 25°C. Place the four separate culture bottles in a 25°C incubator, and grow cells with shaking at 250 rpm.
5. After 24 hr, harvest the cells by centrifuging 20 min at $4000 \times g$, 4°C in an Allegra 6R centrifuge with a GH3.8 rotor or equivalent, resuspend the cells in 30 ml of cell suspension buffer, and transfer the cell suspension to a 50-ml centrifuge tube.
6. Bring the volume of the cell suspension to 50 ml with cell suspension buffer and pellet the cells by centrifuging using the same conditions stated above. Store the washed and pelleted cells indefinitely at -80°C.
7. Perform analyses for expression by enzymatic assay, denaturing electrophoresis, or other methods depending on the nature of the protein and interests of the investigator.

Figure 5.23.1 shows the time course for autoinduction of expression of some example proteins as determined by denaturing polyacrylamide gel electrophoresis with Coomassie Blue staining.

ALTERNATE PROTOCOL 1. SELENOMETHIONINE LABELING BY AUTOINDUCTION

This protocol modifies the Basic Protocol so that the expressed protein is labeled with selenomethionine for multi-wavelength anomalous diffraction phasing in X-ray crystallography. The protocol takes advantage of the conditional methionine auxotrophy of *E. coli* B834 by allowing rapid growth in the non-inducing medium supplemented with vitamin B₁₂ and lacking methionine, and continued growth in the autoinduction medium supplemented with selenomethionine and lacking vitamin B₁₂. Expressed proteins obtained from this protocol have a selenomethionine content of ~90%.

Additional Materials (also see the Basic Protocol)

Autoinduction medium (lacking vitamin B12 solution and methionine, supplemented with selenomethionine; see recipe)

For this protocol, follow steps 1 to 3 of the Basic Protocol. Replace step 4 with the following and then return to steps 5 through 7 of the Basic Protocol.

- 4a After 24 hr, transfer 25 ml of the 100 ml culture to each of four 2-liter polyethyleneterephthalate beverage bottles containing 475 ml of autoinduction medium (lacking vitamin B12 solution and methionine, supplemented with selenomethionine) prewarmed to 25°C. Place the four separate culture bottles in a 25°C incubator, and grow cells with shaking at 250 rpm.

ALTERNATE PROTOCOL 2. EXPRESSION SCREENING IN AN AUTOINDUCTION MEDIUM ARRAY

This protocol provides an adaptation of the Basic Protocol to a format for multi-well screening of different medium compositions (Blommel et al., 2007). The concentrations of the carbon sources (glucose, glycerol, lactose) in the autoinduction medium are varied as part of a five-level, three-parameter factorial in the following ranges (w/v): glucose, 0 to 0.1%; lactose, 0 to 0.6%; and glycerol, 0 to 1.2%. Other medium components are maintained at the same levels specified in the Basic Protocol. The workflow for this protocol is to first assemble stock solutions for each well of the 8 × 12 media array. Table 5.23.1 defines how the (w/v) percentages of glucose, lactose, and glycerol are arranged in the growth block format. The methionine-containing autoinduction medium is arranged into an 8 × 8 array (wells A1:H8) in a 96-well growth block, while the selenomethionine-containing autoinduction medium is arranged into an 8 × 4 array (wells A9:H12). The required stock solutions can be prepared in large volume, frozen, and stored for future use. Individual growth experiments are easily assembled from the stock solutions by dispensing the appropriate volume into the growth block used for the array expression experiment. The autoinduction medium for this protocol is assembled without added sugars so that these can be varied in the different positions of the 96-well plate. The preparation of a sugar-free, methionine-containing autoinduction medium and a sugar-free, selenomethionine-containing autoinduction medium and the arrangement of these solutions into a 96-well format are described in Reagents and Solutions.

Additional Materials (also see the Basic Protocol)

Sugar-free methionine-containing autoinduction medium (see recipe and Table 5.23.1)

Sugar-free selenomethionine-containing autoinduction medium (see recipe and Table 5.23.1)

96-well, 2.4 ml per well growth block (Eppendorf)

AeraSeal gas-permeable sealing tape (ISC Bioexpress, T-2421-50)

Thermomixer R (Eppendorf)

50-ml Falcon tubes

1. Transform a sequence-verified expression plasmid into the expression host using heat shock, and plate the transformed cells onto agar plates of non-inducing medium. Place the freshly transformed cells in a 37°C incubator.
2. After 24 to 36 hr, pick a single colony from the agar plate and inoculate into 3 ml of non-inducing medium prewarmed to 37°C in a sterile 16-ml snap-top growth tube. Loosely cap the tube, place it in a 37°C incubator, and shake it at 300 rpm.
3. Prepare a growth block by adding 0.4 ml of each solution of the 8 × 12 media array prepared as described in Table 5.23.2 into the corresponding positions of the 96-well growth block.

Table 5.23.2 defines the volumes of 40% (w/v) glucose, 20% (w/v) lactose, and 40% (w/v) glycerol that are placed in a Falcon tube to make 40-ml aliquots of each well solution. As an example, for assembly of 40 ml of the culture solution in well A1, place 36 ml of the sugar-free methionine-containing autoinduction medium into a 50-ml Falcon tube labeled A1. Add 75 μ l of 40% (w/v) glucose solution, 900 μ l of 20% (w/v) lactose solution, 300 μ l of 40% (w/v) glycerol solution, and 2725 μ l of sterile water. Assemble Falcon tubes containing 40 ml of each of the remaining 95 medium compositions in a similar manner. For robotic operations, it may be convenient to re-array these solutions into different volumes and storage blocks for more convenient handling.

4. Inoculate each well of the growth block containing an expression array medium with 4 μ l of the starting inoculum prepared in step 1. After the inoculation, seal the growth block with gas-permeable sealing tape. Place the cells in an Eppendorf Thermomixer R (or equivalent device) at 37°C with shaking at ~250 rpm for 24 to 48 hr.

The specified thermal block mixer avoids vertical displacement motion, which helps to avoid cross-contamination.

5. Analyze expression using enzyme assays, denaturing electrophoresis, or other approaches enacted in a 96-well plate format.

When a preferred medium composition has been identified by an expression trial using this array, the preferred composition can be used to prepare larger volumes of a customized autoinduction medium with the identified sugar percentages in a manner similar to that described for the Basic Protocol.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2E**; for suppliers, see **SUPPLIERS APPENDIX**.

Bacterial growth reagents, antibiotics, routine laboratory chemicals, and disposable labware are available from major distributors such as Sigma-Aldrich, Fisher, and others.

Amino acids solution, 50×

This solution provides a defined composition of amino acids to the growth medium. It does not contain cysteine, tyrosine, or methionine. Prepare a 1-liter aliquot from 10 g of the L-isomers by adding each of the following amino acids to 900 ml of deionized water in the order indicated and stir until dissolved before adding the next amino acid from the list.

Sodium glutamate

Lysine-HCl

Arginine-HCl

Histidine-HCl

Free aspartic acid

The zwitterionic forms of the following amino acids are added in the following order:

Alanine

Proline

Glycine

Threonine

Serine

Glutamine

Asparagine

Valine

Leucine

Isoleucine

Phenylalanine

Tryptophan

Valine, leucine, and isoleucine have a tendency to float, so vigorous stirring may be needed to aid in their dissolution. Upon addition of tryptophan, the solution may darken in color. When all amino acids have been dissolved, adjust the solution volume to 1 liter, and sterilize it using a 0.4- μ m filter. Store the sterilized solution for no longer than 1 month at 4°C. It is light sensitive, so the storage container should be opaque or wrapped in foil for protection.

Agar plates of non-inducing medium

Prepare agar plates of the non-inducing medium by combining 10 g of Bacto Agar (BD Biosciences or equivalent) and 800 ml of deionized water in a 2-liter Erlenmeyer flask. Sterilize this mixture using an autoclave and cool it to prepare for pouring of the plates. It is convenient to maintain the melted agar in a hot water bath (~60°C) with stirring while the following additions are made to the agar. Each is thoroughly mixed into the melted agar before the next is added in the following order:

1 ml of the 1000 $\times$ MgSO₄ solution (see recipe)

0.2 ml of the 5000 $\times$ trace metals solution (see recipe)

1 ml of the 1000 $\times$ vitamins solution (see recipe)

1 ml of the 1000 $\times$ vitamin B₁₂ solution (see recipe)

12.5 ml of the 40% glucose solution (see recipe)

25 ml of the 40 $\times$ succinate solution (see recipe)

25 ml of the 40% (w/v) glycerol solution (see recipe)

50 ml of the 20× nitrogen, sulfur, and phosphorus solution (see recipe)

10 ml of the 50× amino acids solution (see recipe)

1 ml of the appropriate 1000× antibiotics solution (see recipe)

Adjust the volume to 1000 ml with sterile water

Add the antibiotics solutions only when the agar is ~60°C as some antibiotics are unstable at higher temperature. Pour the plates shortly after the antibiotics solutions is added. Use ~10 ml of the agar solution per 60-mm diameter × 15-mm depth petri plate. Store plates up to 1 month at 4°C.

Antibiotics solution, 1000×

Depending on the selectable marker of the expression plasmid used, both non-inducing and autoinduction media may contain 100 µg/ml of ampicillin, 50 µg/ml of kanamycin, or other appropriate antibiotic. For expression strains that contain the pRARE2 plasmid for rare codon adaptation, all media contain 34 µg/ml of chloramphenicol. Prepare these 1000× antibiotics solutions using standard laboratory procedures.

Autoinduction medium

Prepare a 1-liter aliquot of the autoinduction medium by adding the following solutions to 800 ml of deionized water and thoroughly mixing in the following order:

1 ml of the 1000 × MgSO₄ solution (see recipe)

0.2 ml of the 5000× trace metals solution (see recipe)

1 ml of the 1000× vitamins solution (see recipe)

1 ml of the vitamin B₁₂ solution (see recipe); omit for Alternate Protocol 1

12.5 ml of the 40% (w/v) glucose solution (see recipe)

25 ml of the 40× succinate solution (see recipe)

50 ml of the 20% (w/v) lactose solution (see recipe)

25 ml of the 40% (w/v) glycerol solution (see recipe)

50 ml of the 20× nitrogen, sulfur, and phosphorus solution (see recipe)

10 ml of the 50× amino acids solution (see recipe)

4 ml of the 250 × methionine solution (see recipe); substitute solution with 250× selenomethionine (see recipe) for Alternate Protocol 1

1 ml of the appropriate 1000× antibiotics solution (see recipe)

Adjust volume to 1000 ml using sterile water

Store frozen indefinitely

The autoinduction medium used in Alternate Protocol 1 does not contain the B₁₂ solution. In addition, the autoinduction medium used in Alternate

Protocol 1 uses 4 ml of 250× selenomethionine solution in place of the 250× methionine solution.

Cell suspension solution

Prepare a 1-liter aliquot by combining 200 g of ethylene glycol, 29.2 g of NaCl, and 7.8 g of NaH_2PO_4 in ~900 ml of deionized water. Adjust the solution to pH 7.4 using 4 M NaOH, and sterilize using a 0.4- μm filter. Store the solution for no longer than 6 months at room temperature.

This solution is used to resuspend cells obtained from the autoinduction protocol after their initial harvest by centrifugation.

Glucose solution, 40% (w/v)

Prepare a 100-ml aliquot of this solution by dissolving 40 g of D-glucose in 74 ml of deionized water. This solution may take 45 min or longer to dissolve at room temperature. When dissolved, sterilize the solution by autoclaving and store for no longer than 6 months at room temperature.

Glycerol solution, 40% (w/v)

Prepare a 100-ml aliquot of this solution by transferring 50 g of 80% glycerol into a 250-ml beaker and then stirring deionized water into the glycerol to achieve a final volume of 100 ml. Sterilize the solution using a 0.4- μm filter, and store for no longer than 6 months at room temperature.

This solution is used to provide an additional, non-fermentable carbon source to support bacterial growth throughout much of the culture.

Lactose solution, 20% (w/v)

Prepare this solution by dissolving 20 g of α -lactose in 60 ml of deionized water. This solution may take 2 hr or longer to dissolve at room temperature, and dissolution can be quickened by gentle heating (but not boiling) in a microwave oven. When all of the lactose is dissolved, add deionized water to achieve a total volume of 100 ml. Autoclave to sterilize the solution and store for no longer than 6 months at room temperature.

This solution is used to provide lactose for autoinduction. Lactose is also consumed as a carbon source for continued bacterial growth during the autoinduction phase of the culture.

Methionine solution, 250×

Prepare this solution by dissolving 2.5 g of L-methionine in 100 ml of deionized water. Sterilize the solution using a 0.4- μm filter, and store for no longer than 1 week at room temperature.

This solution provides methionine to the growth medium lacking vitamin B₁₂ when an auxotrophic host strain such as E. coli B834 is used.

MgSO₄ solution, 1000×

Prepare a 100-ml aliquot by dissolving 24.7 g of MgSO₄·7H₂O in sterile water. Sterilize by autoclaving and store indefinitely at room temperature.

Nitrogen, sulfur, and phosphorus solution, 20×

Prepare a 1-liter aliquot of this solution by dissolving 68 g of KH₂PO₄, 71 g of Na₂HPO₄, 53.6 g of NH₄Cl, and 14.2 g of Na₂SO₄ in deionized water. Sterilize the solution using a 0.4-μm filter, and store indefinitely at room temperature. A 20-fold dilution of this solution should have pH ~6.8. If necessary, use 4 M NaOH or 4 M HCl to adjust the pH.

This solution is used to provide nitrogen, sulfate, and phosphorus to the culture medium.

Non-inducing medium

Prepare a 1-liter aliquot of this medium by adding and thoroughly mixing the following solutions with 800 ml of deionized water:

- 1 ml of the 1000 × MgSO₄ solution (see recipe)
 - 0.2 ml of the 5000× trace metals solution (see recipe)
 - 1 ml of the 1000× vitamins solution (see recipe)
 - 1 ml of the 1000× vitamin B₁₂ solution (see recipe)
 - 12.5 ml of the 40% (w/v) glucose solution (see recipe)
 - 25 ml of the 40× succinate solution (see recipe)
 - 25 ml of the 40% (w/v) glycerol solution (see recipe)
 - 50 ml of the 20× nitrogen, sulfur, and phosphorus solution (see recipe)
 - 10 ml of the 50× amino acids solution (see recipe)
 - 1 ml of the appropriate 1000× antibiotics solution (see recipe)
- Adjust volume to 1000 ml using sterile water.

This non-inducing medium is used for all culture passage (stab culture, replication, and other routine handling procedures) and in preparation of the starting inoculum for all growth procedures.

Selenomethionine solution, 250×

Prepare this solution by dissolving 2.5 g of L-selenomethionine (99% purity, Acros) in 100 ml of deionized water. Sterilize the solution using a 0.4-μm filter, and store for no longer than 1 week at room temperature. It is light sensitive, so the storage container should be opaque or wrapped in foil for protection.

This solution provides selenomethionine to the growth medium lacking vitamin B₁₂ when an auxotrophic host strain such as E. coli B834 is used. It allows for the expression of proteins with high percentage incorporation of L-selenomethionine.

Succinate solution, 40×

Prepare a 1-liter aliquot of this solution by dissolving 150 g of sodium succinate in 800 ml of deionized water. Adjust the pH to 6.8 using 4 N NaOH. Sterilize the solution using a 0.4- μ m filter, and store for no longer than 6 months at room temperature.

This solution is used to stabilize pH and provide an additional carbon source that is used throughout cell growth. When present, succinate is fixed at an initial concentration of 0.375% (w/v) in the autoinduction medium.

Sugar-free, methionine-containing autoinduction medium

Prepare this medium by adding to 800 ml of deionized water and thoroughly mixing in the following order:

- 1 ml of the 1000 $\times$ MgSO_4 solution (see recipe)
- 0.2 ml of the 5000 $\times$ trace metals solution (see recipe)
- 1 ml of the 1000 $\times$ vitamins solution (see recipe)
- 1 ml of the 1000 $\times$ vitamin B₁₂ solution (see recipe)
- 25 ml of the 40 $\times$ succinate solution (see recipe)
- 50 ml of the 20 $\times$ nitrogen, sulfur, and phosphorus solution (see recipe)
- 10 ml of the 50 $\times$ amino acids solution (see recipe)
- 4 ml of the 250 $\times$ methionine solution (see recipe)
- 1 ml of the appropriate 1000 $\times$ antibiotics solution (see recipe)
- Adjust volume to 900 ml with sterile water

Sugar-free, selenomethionine-containing autoinduction medium

Prepare this medium by adding the following solutions to 800 ml of deionized water and thoroughly mixing in the following order:

- 1 ml of the 1000 $\times$ MgSO_4 solution (see recipe)
- 0.2 ml of the 5000 $\times$ trace metals solution (see recipe)
- 1 ml of the 1000 $\times$ vitamins solution (see recipe)
- 1 ml of the 1000 $\times$ vitamin B₁₂ solution (see recipe)
- 25 ml of 40 $\times$ succinate solution (see recipe)
- 50 ml of the 20 $\times$ nitrogen, sulfur, and phosphorus solution (see recipe)
- 10 ml of the 50 $\times$ amino acids solution (see recipe)
- 0.4 ml of the 250 $\times$ methionine solution (see recipe)
- 5 ml of the 250 $\times$ selenomethionine solution (see recipe)
- 1 ml of the appropriate 1000 $\times$ antibiotics solution (see recipe)

Adjust volume to 900 ml using sterile water.

Trace metals solution, 5000×

This solution provides a standard composition of trace metals to both the non-inducing and autoinduction medium. Prepare a 100-ml aliquot from the following:

50 ml of 0.1 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dissolved in ~0.1 M HCl

2 ml of 1 M CaCl_2

1 ml of 1 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$

1 ml of 1 M $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$

1 ml of 0.2 M $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$

2 ml of 0.1 M $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$

1 ml of 0.2 M $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$

2 ml of 0.1 M $\text{Na}_2\text{MoO}_4 \cdot 5\text{H}_2\text{O}$

2 ml of 0.1 M Na_2SeO_3

2 ml of 0.1 M H_3BO_3

36 ml of deionized water

Prepare the individual metal solutions in deionized water and sterilize them using a 0.4- μm filter. Sterilize the combined solution also using a 0.4- μm filter. Store both the individual and combined solutions indefinitely at room temperature.

Vitamins solution, 1000×

This solution provides a standard composition of vitamins. Prepare a 100-ml aliquot of this solution from the following:

2 ml of 10 mM nicotinic acid

2 ml of 10 mM pyridoxine-HCl

2 ml of 10 mM thiamine-HCl

2 ml of 10 mM *p*-aminobenzoic acid

2 ml of 10 mM calcium pantothenate

5 ml of 100 μM folic acid

5 ml of 100 μM riboflavin

80 ml of sterile water

Prepare the individual vitamins solutions in deionized water and sterilize them using a 0.4- μm filter. Sterilize the combined solution using a 0.4- μm filter, and store for no longer than 3 months at room temperature. It is light sensitive, so the storage container should be opaque or wrapped in foil for protection.

Vitamin B₁₂ solution, 1000×

Prepare an initial 5 mM vitamin B₁₂ solution by dissolving 68 mg of vitamin B₁₂ in 10 ml of sterile water. Prepare the 1000× vitamin B₁₂ solution by combining 4 ml of the 5 mM vitamin B₁₂ solution and 96 ml of sterile water. Sterilize the 1000× solution using a 0.4-μm filter, and store for no longer than 3 months at room temperature. It is light sensitive, so the storage container should be opaque or wrapped in foil for protection.

The inclusion of vitamin B₁₂ helps to stimulate the growth of *E. coli* B834 during culture, particularly during early stages of inoculum development and during production of unlabeled protein.

COMMENTARY

Background Information

Theory—Lactose induction of *lac* operon proteins was the subject of the 1965 Nobel Prize in Physiology or Medicine awarded to Francois Jacob, Andre Lwoff, and Jacques Monod “for their discoveries concerning genetic control of enzyme and virus synthesis.” As part of this award, Monod’s lecture established the basis for bacterial diauxic growth (Monod, 1972). Bacterial diauxy leads to a preferred hierarchy for use of different carbon sources dependent on culture conditions, medium composition, and expression plasmid construction. The autoinduction medium described in this unit contains glucose, lactose, and other carbon sources. When lactose becomes the preferred carbon source, it is imported into the cell and can simultaneously support growth of the host cells and induce expression of recombinant proteins. This process, which is controlled by growth of the host organism, is called autoinduction.

History

Lactose was first used as an inducer for expression of recombinant proteins from the hybrid *trp-lac* promoter in order to control the timing of induction (Neubauer et al., 1991, 1992). Subsequent work demonstrated the utility and cost-effectiveness of this approach in stirred-vessel fermenters, including a natural shift from glucose consumption to lactose consumption in mixtures of both carbon sources and expression at high-cell density (Hoffman et al., 1995). These studies noted the considerable cost advantage of lactose relative to IPTG as an inducer, and one study also introduced the concept of scalable manipulation of the medium contents to support different levels of expression governed by desired yield and cost of isotopic labels (Hoffman et al., 1995). The possibility for small-scale testing of the suitability of lactose-induced expression of a given protein in a small volume culture was also described (Hoffman et al., 1995).

With advent of the Protein Structure Initiative, autoinduction was introduced as a labor-saving method, and an extensive description of the development of autoinduction for use with the pET system was reported (Studier, 2005) and adapted to the use of T5 promoter plasmids (Sreenath et al., 2005; Tyler et al., 2005). However, some other Protein Structure Initiative groups found that autoinduction was difficult to control in practice and did not lead to correlative performance in small- and large-scale experiments. Later, analysis of the

process of autoinduction and the interplay of carbon source composition, vector elements, and growth conditions provided insight into the irreproducibility of performance obtained from autoinduction in certain circumstances (Blommel et al., 2007).

In general, protein expression vectors that have tight control of basal expression are most desirable for use in autoinduction. Although tight control has been obtained by increased expression of the LacI repressor (Dubendorff and Studier, 1991), this broad-stroke approach can introduce other significant complications to the use of autoinduction (Blommel et al., 2007). Specifically, overexpression of LacI can strongly inhibit or prevent the ability of the bacterial cells to complete the autoinduction process.

Comparison with Other Methods

Batch addition of IPTG is often used for induction of protein expression from the lac operon (Terpe, 2006). This can lead to rapid and strong induction of protein expression. Since IPTG is not metabolized, this induction is irreversible and thus not responsive to control by the cell. In contrast, autoinduction occurs under control of natural cellular networks that sense the energy levels and nutritional status. In the Basic Protocol described in this unit, protein expression occurs over a multi-hour period (Blommel and Fox, 2007; Blommel et al., 2007), which may permit continued growth of the host cell even as expression continues. This increases volumetric productivity of the expression process. Furthermore, experimental results also suggest that autoinduction is compatible with metal and cofactor incorporation (Hoffman et al., 1995; Bailey et al., 2007) and post-translational modifications (Broadwater and Fox, 1998; Haas et al., 2000). The continued growth also allows autoinduced cells to perform other metabolic processes such as cofactor biosynthesis (Sobrado et al., 2007). In one case, however, partial proteolytic processing of a C-terminal membrane anchor was observed from autoinduction but not from IPTG induction (Sobrado et al., 2007), so case-specific evaluation of the outcome of autoinduction must be undertaken for high-value targets.

Figure 5.23.2 shows the results of autoinduction for the expression of His8-MBP-eGFP in the format of Table 5.23.1 (Blommel et al., 2007). The medium composition described in the Basic Protocol and Alternate Protocol 1 [0.03% (w/v) glucose, 0.45% (w/v) lactose, 0.9% (w/v) glycerol] corresponds to positions C3 (unlabeled medium) and C11 (selenomethionine-labeled medium), respectively, in Table 5.23.1 and Figure 5.23.2. The improved performance of the selenomethionine-containing medium apparently derives from a weakening of catabolite repression, which shifts both the timing and extent of lactose consumption, leading to better expression (Blommel et al., 2007). For comparison, the medium composition originally reported [0.05% (w/v) glucose, 0.2% (w/v) lactose, 0.5% (w/v) glycerol (Studier, 2005)] is most closely approximated by positions F2 through H2 and F10 through H10.

The array format for the various autoinduction media provides a simple way to screen combinations of other plasmid vectors and host strains for conditions that maximize expression of the protein of interest. Based on observed expression levels, other applications might also be supported by small-scale autoinduction experiments. For example, cultures obtained from autoinduction can yield sufficient purified protein for automated protein

purification (Frederick et al., 2007), microfluidics-based crystallization screening (van der Woerd et al., 2003; Segelke, 2005), initial nanoliter-scale crystallization trials (Lion et al., 2004; Zheng et al., 2005), ^{15}N HSQC NMR measurements (Kennedy et al., 2002; Galvao-Botton et al., 2003), or functional and enzymatic characterizations (Kumar and Clark, 2006; Blommel and Fox, 2007).

Critical Parameters

It is necessary that all required parts of the autoinduction medium are added and that the sequence of events described in the protocols is followed precisely. It is also necessary to use non-inducing medium at all stages of culture scale-up except during the final growth phase, where induction is desired.

The growth of the culture must continue until lactose becomes the preferred carbon source and it is imported into the cell. As long as glucose is present and preferentially being used as the carbon source for energy and growth, lactose will not be imported and autoinduction cannot occur.

Troubleshooting

It is good experimental practice to verify the DNA sequence of the expression plasmid, including both the regulatory region and target gene, particularly if the behavior of the target protein is not known from previous studies. Faults in the gene sequence may complicate successful interpretation of the autoinduction experiment.

Improper assembly of medium components may result in their partial removal by filtration, and this must be avoided. To avoid precipitation during the preparation of all media, the $1000\times$ MgSO_4 and $5000\times$ trace metals mix should be added and mixed thoroughly before the $20\times$ nitrogen, sulfur, and phosphorus solution is added. Care must also be taken to ensure that all amino acids in the $50\times$ amino acids solution are dissolved before filter sterilization.

Aerobic growth strongly enforces the pattern of diauxy observed during autoinduction (Blommel et al., 2007), which means that lactose consumption (and autoinduction of protein expression) will not occur until a higher cell density and a potentially longer culture time-period have been achieved. Contrary to expectation, early onset of autoinduction and better expression may be obtained by microaerobic or anaerobic culture growth. It is noted that the reproducible definition of the dissolved O_2 concentration in the shaken flask culture is difficult, but this can be approximated by empirical variation in agitation rate, or defined more precisely by the use of stirred-vessel fermenters having feedback control of dissolved O_2 concentration (Hoffman et al., 1995; Studts and Fox, 1999; Blommel and Fox, 2007).

Anticipated Results

The use of autoinduction has been documented in many growth trials reported by centers of the Protein Structure Initiative to the TargetDB and PepcDB databases of the NIH Knowledgebase (<http://kb.psi-structuralgenomics.org/KB/>).

Work from the authors' laboratory has shown autoinduction is effective in 2-liter shaken bottles, 96-well growth blocks, and automated stirred-vessel fermenters (Blommel and Fox,

2007; Frederick et al., 2007). The different autoinduction results presented here include the use of shaken bottles (Fig. 5.23.1A and B), growth blocks (Fig. 5.23.2), and fermenters (Fig. 5.23.1C and Fig. 5.23.3). In each case, the combination of the designed autoinduction medium and matched expression plasmid yielded strong expression, demonstrating utility in several different formats used to grow bacterial cells. Importantly, consideration of the relationship between plasmid copy number, LacI repressor levels, medium composition, and culture conditions led to better correlation of results in different-sized culture vessels (Blommel et al., 2007).

Some additional results suggest customized modifications to the autoinduction medium composition, and LacI dosing may have general utility in improving the level of expression for specific proteins. Figure 5.23.3 shows the time course for autoinduction of the tobacco etch virus protease using an expression vector arrangement that allows for self-cleavage of the protease from a fusion with maltose-binding protein during cell growth (Blommel and Fox, 2007). The combination of a T5-*lacI* expression plasmid (attenuated expression of the LacI repressor) with a Terrific Broth medium (supplemented with an autoinduction mixture of 0.015% glucose, 0.5% lactose, 0.8% glycerol, 0.375% aspartic acid, and 2 mM MgSO₄) gave an ~5-fold increase in expression of soluble tobacco etch virus protease (Blommel and Fox, 2007) when compared to previous reports (van den Berg et al., 2006; Fang et al., 2007). This composition was identified by exploration of the expression array composition using a fluorescence-based catalytic assay for the protease (Blommel and Fox, 2005).

Factorial approaches to explore the relationship between medium composition and expression behavior, detailed in Alternate Protocol 2, are executable in plate format and lead to protein expression at different cell densities, different levels of total expressed protein, and at different times in the cycle of cell growth from exponential growth to stationary phase. Each of these different conditions may be advantageous for the expression of particular proteins.

The block arrangement described in Alternate Protocol 2 provides internal controls and several patterns that are useful for comparative studies. Thus, wells A1-H6 provide the factorial array of sugar concentrations. Within these, wells E4-F6 and H4-H6 have no lactose, so expression observed in these positions corresponds to basal expression. Wells A7-B8 are replicates of the center point of the factorial, and provide information on reproducibility of growth and expression. Wells C7-D8 provide variation of glucose over the full range of the factorial, wells E7-F8 provide variation of lactose over the full range of the factorial, and wells G7-H8 provide variation of glycerol over the full range of the factorial, with the other sugars held at the center point.

The 8 × 4 selenomethionine-containing array in wells A9-H12 is a duplication of wells A1-H3 and A7-H7. By inclusion of selenomethionine, screening for best conditions for labeling for MAD phasing in X-ray crystallography can be completed. Alternatively, if selenomethionine is replaced by methionine, the comparable 8 × 4 layout of cells A1-H3 with A7-H7 and A9-H12 would allow two screening experiments in the same conditions. This may have utility in evaluation of mutations or in increasing the number of replicates used for statistical analysis of expression.

Autoinduction using the media compositions identified in smaller scale work can be effectively executed in stirred-vessel fermenters (Blommel and Fox, 2007), which has advantages for the reproducible control of dissolved O₂ concentration. Inocula for stirred-vessel fermentation are prepared in the same manner described in the Basic Protocol. Depending on the size of the fermenter vessel, one or more additional scale-up steps may be needed to prepare a suitable volume of starting inoculum (typically 3% to 10% v/v). All scale-up steps are carried out using the non-inducing medium.

With the automated control of culture aeration and pH provided by the fermentation vessel, higher volumetric productivity for expressed protein could be obtained by driving the cell mass to high levels before allowing strong induction of protein expression. The medium described here has autoinduction typically beginning at OD₆₀₀ of ~5, and is sufficient to allow an OD₆₀₀ of ~25. Increases in cell mass above this level require further factorial evolution of the growth medium, which can be undertaken as previously reported (Blommel et al., 2007).

Time Considerations

In a suitably equipped and stocked laboratory, the preparation of the necessary reagents and solutions can be completed in a day or two. The shelf life of the reagents and solutions indicated in Reagents and Solution are conservative estimates. If sufficient freezer space is available, all of the reagents and solutions can be prepared in larger quantities and stored indefinitely at –80°C. If frozen media are used, care should be taken to ensure that all medium constituents are dissolved after the frozen media has been thawed. This can be aided by gentle heating in a sonicating water bath.

The approximate work cycle for execution of an autoinduction experiment is indicated in Basic Protocol and Alternate Protocol 1. In general, the total time required for scale-up and growth of a transformed clone from an agar plate of non-inducing medium to the harvest of cells after completion of the growth in a 2-liter bottle is ~3 days, with the final autoinduction growth in a multi-well growth block or shaken flask typically accounting for 24 to 36 hr of the total elapsed time (see Fig. 5.23.1A or B). The time for the final autoinduction growth can be considerably shorter in a stirred-vessel fermenter (see Fig. 5.23.1C or Fig. 5.23.3).

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Figure 5.23.1.

Denaturing electrophoresis gels showing results from autoinduced expression, OD₆₀₀ values, and elapsed time of the growth. **(A)** Toluene 4-monooxygenase hydroxylase [210 kDa with (αβγ)₂ quaternary structure composed of TmoA (55 kDa), TmoB (10 kDa), and TmoE (35 kDa) polypeptides (Studts et al., 2000)]. Vector pVP58K was derived from pQE80 to include the Flexi Vector cloning cassette (Promega) and a kanamycin selectable marker. The OD₆₀₀ values obtained during the autoinduction in a shaken-flask culture are shown. Enzyme produced in this way had k_{cat} of $\sim 3 \text{ sec}^{-1}$, which is comparable to the best preparations obtained from any induction system. **(B)** Soluble Rieske-type ferredoxin sMR from mouse (gene Mm266515) expressed in a shaken-flask culture as a fusion to the C-terminus of His8-maltose binding protein [63 kDa, PDB ID 3D89, (Levin et al., 2008)]. Vector pVP16A was derived from pQE80 to include the Gateway cloning cassette (Invitrogen) and an ampicillin selectable marker (Thao et al., 2004). Tight control of basal expression at OD₆₀₀ of 2 and 3 and high-level expression at OD₆₀₀ of 33 are demonstrated. The harvested cells were dark brown, corresponding to incorporation of an iron-sulfur center into the expressed ferredoxin. **(C)** Toluene 4-monooxygenase hydroxylase expressed from pVP58K in a fermenter with autoinduction medium enriched with ⁵⁷Fe for studies using Mössbauer spectroscopy. The dissolved O₂ was fixed at 10% and early onset of expression (between OD₆₀₀ of 2 and 3) and continued accumulation of active enzyme up to an OD₆₀₀ of 14 were observed. Approximately 90 g of wet cells were obtained from 10 liters of autoinduction medium. The purified enzyme yield was $\sim 14 \text{ mg}$ per gram of cells, or $\sim 130 \text{ mg}$ per liter of medium.

Figure 5.23.2.

A picture of a 96-well plate containing diluted lysates obtained from autoinduction of enhanced green fluorescent protein expression using the expression media array defined in Tables 5.23.1 and 5.23.2. The plate was illuminated with a 340-nm light source.

Figure 5.23.3.

Time course of autoinduction of the expression of a fusion of tobacco etch virus (TEV) protease to maltose-binding protein (MBP) in a fermenter. The expressed fusion protein had a protease cleavage site located in the linker region between maltose-binding protein and the protease, which yielded self-cleavage of the fusion protein during the cell growth. The times when samples were obtained and their OD₆₀₀ values are indicated. The samples at 8.7 hr are the total cell lysate (T) and the soluble fraction (S) obtained from lysed cells; >95% of the total protease was found in the soluble fraction.

Table 5.23.1

Percentages (w/v) of Glucose, Lactose, and Glycerol Present in Each Well of an Expression Media Array Shown in the 96-Well Plate Format^a

	Methionine							Selenomethionine				
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
	0.450	0.450	0.450	0.600	0.600	0.600	0.300	0.300	0.450	0.450	0.450	0.300
	0.300	0.300	0.300	0.000	0.000	0.000	0.600	0.600	0.300	0.300	0.300	0.600
	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
B	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300
	0.900	0.900	0.900	1.200	1.200	1.200	0.600	0.600	0.900	0.900	0.900	0.600
	0.075	0.050	0.025	0.100	0.050	0.000	0.025	0.000	0.075	0.050	0.025	0.025
	0.450	0.450	0.450	0.600	0.600	0.600	0.300	0.300	0.450	0.450	0.450	0.300
C	0.900	0.900	0.900	1.200	1.200	1.200	0.600	0.600	0.900	0.900	0.900	0.600
	0.075	0.050	0.025	0.100	0.050	0.000	0.075	0.100	0.075	0.050	0.025	0.075
	0.450	0.450	0.450	0.600	0.600	0.600	0.300	0.300	0.450	0.450	0.450	0.300
	0.075	0.050	0.025	0.100	0.050	0.000	0.075	0.100	0.075	0.050	0.025	0.075
D	0.450	0.450	0.450	0.600	0.600	0.600	0.300	0.300	0.450	0.450	0.450	0.300
	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600
	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
	0.150	0.150	0.150	0.000	0.000	0.000	0.450	0.600	0.150	0.150	0.150	0.450
E	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600
	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
	0.150	0.150	0.150	0.000	0.000	0.000	0.450	0.600	0.150	0.150	0.150	0.450
	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600
F	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
	0.150	0.150	0.150	0.000	0.000	0.000	0.150	0.000	0.150	0.150	0.150	0.150
	0.300	0.300	0.300	0.000	0.000	0.000	0.600	0.600	0.300	0.300	0.300	0.600
	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
G	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300
	0.300	0.300	0.300	0.000	0.000	0.000	0.900	1.200	0.300	0.300	0.300	0.900
	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
	0.150	0.150	0.150	0.000	0.000	0.000	0.300	0.300	0.150	0.150	0.150	0.300
H	0.900	0.900	0.900	1.200	1.200	1.200	0.300	0.000	0.900	0.900	0.900	0.300
	0.075	0.050	0.025	0.100	0.050	0.000	0.050	0.050	0.075	0.050	0.025	0.050
	0.150	0.150	0.150	0.000	0.000	0.000	0.300	0.300	0.150	0.150	0.150	0.300
	0.900	0.900	0.900	1.200	1.200	1.200	0.300	0.000	0.900	0.900	0.900	0.300

^aThe media in wells A1:H8 contain methionine, while the media in wells A9:H12 contain selenomethionine. This expression media array was used to obtain the GFP expression results shown in Figure 5.23.2. Wells C3 and C11 contain the sugar compositions of the media used in the Basic Protocol and Alternate Protocol 1, respectively.

Table 5.23.2

Volumes (μl) of Glucose, Lactose and Glycerol Solutions, and Water Added to Sugar-Free Autoinduction Medium to Create 40-ml Aliquots of the Expression Media Array

	8×8 methionine-containing array ^a								8×4 selenomethionine array ^b			
	1	2	3	4	5	6	7	8	9	10	11	12
A	75	50	25	100	50	0	50	50	75	50	25	50
	900	900	900	1200	1200	1200	600	600	900	900	900	600
	300	300	300	0	0	0	600	600	300	300	300	600
	2725	2750	2775	2700	2750	2800	2750	2750	2725	2750	2775	2750
B	75	50	25	100	50	0	50	50	75	50	25	50
	600	600	600	600	600	600	600	600	600	600	600	600
	900	900	900	1200	1200	1200	600	600	900	900	900	600
	2425	2450	2475	2100	2150	2200	2750	2750	2425	2450	2475	2750
C	75	50	25	100	50	0	25	0	75	50	25	25
	900	900	900	1200	1200	1200	600	600	900	900	900	600
	900	900	900	1200	1200	1200	600	600	900	900	900	600
	2125	2150	2175	1500	1550	1600	2775	2800	2125	2150	2175	2775
D	75	50	25	100	50	0	75	100	75	50	25	75
	900	900	900	1200	1200	1200	600	600	900	900	900	600
	600	600	600	600	600	600	600	600	600	600	600	600
	2425	2450	2475	2100	2150	2200	2725	2700	2425	2450	2475	2725
E	75	50	25	100	50	0	50	50	75	50	25	50
	300	300	300	0	0	0	900	1200	300	300	300	900
	600	600	600	600	600	600	600	600	600	600	600	600
	3025	3050	3075	3300	3350	3400	2450	2150	3025	3050	3075	2450
F	75	50	25	100	50	0	50	50	75	50	25	50
	300	300	300	0	0	0	300	0	300	300	300	300
	300	300	300	0	0	0	600	600	300	300	300	600
	3325	3350	3375	3900	3950	4000	3050	3350	3325	3350	3375	3050
G	75	50	25	100	50	0	50	50	75	50	25	50
	300	300	300	300	300	300	300	300	300	300	300	300

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8 × 8 methionine-containing array ^a								8 × 4 selenomethionine array ^b					
1	2	3	4	5	6	7	8	9	10	11	12		
H	300	300	0	0	0	900	1200	300	300	300	900	glycerol	
	3325	3350	3375	3600	3650	3700	2750	2450	3325	3350	3375	2750	water
	75	50	25	100	50	0	50	50	75	50	25	50	glucose
	150	150	150	0	0	0	300	300	150	150	150	300	lactose
	900	900	900	1200	1200	1200	300	0	900	900	900	300	glycerol
	2875	2900	2925	2700	2750	2800	3350	3650	2875	2900	2925	3350	water

^aThe indicated volumes are added to 36 ml of the sugar-free, methionine-containing autoinduction medium for wells A1:H8.

^bThe indicated volumes are added to 36 ml of the sugar-free, selenomethionine-containing autoinduction medium for wells A9:H12.