

Chapter 1

A Novel Bacterial Expression Method with Optimized Parameters for Very High Yield Production of Triple-Labeled Proteins

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Abstract

The Gram-negative bacterium *Escherichia coli* offer a means for rapid, high-yield, and economical production of recombinant proteins. However, when preparing protein samples for NMR, high-level production of functional isotopically labeled proteins can be quite challenging. This is especially true for the preparation of triple-labeled protein samples in D_2O ($^2H/^{13}C/^{15}N$). The large expense and time-consuming nature of triple-labeled protein production for NMR led us to revisit the current bacterial protein expression protocols. Our goal was to develop an efficient bacterial expression method for very high-level production of triple-labeled proteins that could be routinely utilized in every NMR lab without changing expression vectors or requiring fermentation. We developed a novel high cell-density IPTG-induction bacterial expression method that combines tightly controlled traditional IPTG-induction expression with the high cell-density of auto-induction expression. In addition, we optimize several key experimental protocols and parameters to ensure that our new high cell-density bacterial expression method routinely produces 14–25 mg of triple-labeled proteins and 15–35 mg of unlabeled proteins from 50-mL bacterial cell cultures.

Key words: High yield protein production, Bacterial expression, Isotopic labeling, NMR

1. Introduction

To perform NMR structural studies of proteins, we have to produce proteins that are isotopically labeled with ^{13}C and ^{15}N for small proteins (<20 kDa) and with ^{13}C , ^{15}N , and 2H for larger proteins (>20 kDa). Among the many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains one of the most attractive hosts (1, 2). This is especially true for isotopically labeling proteins since bacterial expression

provides the cheapest way to prepare these proteins for NMR studies (3). Protein expression and purification is a routine practice in many NMR labs, but it is not uncommon to see a drastic reduction in protein yield when isotopically labeling the proteins, especially when D₂O must be used.

To overcome these difficulties, we have developed a novel bacterial expression method that combines the tightly controlled traditional IPTG-induction bacterial expression with the high cell-density of the auto-induction method (4). To summarize our procedure, we first determine how to make a proper starting culture, followed by double colony selection and finally high cell-density expression. With these optimized protocols and parameters, our new bacterial expression method offers near gram quantity production of triple-labeled proteins from one-liter bacterial cell cultures, without changing expression vectors and without fermentation (5). Thus, every NMR laboratory can easily apply this novel bacterial expression method on a routine basis for the production of a very high yield of triple-labeled proteins for their NMR structural studies of proteins.

2. Materials

2.1. Sample Preparation and SDS-PAGE

1. 4× SDS loading buffer: 200 mM Tris-HCl, pH 6.8, 8% (w/v) sodium dodecyl sulfate (SDS), 0.4% (w/v) bromophenol blue, 40% glycerol. Store at room temperature.
2. Dithiothreitol (DTT): Prepare 1 M solution using sterile water. Filter through a 0.22-μm pore size membrane (syringe filter) and store 1-mL aliquots at -20°C.
3. 30% Acrylamide/bis solution (29:1).
4. 1.5 M Tris-HCl, pH 8.8.
5. 10% SDS.
6. 10% ammonium persulfate.
7. TEMED.
8. M Tris-HCl, pH 6.8.
9. Protein molecular weight markers.
10. 5× SDS running buffer: 0.5% SDS, 125 mM Tris base, 1.25 M glycine. Store at room temperature. Do not adjust pH.
11. Coomassie blue-staining solution: 0.25% (w/v) Coomassie brilliant blue, 45% methanol, 10% acetic acid. Add brilliant blue to methanol and stir for 60 min. Add water and acetic acid and stir for another 30 min. Store at room temperature (see Note 1).
12. Destaining solution: 30% methanol, 10% acetic acid. Store at room temperature.

2.2. Protein Expression

1. LB medium (Miller): Dissolve 25 g of powdered LB medium in 1 L of distilled water or D₂O. Adjust the pH to 7.4 using NaOH. Autoclave and store at room temperature (see Note 2). Add antibiotics (KAN or AMP) prior to use.
2. Kanamycin monosulfate stock solution (KAN): Dissolve KAN monosulfate to a concentration of 30 mg/mL in distilled water. Syringe filter and store 1 mL aliquots at -20°C.
3. Ampicillin sodium sulfate stock solution (AMP): Dissolve AMP sodium sulfate to a concentration of 50 mg/mL in distilled water. Syringe filter and store 1 mL aliquots at -20°C.
4. LB agar plates (Miller): Dissolve 40 g of LB agar (Miller) in 1 L of distilled water and/or D₂O in a 2-L flask. Cover with foil and autoclave. Monitor the temperature as it cools. When the temperature reaches ~50°C, add 1 mL of the KAN or AMP stock solution. Pour ~10 mL into 100×10-mm Petri dishes and swirl to coat the plate. Let the LB agar solidify at room temperature. Place plates back into a plastic bag, seal with tape, and store at 4°C (see Note 2).
5. Isopropyl-β-D-thiogalactopyranoside (IPTG): Prepare a 1 M solution using distilled water. Syringe filter and store 1 mL aliquots at -20°C.
6. 100% Glycerol: Autoclave to sterilize. Store at room temperature.
7. 5× M9 Salts (1 L): Dissolve 64 g of Na₂HPO₄, 15 g of KH₂PO₄, 5 g of NH₄Cl, and 2.5 g of NaCl in distilled water or D₂O, adjust the volume to 1 L. Autoclave to sterilize and store at room temperature (see Note 2). Omit NH₄Cl from this recipe when 5× M9 salts are used for isotope-labeling. Do not adjust pH.
8. 20% Glucose: Dissolve 20 g glucose in distilled water, adjust volume to 100 mL. Sterilize by filtration and store at 4°C.
9. MgSO₄: Prepare a 1-M solution, autoclave, and store at room temperature.
10. CaCl₂: Prepare 1-M solution, autoclave, and store at room temperature.
11. M9 minimal medium for traditional IPTG method and double colony selection (100 mL): 78 mL of distilled, sterilized water, 20 mL of 5× M9 salts, 2 mL of 20% glucose, 200 μL of 1 M MgSO₄, 10 μL of 1 M CaCl₂, and 100 μL of antibiotic. Add the CaCl₂ last and immediately swirl the flask to dissolve the cloudy precipitate. Adjust the pH to 7.4 using NaOH.
12. M9 minimal medium for high cell-density IPTG-induction method (100 mL): 75 mL of distilled, sterilized water, 20 mL of 5× M9 salts, 5 mL of 20% glucose, 200 μL of 1 M MgSO₄, 10 μL of 1 M CaCl₂, and 100 μL of antibiotic. Add the CaCl₂ last and immediately swirl the flask to dissolve the cloudy precipitate (see Note 3). Adjust the pH to 7.4 using NaOH (see Note 4).

13. M9 for double-labeling (100 mL): 80 mL of distilled, sterilized water, 20 mL of 5× M9 salts without NH_4Cl , 100 mg of $^{15}\text{NH}_4\text{Cl}$ and 0.2 g of ^{13}C -glucose (for traditional IPTG method) or 1 g of ^{13}C -glucose (for high cell-density IPTG-induction method), 200 μL of 1 M MgSO_4 , 10 μL of 1 M CaCl_2 , and 100 μL of antibiotic. Add the CaCl_2 last and immediately swirl the flask to dissolve the cloudy precipitate (see Note 3). Adjust the pH to 7.4 using NaOH (see Note 4). Use a filtration unit with a 0.22- μm pore size to sterilize the medium (see Note 5).
14. M9 for triple-labeling (100 mL): 80 mL of 99% D_2O , 20 mL of 5× M9 salts without NH_4Cl in D_2O , 100 mg of $^{15}\text{NH}_4\text{Cl}$, and 0.2 g of $^{13}\text{C}/^2\text{H}$ -glucose (for traditional IPTG-induction method), or 1 g of $^{13}\text{C}/^2\text{H}$ -glucose (for high cell-density IPTG-induction method), 200 μL of 1 M MgSO_4 , 10 μL of 1 M CaCl_2 , and 100 μL of antibiotic. You can also use ^{13}C -glucose; however, this usually generates ~90% deuterated triple-labeled protein samples. Add the CaCl_2 last and immediately swirl the flask to dissolve the cloudy precipitate (see Note 3). Adjust the pH to 7.4 using NaOH (see Note 4). Use a filtration unit with a 0.22- μm pore size to sterilize the medium (see Note 5).
15. 1000× Trace metals (100 mL): Dissolve 811 mg of FeCl_3 (50 mM), 222 mg of CaCl_2 (20 mM), 125.8 mg of MnCl_2 (10 mM), 161.5 mg of ZnSO_4 (10 mM), 26 mg of CoCl_2 (2 mM), 26.9 mg of CuCl_2 (2 mM), 25.9 mg of NiCl_2 (2 mM), 41.2 mg of Na_2MoO_4 (2 mM), 34.6 mg of Na_2SeO_3 (2 mM), and 12.4 mg of H_3BO_3 (2 mM) in 60 mM HCl. Autoclave to sterilize. Store at room temperature.
16. BME vitamins (see Note 3).

2.3. Protein Purification

1. Affinity resin: His-Bind Resin.
2. 8× Charge buffer: 400 mM NiSO_4 . Store at 4°C.
3. 8× Binding buffer: 160 mM Tris-HCl, pH 7.9, 2 M NaCl, 20 mM imidazole. Store in an amber bottle at room temperature.
4. 8× Wash buffer: 160 mM Tris-HCl, pH 7.9, 2 M NaCl, 240 mM imidazole. Store in an amber bottle at room temperature.
5. 4× Elute buffer: 80 mM Tris-HCl, pH 7.9, 1 M NaCl, 4 M imidazole. Store in an amber bottle at room temperature.
6. $(\text{NH}_4)_2\text{CO}_3$: Dissolve 474.4 g of $(\text{NH}_4)_2\text{CO}_3$ in 3.5 L of water. Once dissolved, adjust the volume to 4 L (final concentration 1.23 M) and store at room temperature. Do not adjust pH.
7. Urea.

3. Methods

When triple-labeling proteins, bacteria have to be grown in D_2O , usually causing a significant reduction in protein yields. We sought to overcome this obstacle. Our strategy mainly focuses on increasing the cell density of bacterial expression without manipulation of the expression vector or use of a fermenter. Unfortunately, bacterial expression at a high cell-density in D_2O usually causes several major problems, including (1) plasmid loss, (2) significant reduction in the pH of the growth medium due to cell metabolites, and (3) limited availability of dissolved oxygen. These problems often result in a low or even no protein production with high cell-density bacterial expression.

We developed several practical protocols that solved these problems, including (1) preparation of a proper starting culture, (2) double colony selection in D_2O , (3) optimization of bacterial expression conditions, and (4) better control of the pH of the medium. We further developed a high cell-density IPTG-induction bacterial expression method that combines the tightly controlled traditional IPTG-induction expression with high cell-density auto-induction expression. Our optimized protocols ensure plasmid stability inside bacterial cells, resulting in routine production of 14–25 mg triple-labeled proteins from a 50-mL bacterial cell culture. Importantly, this novel bacterial expression method uses the same expression vectors as the traditional IPTG-induction method and does not require a fermenter. Thus, every NMR laboratory can easily adopt this novel bacterial expression method to produce large quantities of triple-labeled proteins.

3.1. PAGE Sample Preparation

PAGE samples come either from the cell lysate isolated immediately after bacterial expression or from the column flow through during protein purification.

1. For samples collected directly from bacterial cell culture: Collect 500 μL of cells and place in a 1.7-mL microcentrifuge tube. Spin down at $12,000\times g$ for 5 min at room temperature using a microcentrifuge, discard the supernatant, and tap out the excess on a paper towel.
2. Add 25 μL of 4 \times SDS loading buffer and 25 μL of water and resuspend the pellet. Store the samples in a freezer until ready to run a gel.
3. Before running the culture samples on a gel, place the samples on a 90°C heat block for 30 min. Remove from the heat block; add 50 μL of water and vortex for 30 s (see Note 6).
4. For samples collected from flow through during protein purification, mix 60 μL of column flow through with 20 μL of

4× SDS loading buffer and mix thoroughly by repeated pipetting up and down. Store the samples in a freezer until ready to run a gel.

5. Before running the flow through samples on a gel, place the samples on a 90°C heat block for 5 min (see Note 6).
6. Before loading samples on a gel, centrifuge at 12,000×*g* for 10 min at room temperature using a microcentrifuge to pellet cellular debris (see Note 7).

3.2. SDS-PAGE

1. Depending on the protein size, choose an appropriate acrylamide percentage for the resolving gel (see Note 8).
2. To prepare a 10% SDS-PAGE mini-resolving gel (5 mL), using a mini-gel apparatus: Mix 1.9 mL of water, 1.7 mL of 30% acrylamide/bis solution, 1.3 mL of 1.5 M Tris-HCl, pH 8.8, 50 µL of 10% SDS, 50 µL of 10% ammonium persulfate, and 2 µL of TEMED. Mix well and pour between glass plates set in a loading cassette. Leave about 1.5-cm space on top for the stacking gel. Gently pipet water on top and let the gel set (about 20 min).
3. Pour a stacking gel (2 mL) once the resolving gel has set. Mix 1.4 mL of water, 330 µL of 30% acrylamide/bis solution, 250 µL of 1 M Tris-HCl, pH 6.8, 20 µL of 10% SDS, 20 µL of ammonium persulfate, and 2 µL of TEMED. Pour the water off the top of the resolving gel, remove excess water with filter paper, and pour the stacking gel. Insert a comb containing the appropriate number of lanes and let the stacking gel set (about 20 min).
4. Prepare 1× running buffer by diluting 100 mL of 5× running buffer solution with 400 mL of distilled water. Make sure to mix the solution well. Pour the buffer into the inner and outer chambers of the gel apparatus. For cell culture samples, load 7.5 µL of each sample into the lanes. For column flow through samples, load 20 µL into each lane. Make sure to load 5 µL of molecular weight markers in one lane.
5. Secure the lid on the gel box and plug into a power supply. Run the gel at 88 V for ~2 h. Turn off the power supply when the blue dye front reaches the bottom of the gel.
6. Remove the gel from the glass plates and place it in a small box with 20 mL of Coomassie blue-staining solution. Allow the gel to stain for at least 30 min (see Note 1). Pour out the stain, rinse the gel with water to remove excess stain and then add 30 mL of destaining solution. Place a small piece of paper towel in the box to accelerate the destaining process. This process may take a few hours; however, you can start to detect bands within 30–60 min.

3.3. A Proper Starting Culture

A critical consideration for high-level bacterial expression is the preparation of a proper starting culture in a rich medium for scaling up in minimal medium. The general practice in NMR laboratories is to grow an overnight culture using a rich medium, such as LB, at 37°C. We observe that an overnight culture usually reaches saturation by the next morning, and may result in plasmid instability and loss due to several factors including basal leakage of the T7 expression system that expresses the toxic target proteins to the host cells under this overgrowth condition (4, 6, 7). This usually results in a poor yield of target protein. Figure 1 shows a growth curve for *E. coli* BL21 (DE3) cells carrying the LCAT/pET30a vector, in H₂O-based LB, suggesting that the bacteria are in the exponential or log phase of growth between 6 and 7.5 h at 37°C (see Note 9). We placed particular emphasis on double colony selection (Subheading 3.5) to ensure that a high percentage of bacterial cells within this colony contain the DNA expression plasmid.

1. Perform a time course of bacterial growth of a new protein expression vector in rich (LB) medium by measuring the OD₆₀₀ every 30 min for ~10–12 h in water-based rich medium and ~16–18 h in D₂O-based rich medium (see Note 10). The OD₆₀₀

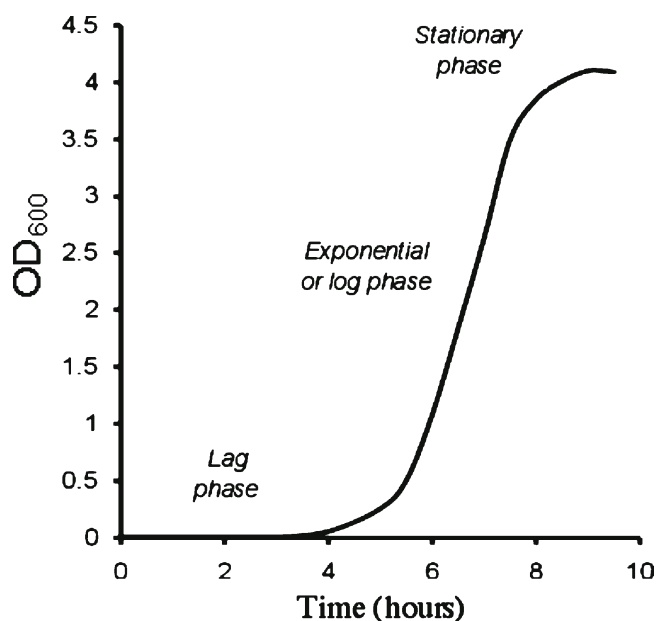


Fig. 1. Plot of *E. coli* growth in 5 mL of LB medium over a 10-h period at 37°C starting with a glycerol stock. The bacterial strain, BL-21(DE3), contains a pET30a vector expressing the gene for lecithin:cholesterol acyltransferase (LCAT). Based on this plot, the log phase of the culture is between an OD₆₀₀ of 1 and 3.5. Note: The growth curve is vector, protein, and bacterial strain dependent. Reproduced from Murray 2010 with permission from Cold Spring Harbor.

of the log phase and the time to reach saturation are vector, protein, and bacterial strain-dependent; therefore, we suggest performing this experiment before actually expressing protein for purification and labeling (see Note 11).

2. Once the optimal OD_{600} of the log phase of the starting culture is determined, this will be the OD_{600} for all future starting cultures using both traditional IPTG expression and high cell-density expression methods.

3.4. Traditional IPTG Method

This method can be scaled up or down to suit your needs. This following protocol is used to make a 100-mL expression culture.

1. Prepare a 5-mL proper starting culture as described in Subheading 3.3 in a 50-mL conical tube with holes poked in the lid.
2. Prepare 100 mL of M9 minimal medium in a 250-mL flask. Add 1.5 mL of the starting culture and measure the OD_{600} . (To check the OD_{600} at this point, aliquot 1 mL of the cell culture into a cuvette and measure the OD_{600} .) We suggest a starting OD_{600} between 0.05 and 0.10 for healthy bacterial cell growth. Place the flask in a 37°C incubator with a shaking speed of 200 rpm.
3. Start to monitor the OD_{600} after 3 h (to check the OD_{600} at this point, dilute 100 μ L of cell culture into a cuvette containing 900 μ L of distilled water and measure the OD_{600} . The final OD_{600} value is ten times the spectrophotometer reading). Once the OD_{600} reaches between 0.8 and 1.2 (see Note 12), remove 2 mL of cell culture and place it in a 15-mL culture tube (for a non-induced reference). Induce the remaining culture with 0.5 mM IPTG. Place both the flask and the 15-mL culture tube in a 20°C incubator overnight with a shaking speed of 200 rpm.
4. The following morning, measure the final OD_{600} of the cell culture. If the protein expression is induced at an OD_{600} of 1, the OD_{600} of the bacterial cell culture will be around 2–3, indicating healthy bacterial cell growth. Harvest the cells by spinning down at 10,000 $\times g$ for 10 min at 4°C using a benchtop centrifuge. Remove the supernatant and either store the cell pellet at –80°C or use immediately for protein purification.
5. To check protein expression levels, take 500 μ L samples of each culture (non-induced and IPTG-induced), follow the sample preparation protocol (Subheading 3.1) and run an SDS-PAGE (Subheading 3.2) to compare non-induced and IPTG-induced samples.

3.5. Double Colony Selection

Since plasmid loss is encountered during bacterial expression in D_2O much more frequently than in H_2O , we describe a double colony selection procedure for triple-labeling protein in D_2O . Based on our

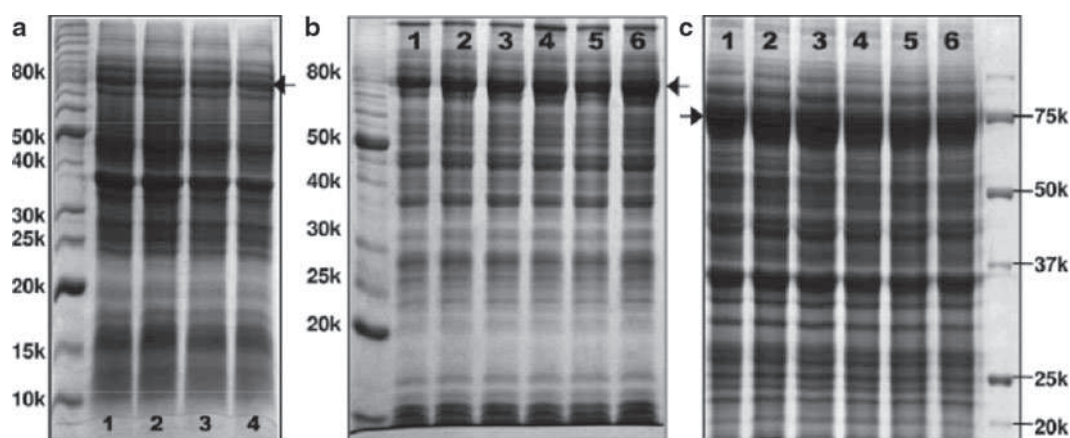


Fig. 2. SDS-PAGE of protein expression of apoE(1-215)/pTYB1 in D_2O before (a), during (b) and after (c) double colony selection. Arrows indicate the expected protein band (~ 80 kDa). (a) Shows four different colonies before colony selection. (b) Shows the results of three different colonies selected from the single colony selection (*lanes 1-3*) and another three colonies selected from the double colony selection (*lanes 4-6*). The second colony selection was based on Colony 3 (*lane 3, b*). (c) Shows the results of six colonies from the double colony selection, indicating a high level of protein expression for all six colonies. Reproduced from Sivashanmugam 2009 with permission from Wiley Interscience.

experience, this is a critical protocol that significantly increases the yield of triple-labeled proteins. Figure 2 shows the result of a typical double colony selection of apoE(1-215)/pTYB1 in D_2O , demonstrating that high protein expression levels are achieved after double colony selection. This high-level expression of apoE(1-215)/pTYB1 in D_2O has been stable for more than 2 years.

Usually, we perform double colony selection before we optimize the expression conditions. Thus, the traditional IPTG-induction method is the default method with which to start double colony selection. The procedure, described in Subheading 3.4, can be applied here for double colony selection, except for culture volume and the use of D_2O .

1. Prepare LB agar plates with 50% D_2O .
2. Perform a bacterial transformation using LB agar plates prepared with D_2O .
3. Next afternoon, choose nine colonies to make starting cultures of 5 mL of LB in 50% D_2O and 5 μ L of antibiotic in 50-mL conical tubes, and prepare a master plate (see Note 13). Punch holes in the lids of the tubes. Eight of the nine colonies will be induced with IPTG and the ninth colony will be used as a negative, non-induced control.
4. When the starting culture is ready, add 50–100 μ L to 5 mL of M9 minimal medium in 70% D_2O and 5 μ L of antibiotic in 50-mL conical tubes. Ensure that the starting OD_{600} is between 0.05 and 0.10. Place the tube in a 37°C incubator with a shaking speed of 200 rpm.

5. When the OD_{600} of the culture reaches between 0.8 and 1 (see Note 14), add 0.5 mM IPTG to eight of the nine cultures to induce protein expression, clearly marking which culture is serving as a negative control. Place the tubes in a 20°C incubator overnight with a shaking speed of 200 rpm.
6. The following morning, remove 500 μ L of cell culture from each tube and place in a microcentrifuge tube. Centrifuge at $12,000\times g$ for 10 min at room temperature and discard the supernatant. Prepare SDS-PAGE samples and run a gel.
7. Choose the colony expressing the biggest protein band (using the negative control as reference) and prepare 5 mL of LB culture in either 70 or 99% D_2O and 5 μ L of antibiotic containing the colony from the master plate. Grow at 37°C until the OD_{600} reaches 0.7–0.9 and spread 150 μ L on an LB plate prepared in 50% D_2O . Invert the plate and incubate it at 37°C overnight.
8. The following day, repeat steps 3–6 using 70 or 99% D_2O for the second round of colony selection (see Note 14). When completed, choose the colony expressing the biggest protein band to make a 5-mL LB culture in 100% D_2O and 5 μ L of antibiotic. Grow the culture halfway through its log phase. Add 800 μ L of culture to 200 μ L of 100% sterile glycerol in a 2-mL cryogenic tube with a screw top cap. Pipet up and down to mix thoroughly and flash freeze the tubes by dipping them in liquid nitrogen, store at -80°C . If the protein expression level is extremely high, we suggest that you make at least 5–10 glycerol stocks of this double selected colony for future use.

3.6. High Cell-Density IPTG-Induction Method

This is a hybrid method combining traditional IPTG-induction and auto-induction bacterial expression methods. It takes advantage of tightly controlled IPTG-induction and the high cell-density of the auto-induction bacterial expression. We use rich media, such as LB and 2 \times YT, to reach a high cell-density before IPTG-induction and then switch the culture medium by gently spinning-down the cells and resuspending them into an equal volume of minimal medium. However, many problems may occur during a high cell-density bacterial expression that can cause a significant reduction in protein yield, such as reduced pH of the expression medium, poor aeration, and/or plasmid loss during expression. We describe the following procedure to avoid these problems, ensuring a high yield of triple-labeled protein.

1. Using the glycerol stock prepared after double colony selection (Subheading 3.5), make a starting culture in 50 mL of LB medium containing 50 μ L antibiotic in a 250-mL flask (Dip the pipet tip in the glycerol stock and scratch the surface. Place the tip into the culture medium, pipet up and down a few

- times and remove the tip. Immediately return the glycerol stock to the -80°C freezer). Place the flask in a 37°C incubator with a shaking speed of 200 rpm and incubate until the OD_{600} is halfway through the log phase (Subheading 3.3, step 1). DO NOT let the starting culture grow overnight since the saturation of cell growth may cause plasmid loss.
2. Transfer the cells into sterile tubes and spin down the culture at $5,000\times g$ for 7 min at room temperature. Remove the supernatant and tap the tubes on paper towels to remove as much LB as possible.
 3. Gently resuspend the pellets in 50 mL of M9 minimal medium (see Note 4) and transfer the resuspended cells to a 250-mL sterile flask. Place the flask in an incubator that is set at the optimal induction temperature (see Subheading 3.7). Maintain the shaking speed at 200 rpm for efficient aeration and keep the culture at the optimal temperature for 1–1.5 h to allow the cells to adapt to the new medium. An enhancement of approximate 0.5–1 U of OD_{600} should be observed at the end of this time. For example, if the OD_{600} is 3 right after medium exchange, you can expect the OD_{600} to reach 3.5–4 at the end of the 1–1.5 h incubation, indicating healthy cell growth (this increase in OD_{600} will be slightly less when expressing in D_2O).
 4. Add the optimal concentration of IPTG (Subheading 3.7) to induce protein expression and keep the culture in the incubator for an optimal period of time (Subheading 3.7) at a shaking speed of 200 rpm.
 5. Measure the final OD_{600} before harvesting cells. You can expect to see a two- to fourfold enhancement at the end of the expression. For example, if your OD_{600} is 4 after the 1–1.5 h incubation, the final OD_{600} will be between 8 and 16, indicating healthy bacterial growth.
 6. Harvest the cells by spinning down the culture at $10,000\times g$ for 10 min at 4°C . Remove the supernatant and either store the cell pellet at -80°C , or immediately use for protein purification.

3.7. Optimization of Various Conditions

Another important step for high-level protein production using high cell-density bacterial expression is to optimize the expression conditions such as culture temperature and the induction time. These steps are critical for the initial expression of a protein using the high cell-density expression method. We usually use the traditional IPTG-induction method first to check if a new protein can be expressed by using bacteria. Once the protein expression is confirmed by the traditional IPTG-induction method, we can optimize the high cell-density expression method to produce high-yield isotopically labeled proteins.

We usually carry out time courses at different temperatures, such as 15, 20, room temperature, 30 and 37°C. We normally prepare a 5-mL starting culture either in D₂O or in water for the time course and closely monitor the following parameters during expression: OD₆₀₀, pH, and target protein production. The detailed procedure of optimization follows:

1. Prepare 10 mL starting cultures in 50-mL flasks: 10 mL of LB medium in 99% D₂O, 10 µL of antibiotic and bacterial cells from a glycerol stock after double colony selection. Incubate at 37°C with a shaking speed of 200 rpm.
2. Once the optimal OD₆₀₀ has been reached, gently centrifuge the cells at 5,000×*g* for 7 min at room temperature and discard the supernatant. Resuspend the cell pellets with 10 mL of M9 minimal medium in 99% D₂O in 50-mL flasks and incubate at various temperatures, such as 15, 20, room-temperature, 30° and 37°C, for 1 h. Check the OD₆₀₀ of the cultures before and after this 1-h cell incubation.
3. If the OD₆₀₀ of each culture after the 1-h incubation increases by 0.5–1 OD₆₀₀ units, this indicates that the cells are healthy and growing after the medium exchange. Induce protein expression by adding 0.5 mM IPTG. Be sure to choose one culture without IPTG-induction to serve as the negative control. Return the tubes to appropriate shakers at different temperatures. We found that 0.5 mM IPTG usually gives a reasonable protein production, thus we always used this IPTG concentration as our starting point. However, an independent optimization of IPTG concentration can be carried out and is discussed in step 6 of this section.
4. For cultures growing below 25°C, let them grow overnight (~14–16 h). The following morning, collect 500 µL of cell culture samples every 2 h (typically collect between 16 and 28 h and one more sample the next morning). For the cultures growing above 25°C, start to collect samples every 2 h after induction for at least 8 h and one the next morning. At each time point, check the OD₆₀₀ and the pH of the cell culture (see Note 4). Be sure to keep the collected cell pellets at –20°C.
5. When all of the samples have been collected, prepare samples for SDS-PAGE analysis. A comparison with the negative control (noninduced culture) allows you to determine which temperature and induction time give you the best protein yield (see Note 15).
6. Repeat the above procedure to optimize the IPTG concentration using the optimized temperature and incubation time. We usually test IPTG concentrations of 0.1, 0.25, 0.5, 0.75, and 1 mM.
7. Once the optimal conditions have been determined, you can now perform high cell-density bacterial expression on a larger

scale. Be sure to use a large flask for better aeration. We usually use a 250-mL flask for a 50-mL cell culture and a 500-mL flask for a 100-mL cell culture. If you want to grow a 200-mL cell culture, we suggest dividing the culture into 2×100 mL cultures in two 500-mL flasks.

Figure 3 shows a typical time course experiment performed during optimization of experimental conditions. Table 1 shows the expression parameters for the time course during our optimization of the expression of human apolipoprotein A-I (apoAI). It is clear that at the maximum OD_{600} the bacterial expression produces the highest yield of triple-labeled apoAI, as confirmed by a Western blot (Fig. 3).

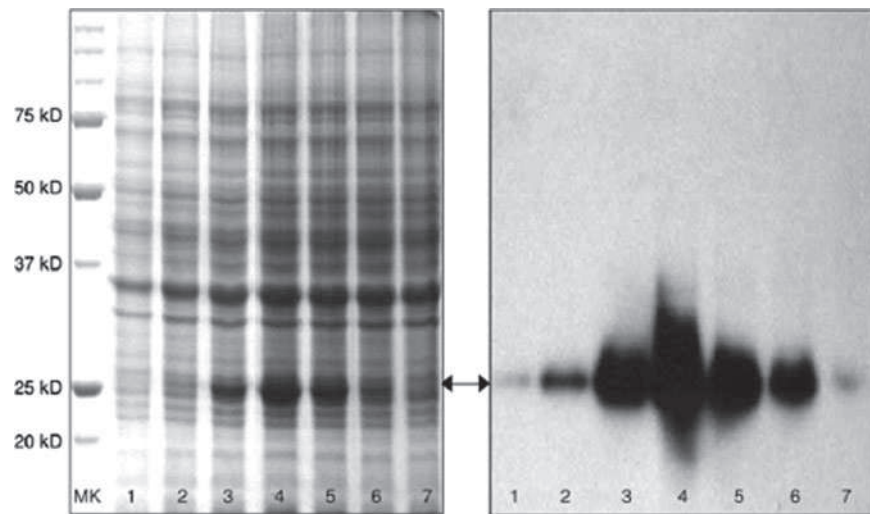


Fig. 3. *Left panel*: An SDS-PAGE showing auto-induction time course of triple-labeled human apoAI expression in D_2O at room temperature. *Lanes 1–7* = 24, 28, 32, 36, 40, 44, and 54 h, respectively. *Right panel* is a Western blot of the same time course using anti-apoAI monoclonal antibody. Reproduced from Sivashanmugam 2009 with permission from Wiley Interscience.

Table 1
Parameters of the time course of human apolipoprotein A-I expression

Time	24 h	28 h	32 h	36 h	40 h	44 h	54 h
OD_{600}	2.5	3.9	7.2	9.1	8.4	8	8.1
pH	6.6	6.5	6.3	6	6	6	6.1
Protein yield	–	+	++	+++	++	++	–

3.8. Protein Purification

Protein purification depends on the fusion tag that is used, as different tags serve different purposes. In our laboratory, we generally use histidine tags. In this section, we describe a typical protein purification procedure using a His-Bind Resin column.

1. Prepare 1× dilutions of all buffers. Recheck the pH to ensure that they are still 7.9.
2. Centrifuge the cell culture at 10,000×*g* for 10 min at 4°C. Remove the supernatant and resuspend the pellets in 20 mL of 1× binding buffer. If the protein is in inclusion bodies and can be refolded readily during dialysis, you can resuspend the pellet in 20 mL of 1× binding buffer containing 6 M urea.
3. Lyse the cells by using either sonication or a French press. Centrifuge the lysate at 16,000×*g* for 20 min at 4°C. Collect the supernatant and store it on ice.
4. Add 10 mL of 1× binding buffer and repeat step 2 at least twice, and then combine all the supernatants. Depending on the protein, you may need to add additional binding buffer and repeat step 2 3–5 times to completely extract the protein from the cells.
5. Equilibrate the affinity column with 50 mL of 1× charge buffer (see Note 16). Remove the charge buffer and equilibrate the column with 50 mL of 1× binding buffer. The column should be a light blue color after equilibration.
6. Load the column with the clear lysate from step 3. The flow rate should be ~1 mL/minute (see Note 17). Collect the flow through and remove a 60-μL sample for SDS-PAGE.
7. Wash the column with 200 mL of 1× binding buffer, followed by an additional 100 mL of 1× wash buffer. The flow rate of wash buffer should also be ~1 mL/min. Elute the column with 100 mL of 1× elution buffer. Collect the last drop of elution for SDS-PAGE to make sure that all of the protein has been eluted from the column.
8. Perform SDS-PAGE analysis with all of the collected samples to assess the purification.
9. Place the eluted protein into a dialysis bag and dialyze extensively against water containing 20 mM (NH₄)₂CO₃ to remove imidazole, salts and possibly urea. After dialysis, freeze the protein sample with liquid nitrogen and lyophilize to obtain pure triple-labeled protein powder. Run a gel to assess the purity of the protein powder.

3.9. Conclusion

With the high cell-density IPTG-induction bacterial expression method and the practical protocols described above, we routinely produce 14–25 mg of triple-labeled proteins and 15–35 mg of unlabeled proteins from a 50-mL cell culture for all the proteins we tested. Table 2 lists the final yields of unlabeled and triple-labeled proteins obtained using high cell-density bacterial expression

Table 2
Final yields of unlabeled and triple labeled proteins: high cell-density vs. traditional IPTG method

Protein	High cell density ^b (mg)	IPTG ^b (mg)	M.W. (Cal) (Da)	M.W. (MS) (Da)	%D ^c
<i>Triple-labeled</i>					
RAP(1–210)	20 ± 3	0.5	33,801	33,525 ± 195	~92
RAP(91–323)	25 ± 3	0.8	36,633	36,376 ± 200	~93
ApoE(1–183) ^a	18 ± 4	2	22,866	22,686 ± 116	~89
Mouse apoAI(1–216)	15 ± 2	0.8	28,014	27,732 ± 125	~90
Human apoAI	14 ± 1	0.6	32,814	32,401 ± 150	~88
<i>Unlabeled</i>					
Human apoAI	34 ± 1	1			
Human apoE	17 ± 2	0.2			

M.W. molecular weight

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^aApoE(1–183) was expressed in 40% D₂O, the rest are expressed in 99.7% D₂O

^bHigh cell density (50-mL culture volume): high cell-density expression methods, including auto-induction and high cell-density IPTG-induction; IPTG: the optimized traditional IPTG-induced expression. We repeated the expressions at least three times for all proteins, the yield shown is the average ± standard deviation

^cEstimated percentage of deuteration, assuming 100% ¹³C and ¹⁵N-labeling. For apoE(1–183), the %D is the estimated percentage of deuteration based on 40% D₂O. For the other four proteins, the %D is the estimated percentage of deuteration based on 99.7% D₂O

compared with the yields obtained by using the traditional IPTG-induction method in a 50-mL cell culture, the results suggest a 5–100-fold enhancement in protein yield. In addition, the protocols described produce a consistent high-level of triple-labeled protein, which is always reproducible. Table 2 also gives mass spectroscopic data for the triple-labeled protein, indicating the efficiency of deuteration for triple-labeled protein using auto-induction expressions. Overall, the deuteration efficiency is around 90% if we assume the ¹³C and ¹⁵N-labeling are 100%. This is because we used 99.7% D₂O and nondeuterated ¹³C-glycerol or ¹³C-glucose in high cell-density expressions. This result is comparable to the deuteration efficiency of the traditional IPTG-induction expression method using single labeled ¹³C-glucose.

4. Notes

1. The stain can be reused multiple times. When using fresh stain, you only need to stain gels for 15–30 min. Pour the used stain into a separate container. When reusing stain, you may need to stain gels longer.

2. When using D₂O to replace water in LB agar, broth or M9 salts, solutions CANNOT be autoclaved to sterilize. When making LB broth, follow the directions as stated, but instead of autoclaving, use a filtration unit with a 0.22- μ m pore size to sterilize. When making LB agar plates, use a microwave to bring the solution just to a boil (to dissolve agar), add antibiotics once the agar temperature cools to ~50°C, and pour into plates. When making 5 \times M9 salts, follow the directions as stated, however omit NH₄Cl for isotopic-labeling. Once again, do not autoclave; sterilize using a filtration unit with a 0.22- μ m pore size.
3. For the auto-induction method, Studier suggests using vitamins and trace metals (4). We found vitamins and trace metals help promote healthy bacterial growth when using our high cell-density IPTG-induction bacterial expression. We purchased the trace metals and BME vitamins stock solution from Sigma. The trace metals used in our laboratory is based on Studier's recipe provided in the supplement materials of his elegant paper on the auto-induction bacterial expression method (4). For our optimized high cell-density IPTG-induction minimal medium, we added 0.25 \times vitamins and 0.25 \times trace metals (see Table 1 in ref. 5).
4. While monitoring OD₆₀₀ during the optimization time course (Subheading 3.7), the pH should be monitored as well. As the cell density increases, the pH of the culture lowers due to the release of cell metabolites. If the pH becomes too low (pH < 6), it will affect bacterial cell health and protein production. If the pH drops below 6 during the time course, we increase the pH of the M9 minimal medium to 8 using NaOH to allow for a larger buffering capacity of the culture medium.
5. M9 minimal medium containing isotopes (²H, ¹³C, or ¹⁵N) cannot be autoclaved. It must be sterilized using a filtration unit containing a 0.22- μ m pore size.
6. If the protein contains cysteine residue(s), add 10–20 mM DTT after adding water. Vortex the sample well and let it sit for 30–60 min at room temperature.
7. The release of DNA can cause the sample to become quite viscous, making it hard to load on the gel. If you notice this, you can simply sonicate your sample at a low wattage (4–6 W) for 5–10 s. Afterward, spin down the sample at 12,000 $\times g$ for 2 min at room temperature. Also, when loading the gel, remove the sample from the top portion of the supernatant to avoid the pelleted cellular debris at the bottom.
8. To determine which percentage gel to use, follow the guidelines found in Table A8-8 in Sambrook and Russell (8). Also, if you are working with proteins that weigh less than 15 kDa,

12% Tricine gels or gradient gels are highly recommended for better resolution in the molecular weight range of 5–15 kDa.

9. Bacteria display a four-phase pattern of cell growth in liquid media. First, there is an initial lag phase when bacteria are adapting to the growth conditions; at this point, an increase in OD_{600} will not be seen. Second, bacteria enter their exponential or log phase at which point the bacterial cells start dividing (doubling in number). The OD_{600} during this log phase climbs steadily. Third, bacteria enter the stationary phase during which the rate of cell growth significantly slows due to a decrease in available nutrients and an accumulation of toxins. The OD_{600} will level off during this phase. Finally, if fresh medium is not made available and toxins are not removed, bacteria will enter the death phase and a noticeable drop in OD_{600} will be observed. The key is to utilize a starting culture during the exponential or log phase of their growth curve (see Fig. 1).
10. When performing a time course using rich media, such as LB or 2× YT in D_2O , bacteria grow much more slowly than in water.
11. Based on our experience, we suggest the appropriate OD_{600} range for the starting culture is between 3 and 5 in LB medium and between 5 and 7 in 2× YT medium. However, the OD_{600} of the log phase is vector, protein, and bacterial strain-dependent. Thus, the best way to determine the middle point of the log phase is to perform a time course of bacterial growth for each new protein expression vector.
12. Based on our experience, for healthy bacterial cell growth, the culture is expected to reach an OD_{600} of 0.8–1.2 within 4–6 h for bacterial expressions in water and within 6–9 h for bacterial expressions in D_2O if the starting OD_{600} is between 0.05 and 0.10.
13. When performing colony selection, it is helpful to make a “master plate.” Take a KAN or AMP plate and make a 9-square grid under the agar plate. Label boxes 1–9. When you are ready to inoculate the medium, take a sterilized tip, gently touch the selected colony, and then gently touch the agar of the master plate in the corresponding box. Go back to the original plate, retouch the same colony, and drop it into a tube. Repeat this procedure for all selected colonies. Once finished, put the lid back on the plate, invert and incubate at 37°C for about 8 h. Colonies should be about 1–2-mm in diameter. Cut a long strip of parafilm, wrap the edges of the plate, and store inverted at 4°C. You can use this plate to regrow the colonies for future cell cultures. However, the plate is only good for about 2 weeks, so be sure to also make glycerol stocks.
14. If the bacteria do not grow well in 70% D_2O , then they must be trained to adapt to D_2O medium. For this purpose, pick a colony off a D_2O plate and start a 5-mL bacterial culture of LB

medium in 25% D₂O. Once the OD₆₀₀ of the culture reaches 1 at 37°C, transfer 100 µL of the cell culture into 5 mL of LB medium in 50% D₂O. The starting OD₆₀₀ of this new culture is about 0.1. Let the cell culture grow at 37°C until the OD₆₀₀ reaches 1 and transfer 100 µL of the cell culture into 5 mL of LB medium in 75% D₂O and let the culture to grow at 37°C until the OD₆₀₀ reaches 2–3. Use this cell culture as your starting culture.

15. A Western blot of this time course will further allow an unambiguous determination of the time point that produces the best protein yield.
16. Typically, we prepare columns containing 5 mL of affinity resin for a 50-mL cell culture since 2.5 mL of resin can bind about 20 mg of protein. This can be scaled up or down to suit your needs based on the expected protein yield.
17. If the flow rate is too slow, we have found that using a 1.5-µm syringe filter to remove cellular debris from the clear lysate prevents columns from becoming clogged and running slow.

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