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COMMUNICATION

A simple and robust protocol for high-yield expression of perdeuterated proteins in *Escherichia coli* grown in shaker flasks

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Abstract We present a simple, convenient and robust protocol for expressing perdeuterated proteins in *E. coli* BL21(DE3) cells in shaker flasks that reduces D_2O usage tenfold and d_7-glucose usage by 30 %. Using a modified M9 medium and optimized growth conditions, we were able to grow cells in linear log phase to an OD_{600} of up to 10. Inducing the cells with isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 10, instead of less than 1, enabled us to increase the cell mass tenfold per unit volume of cell culture. We show that protein expression levels per cell are the same when induced at an OD_{600} between 1 and 10 under these growth conditions. Thus, our new protocol can increase protein yield per unit volume of cell culture tenfold. Adaptation of *E. coli* from H_2O-based to D_2O-based medium is also key for ensuring high levels of protein expression in D_2O. We find that a simple three-step adaptation approach—Luria–Bertani (LB) medium in H_2O to LB in D_2O to modified-M9 medium in D_2O is both simple and reliable. The method increases the yield of perdeuterated proteins by up to tenfold using commonly available air shakers without any requirement for specialized fermentation equipment.

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Introduction

Protein expression in *E. coli* is routinely employed to produce large quantities of protein for structural and functional studies. Several structural techniques, including NMR, EPR and small angle neutron scattering make use of perdeutrated proteins. To produce fully perdeuterated proteins, deuterium oxide is one of the major costs. In the literature, most laboratories choose to use M9 minimal medium, adapted from Anderson's original work (Anderson 1946) in conjunction with d7-glucose and ammonium chloride as the sole carbon and nitrogen sources, respectively. In almost all cases, cells are induced at low cell densities (OD_{600} of 1 or less). Extremely high densities can be achieved in a fermentor, where the pH of the medium, oxygen level, and other factors can be regulated and kept optimal, there by allowing linear growth to very high cell densities. Duff et al. (2015) recently proposed a robust fermenter protocol to increase the yield of perdeuterated proteins by inducing cells at an OD_{600} of 30, thereby increasing the cell mass and protein yield proportionally. This fermenter-based method is not generally applicable to most laboratories that lack access to a fermenter. In the process of optimizing conditions to express a silk-elastin-like protein in shaker flasks, Collins et al. (2013) reported that under their optimized conditions in rich Luria–Bertani (LB) medium, maximum cell mass and protein yield was obtained at an OD_{600} of $\sim$2, corresponding to the end of the linear growth phase and the beginning of the stationary phase. In the current report, we describe a convenient

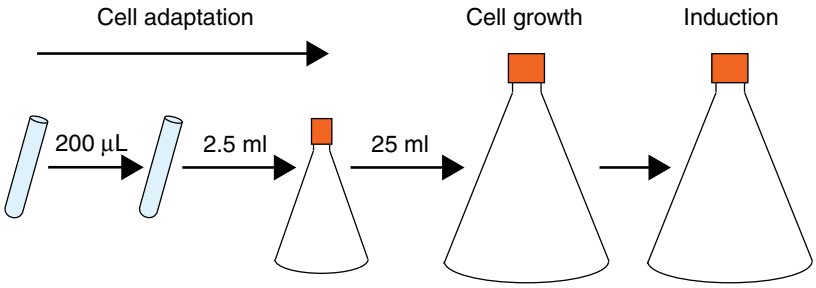
Table 1 Composition of regular M9 and the new M9+ medium for perdeuterated protein expression

Regular M9 medium		M9+ medium	
K_2HPO_4	10.0 g	K_2HPO_4	19.0 g
KH_2PO_4	13.0 g	KH_2PO_4	5.0 g
Na_2HPO_4	9.0 g	Na_2HPO_4	9.0 g
K_2SO_4	2.4 g	K_2SO_4	2.4 g
D-d_7-Glucose[a]	2.5 g	D-d_7-Glucose[a]	18.0 g
NH_4Cl[a]	1.0 g	NH_4Cl[a]	5.0 g
Trace element solution[b]	10.0 ml	Trace element solution[b]	1.0 ml
MEM[c]	10.0 ml	MEM[c]	10.0 ml
1 M $MgCl_4$	10.0 ml	$MgCl_2$	0.95 g

[a] d_7- or d_7/^{13}C-D-glucose is used as the sole carbon source; $^{15}NH_4Cl$ is used as the sole nitrogen source

[b] The trace element solution is that described by Cai et al. (1998) and comprises the following per 100 ml: 0.6 g $FeSO_4$ ($7H_2O$), 0.6 g $CaCl_2$ ($2H_2O$), 0.12 g $MnCl_2$ ($4H_2O$), 0.08 g $CoCl_2$ ($6H_2O$), 0.07 g $ZnSO_4$ ($7H_2O$), 0.03 g $CuCl_2$ ($2H_2O$), 2 mg H_3BO_4, 0.025 g $(NH_4)_6Mo_7O_{24}$ ($4H_2O$), 0.5 g ethylenediaminetetraacetic acid (EDTA)

[c] MEM vitamins 100× solution from Corning (VWR catalog number 45000-702) was lyophilized and dissolved back in the same volume of D_2O



Vessel volume	15 ml	15 ml	250 ml	2.8 L	2.8 L
Media	LB/H_2O	LB/D_2O	10% LB/D_2O 90% M9+/D_2O	M9+/D_2O	M9+/D_2O
Culture Volume	1 ml	2.5 ml	25 ml	250 ml	250 ml
Growth time	~3 h	~5 h	~15 h	~10 h	~20 h
Cell Density (OD_{600})	0.5-1.0	0.5-1.0	10	10	20
Temperature	37°C	37°C	37°C	37°C	25°C

Fig. 1 Schematic of the high-yield protein expression protocol in M9+/D_2O medium

shaker flask-based protocol that increases protein yields tenfold per unit volume. We modified M9 minimal medium to maintain an optimal pH for *E. coli* growth over a wide cell density range. We systematically measured growth curves of *E. coli* BL21(DE3) cells harboring the pET11a plasmid encoding the *Neisseria Gonorrhoeae* MinE protein under different conditions and growth media. We were able to maintain linear growth to high cell density (OD_{600} ~ 10) in a shaker flask. We demonstrate that the expression level per cell is the same between an OD_{600} of 1 and 10. By inducing the cells at OD_{600} = 10 instead of 1, we increased the quantity of cell mass and protein yield tenfold. Growing cells in D_2O medium requires that the *E. coli* cells be adapted to D_2O via multistep sub-culturing steps (Gardner and Kay 1998; Moore 1979; Paliy et al. 2003). We find that the adaptation sequence—LB/H_2O to LB/D_2O to modified-M9/D_2O is both simpler and more reliable than most commonly used sequences. We applied our procedure to the expression of five different perdeuterated proteins. For four of the five proteins, the protein yield was increased approximately tenfold in the same volume of culture medium compared to the conventional procedure; we were

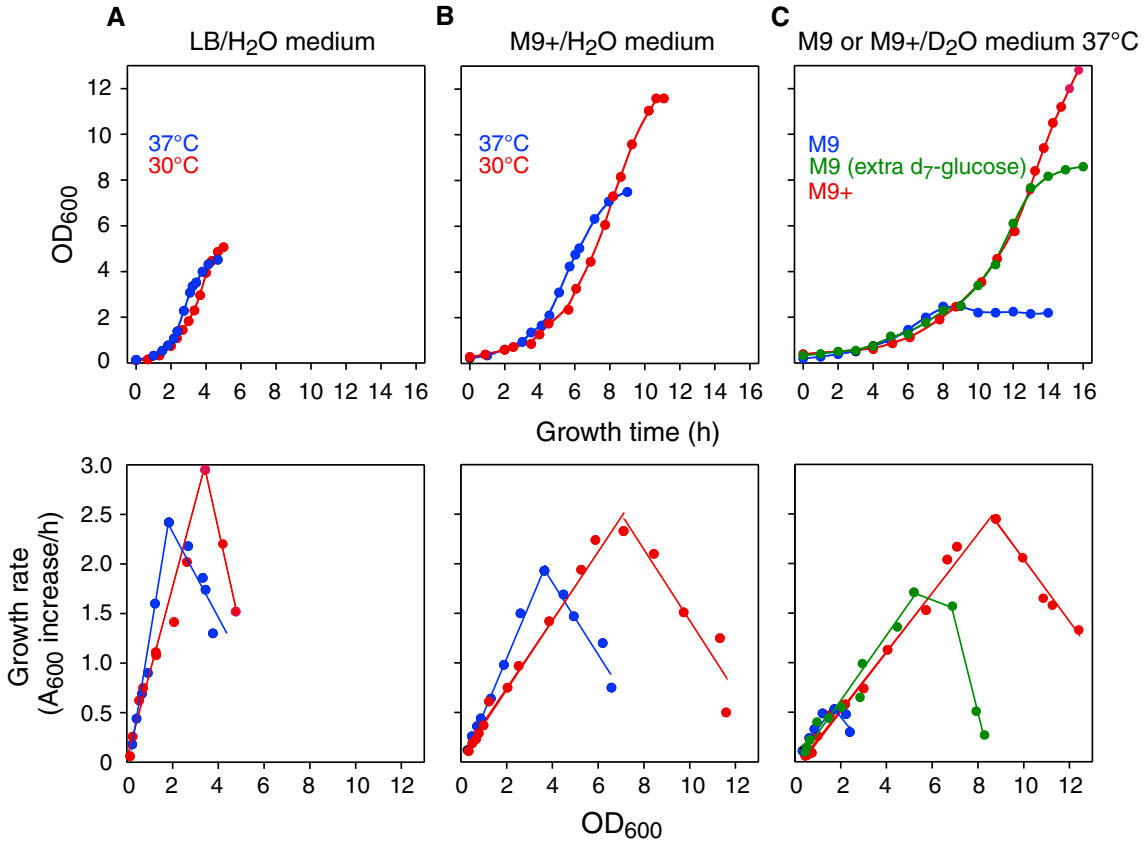


Fig. 2 Growth curves for *E. coli* BL21 (DE3) transfected with ngMinE in pET21a. **a** LB/H_2O, **b** M9+/H_2O, and **c** M9 or M9+ (as indicated)/D_2O media. The *upper panels* show cell density measured by OD_{600} as a function of time. The *lower panels* show growth rate (OD_{600} increase per hour) as a function of OD_{600}. In **a** and **b**, the *blue circles* are from data at 37 °C, the *red circles* from data at 30 °C. In **c** the *blue circles* are from regular M9/D_2O medium; the *green circles* from regular M9/D_2O medium augmented with 12 g/L d_7-glucose) and 4 g/L $^{15}NH_4Cl$; and the *red circles* from M9+/D_2O (cf. Table 1) medium

unable grow cells expressing the fifth protein to high cell density, perhaps due to cell toxicity at basal expression levels prior to induction.

Materials and methods

Growth curve and growth condition optimization

The following six media were used to grow *E. coli* BL21(DE3) cells: (1) LB/H_2O, LB capsules (MP Biochemicals, catalog number 113002031) dissolved in deionized water and sterile filtered following the manufacturers instructions; (2) LB/D_2O, same as LB/H_2O but substituting 99 % D_2O for H_2O; (3) M9/H_2O, regular M9 medium (Cai et al. 1998) in H_2O (see Table 1); (4) M9/D_2O, same as M9/H_2O but substituting 99.9 % D_2O for H_2O; (5) M9+/H_2O, newly modified M9 medium (as shown in Table 1) prepared in H_2O; and (6) and M9+/D_2O, same as M9+/H_2O but in 99.9 % D_2O. The key differences between M9+ and M9 resides in (a) a larger amount of d7-glucose and (b) an alteration in the amounts and ratios of K_2HPO_4 and KH_2PO_4 buffer to ensure that the pH remains at or above 6.5 up to high cell density (OD_{600} ~10). All experiments were carried out in a shaker at a shaking speed of 200 rpm in 2.8 L baffled bottom PYREX flasks (VWR catalog number: 22877-168) with the culture volume equal to or less than 280 ml (unless otherwise specified).

As an example, *Neisseria Gonorrhoeae* MinE (ngMinE), a small 87 amino acid dimeric protein cloned in pET11a, was used for optimization of growth conditions. Cell densities were determined by measuring the optical density at 600 nm (OD_{600}). The ratio of the flask volume to the medium volume, shaking speed and selection of flasks were first determined using LB medium. We found that flasks with a baffled bottom were better than ones without, and the optimal shaking speed was 200 rpm or higher with a ratio of flask to medium volume of 10 or greater, consistent with previously reported results (Collins et al. 2013). To investigate factors that affect the length of log phase (linear cell growth phase), growth curves were measured in LB/H_2O, M9/H_2O and M9/

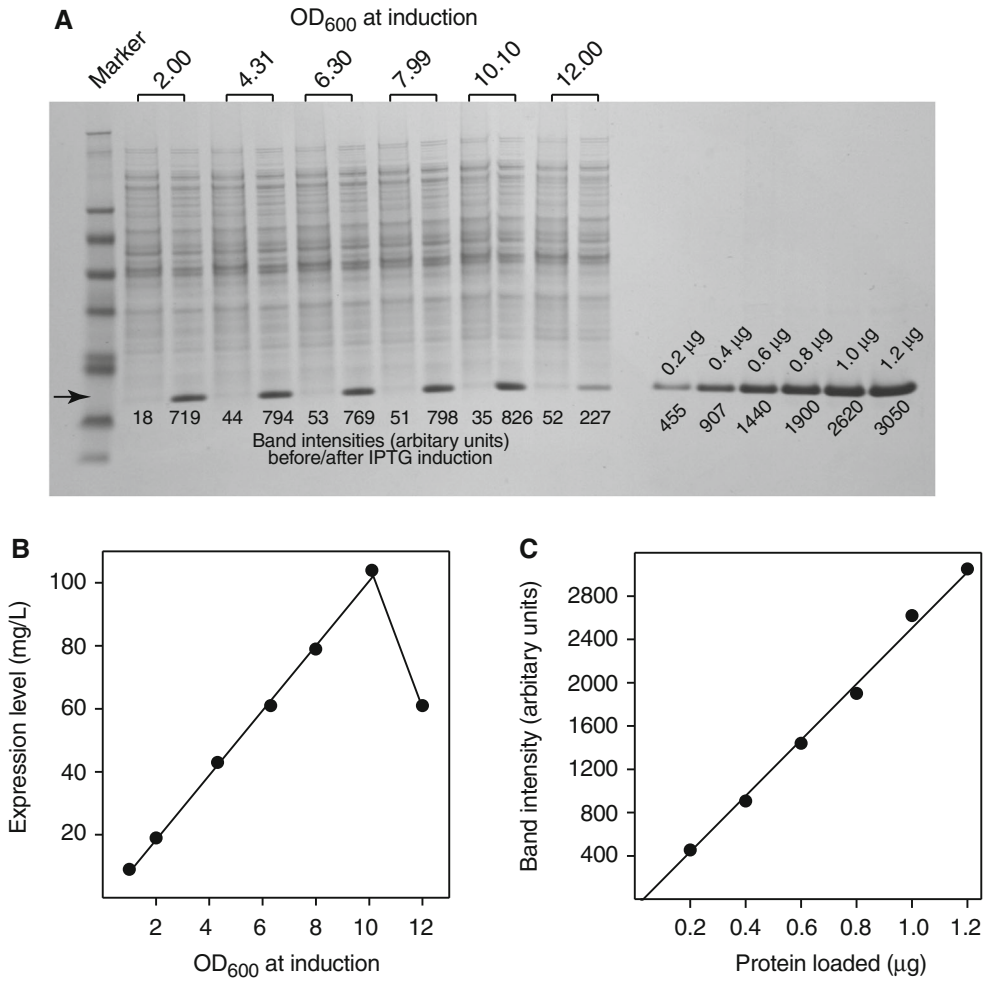


Fig. 3 Protein expression levels of ngMinE at different cell densities. **a** SDS-PAGE of samples induced at different OD_{600} values (indicated at the *top* of each *lane*) before (*left*) and after (*right*) induction for 20 h. The band corresponding to ngMinE is indicated by an *arrow* on the *left side* of the gel, and the corresponding ngMinE band intensities are indicated *below* each *lane*. For the sample induced at an OD_{600} value of 2.0, 0.7 ml of culture before (*left*) and after (*right*) induction was collected, pelleted and resuspended in 0.5 ml 50 mM TrisHCl, pH 7.5 and 200 mM NaCl buffer. 8 µl of 4× SDS loading buffer was added to 3 µl of the above suspension, and the sample was heated at 90 °C for 10 min. After centrifugation at 13,000 rpm in a benchtop microfuge for 5 min, 15 µl was loaded onto the gel. Samples induced at other OD_{600} values were first diluted to a cell density of $OD_{600} = 2.0$ and then treated in the same way. Samples on the *right-hand side* of the gel are known amounts of ngMinE obtained after a two-step (Ni-affinity column and Superdex size exclusion column) purification. **b** Protein yields estimated on the basis of gel band intensities as a function of OD_{600} at the time of induction. **c** Standard curve of gel band intensities on SDS-page plotted as a function of protein loaded

D_2O. Growth curves were measured at both 30 and 37 °C for LB/H_2O and M9/H_2O media. Since the cells grow too slowly at 30 °C in M9 or M9+/D_2O, growth curves were only measured at 37 °C in the D_2O media.

Protein expression levels were measured at different cell densities. Cells were allowed to grow to an OD_{600} of between 1 and 12, and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C for 20 h. Protein expression levels were evaluated by either SDS-PAGE gel electrophoresis (gel band intensities were scanned and measured using the Li-COR OYSSEY CLx system) and/or by measuring the protein yield from the 280 nm absorption after Ni affinity column purification. Optimal induction time was determined by collecting samples every 2 h after IPTG induction, and the levels of expression were compared via protein band intensities on a SDS gel. Levels of perdeuteration were evaluated by electrospray ionization mass spectrometry.

Optimized protocol

The final optimized protocol for protein expression in D_2O medium is schematically shown in Fig. 1. The protocol comprises four major steps: cell adaptation from H_2O medium to D_2O medium, pre-culturing, cell growth, and IPTG induction. To grow a 250 ml D_2O (99.9 %) cell

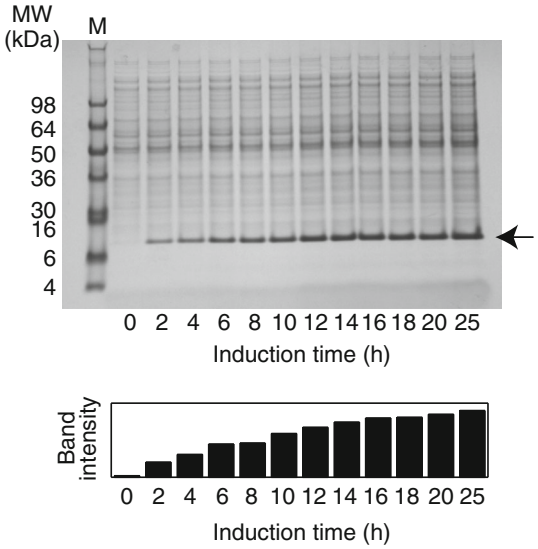


Fig. 4 Expression levels of ngMinE as a function of induction time. The arrow indicates the position of the ngMinE band

culture, a small LB/H_2O culture was started from a fresh agar plate or glycerol stock early in the morning. After about 3 h of growth at 37 °C, 2.5 ml of LB/D_2O culture was inoculated with 200 µl of the LB/H_2O culture. The cell density of this culture should reach an OD_{600} between 0.5 and 1.0 in about 5 h. The overnight pre-culture was started by transferring all of the above 2.5 ml LB/D_2O culture into a 250 ml baffled flask containing 25 ml M9+/D_2O medium, and allowing it to grow at 37 °C for about 15 h. The OD_{600} of this pre-culture should be ~10 early next morning. All 25 ml of the pre-culture was then poured into a 2.8 L baffled bottom flask containing 250 ml M9+/D_2O medium, and allowed to grow at 37 °C until the cell density reached an OD_{600} of ~10 (which takes 8–10 h). The shaker temperature was then reduced to 25 °C and protein expression was induced by addition of IPTG (final concentration = 0.5 mM). Cells were harvested by centrifugation 20 h after IPTG induction at 25 °C.

Results and discussion

The growth behavior of *E. coli* BL21(DE3) transfected with ngMinE cloned into pET11a cultured in different media and conditions is shown in Fig. 2. Plots of cell density (OD_{600}) as a function of time (Fig. 2, top panels) clearly show the different phases of cell growth (i.e. lag, exponential and stationary phases, followed by cell death). Plots of growth rates (OD_{600} increase per hour) as a function of cell density (Fig. 2, lower panels) provide a straightforward indicator of the linear range of cell growth. As discussed in the following section, inducing cells at the end of the linear growth phase results in the highest protein yield.

The linear range of cell growth is affected by several factors. There is an inverse relationship between the duration of the linear growth phase and cell growth rates. Cells grow fastest in LB medium and have the shortest linear growth range (Fig. 2a, lower panel), while cells grow the slowest in M9+/D_2O media and have the longest linear growth range. Lowering the temperature slows down cell growth rate and elongates the linear growth range (Fig. 2a, b). The shapes of the growth curves and linear growth range are also determined by the amount of nutrients added to the growth media. We have previously shown that in a fermentor, each gram of glucose increases the cell density of *E. coli* BL21(DE3) by an OD_{600} of 1.4 (Cai et al. 1998). In the current work, the maximum cell density that supports linear growth has a relationship of $(n-0.5) \times 1.4$ with the amount of glucose in the medium, where n is quantity in grams of glucose per liter of medium. For example, when cells were grown in M9+/H_2O medium at 30 °C, the linear growth range extends up to OD_{600} of 2 for 2 g of glucose per liter of medium, 4.9 for 4 g/L of glucose and 6.3 for 5 g/L. Beyond this point, increasing the glucose further does not increase the cell's linear growth range as oxygenation becomes the limiting factor. We also previously showed that oxygen demand is greatly reduced under limited or exhausted nutrient conditions (Cai et al. 1998), and that *E. coli* has less oxygen demand when cultured in minimal M9 medium as compared to the rich (LB) medium (unpublished data). In regular M9/D_2O medium (blue circles in Fig. 2c), the linear phase of *E. coli* growth stops at an OD_{600} of around 2.0. This is a result of exhaustion of d_7-glucose. Theoretically, 2 g/L glucose should bring the OD_{600} up to 2.8 but only a value of 2.4 is attained using regular M9 medium. We also noticed that the linear growth range of *E. coli* in regular M9 medium stops before an OD_{600} of 2.0 is reached. This data suggests that *E. coli* needs a minimum of 0.5 g/L of d_7-glucose to grow well and a minimum 0.3 g/L to survive. Depending on the proteins to be expressed, the OD_{600} normally doubled or slightly more than doubled after IPTG induction. To achieve maximum protein yield in regular M9 medium, one has to induce at an OD_{600} of less than 1.0 (most researchers typically induce their cells between an OD_{600} of 0.6 and 1.0). To induce at an OD_{600} of 1 or slightly higher and achieve maximum protein yield, one has to use 2.5 g or more of d_7-glucose per liter of culture. Finally, the pH of the culture medium decreases with increasing cell density. Adjusting pH during cell growth, as is often done in a fermenter, is not practical when cells are grown in a shaker flask. As shown in Fig. 2c, the linear cell growth range cannot reach its potential maximum if cells are only supplied with enough nutrients (4 g NH_4Cl and 12 g d_7-

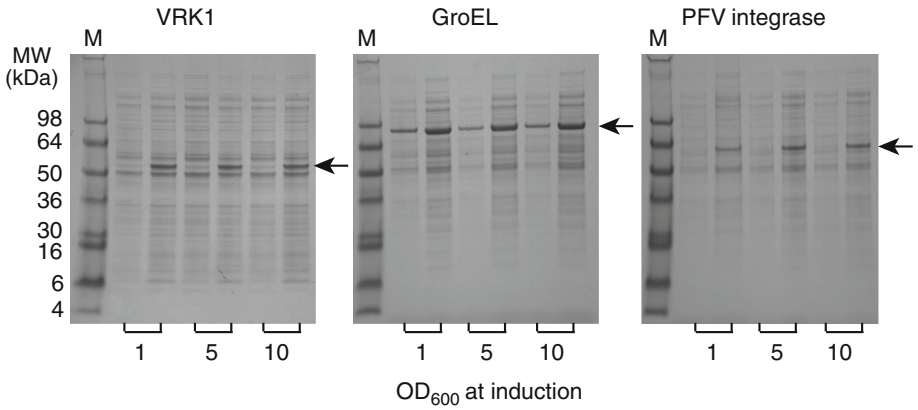


Fig. 5 Expression levels of VRK1, GroEL and PFV integrase at different cell densities (OD_{600}) at the time of induction. *Lanes* are displayed in pairs before (*left*) and after (*right*) induction. Sample preparation and loading amounts are the same as in Fig. 2. The amount of cells loaded onto the gel was adjusted so that the amount of cells were equal for the different OD_{600} induction values. The expression levels of each of the three proteins are the same regardless of the OD_{600} at which the cells were induced

glucose per liter) in regular M9 medium (green circles). We found that the pH drops significantly when the OD_{600} exceeds 7 in regular M9/D_2O medium. To address this problem, we modified the M9 medium (M9+), to maintain the pH within the optimal range up to an OD_{600} of 24. The pH of the $M9^+$ medium (Table 1) at the start point is 7.4 and 6.8 at harvest (OD_{600} between 20 and 24 with induction at OD_{600} $\sim$10).

Expression levels of ngMinE induced at different cell densities are shown in Fig. 3. Protein yields were estimated from band intensities on an SDS/polyacrylamide gel. The same quantity of cells was loaded for each sample. The standard curve was generated by plotting band intensities against a known amount of loaded protein. ngMinE was also purified from cells induced at different OD_{600} values by Ni-affinity chromatography and the yields were about 20 % lower than those estimated from gel intensities. The yield ratios from cells induced at different OD_{600} values are, however, the same whether the yields were obtained from Ni-column purification or from band intensity estimates. The protein yields increase proportionally with cell density up to an OD_{600} of 10.0. The linear growth range is longer at lower temperature as shown in Fig. 2a and b for cells grown in both LB/H_2O and M9+/H_2O media. Inducing cells at a lower temperature keeps them under optimal conditions for a longer period of time leading to a higher final cell density. We did not, however, systematically investigate the expression level as a function of induction temperature, but only compared yields at 25 and 37 °C as a function of time. As shown in Fig. 4, expression level reaches a value of 80 % of maximum at $\sim$12 h and levels off at $\sim$18 h after induction.

To demonstrate the general applicability of the new high yield protocol, we compared expression levels for four additional proteins (Fig. 5): vaccinia-related kinase 1 (VRK1), the p51 subunit of HIV-1 reverse transcriptase (p51-RT), prototype foamy virus integrase (PFV-IN) and *E. coli* GroEL. Of these four proteins, only cells containing the plasmid expressing p51-RT could not be grown to a high cell density as partial cell death was observed at high OD_{600}. This is probably due to leaky expression and resulting toxicity of p51-RT. The other three proteins were expressed at high cell density as expected. As can be seen in Fig. 5, the expression levels of VRK1. GroEL and PFV-IN is the same per unit cell mass whether the cells are induced at an OD_{600} of 1 (regular M9/D_2O medium with 2.5 g/L glucose) or at a high OD_{600} (5 or 10) and grown in M9+/D_2O medium (Table 1).

To test the applicability of the new high yield protocol for any given protein expression system, one simply needs to grow the cells in M9+/H_2O medium at 30 °C using unlabeled glucose and NH_4Cl, and compare expression levels between cultures induced at an OD_{600} of 1 and 6. If the same expression levels are achieved for the cultures induced at lower and higher OD_{600}, one can safely use the protocol for perdeuterated protein expression.

Concluding remarks

The new protocol using M9+ medium to express proteins at high cell density is clearly advantageous compared to the conventional protocol of inducing cells at lower cell density. In a typical case, one needs to grow 2 L of culture using the conventional protocol to produce enough perdeuterated protein for one or two NMR samples. A 2 L culture requires 5 g d_7- or d_7/^{13}C-glucose and 2 L D_2O. To produce the same amount of protein using our new M9+/D_2O-based protocol, only a 200 ml culture using 200 ml D_2O and 3.6 g labeled glucose is required, resulting in a

tenfold reduction in D_2O requirements and a 30 % saving in labeled glucose usage. Using a fermenter, one can keep cells under linear growth conditions up to much higher cell densities, thus further decreasing D_2O usage. However, not everyone has access to a fermenter and most fermenters require a minimum of one or two liters of growth medium. Our M9+/D_2O-based protocol provides a method to greatly save on the cost of labeled D_2O-based medium for perdeuterated protein expression using only commonly available equipment.

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