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Structure Determination by NMR

Isotope Labeling

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1. Introduction

Solution NMR spectroscopy is used widely to determine the structure of proteins. The size of the proteins that can be studied has increased dramatically in the past decade as advances in pulse sequences, probe design, and instrumentation has been made. One major contributing factor to these advances has been the ability to utilize ^{2}H, ^{13}C, and ^{15}N isotopically labeled proteins in residue assignment strategies. For modestly sized proteins, the assignments can be accomplished by standard homonuclear ^{1}H 2D methodology ***(1)***. As the size of the proteins exceeds 10 kDa, the NMR spectra become more crowded with overlapping signals. With ^{13}C and ^{15}N labeling, the heteronuclear experiments have allowed the spectra to spread into two, three, or four dimensions, thus increasing the resolution and decreasing the assignment ambiguities ***(2)***. Another problem accompanying increasing protein size is sensitivity loss as a result of line broadening because of the decrease in ^{13}C and ^{1}H T_2 relaxation times. The most significant contribution to ^{13}C T_2 relaxation is the strong dipolar coupling between the ^{13}C–^{1}H spin pairs. ^{1}H T_2 relaxation arises from proton–proton dipolar couplings. Replacement of ^{1}H by ^{2}H can increase the T_2 relaxation times significantly. Thus, incorporation of ^{2}H into large proteins has been widely used to improve the quality of spectra by a reduction in the number of peaks and concomitant narrowing of linewidths ***(3)***. In addition to structural determination, heteronuclear multidimensional NMR has also been widely used to study protein dynamics and interactions of these molecules ***(3)***.

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In order for these experiments to achieve common use, it is important to be able to isotopically label a protein in an efficient and cost-effective manner and to purify it in good yield, both uniformly and specifically. Practically, if the protein under consideration for NMR studies can be cloned and expressed in *Escherichia coli*, uniform labeling with ^{15}N is relatively straightforward and inexpensive by using defined media containing [^{15}N, 99%] ammonium chloride or ammonium sulfate as the nitrogen source. Uniform (>95%) labeling with ^{13}C is also relatively straightforward by using defined media containing [$^{13}C_6$, 99%] glucose. [$^{13}C_6$, 99%] glucose is the most reliable method in terms of giving high yield and high ^{13}C-incorporation. It is also much less expensive than commercially available ^{13}C-enriched media. Replacement of [$^{13}C_6$, 99%] glucose by other reagents like [1,2–$^{13}C_2$, 99%] acetate is possible ***(4)***. Partial or complete aliphatic ^{2}H incorporation has been obtained by growth of *E. coli* in defined media containing certain percentage of D_2O and backbone $^{1}H_N$ can be exchanged out by dissolving the sample in D_2O. In terms of efficient type-specific labeling, the host bacteria can be grown on a defined medium supplemented with one or more isotope-labeled amino acids or amino acid precursors.

In the past few years, we have enjoyed great success in isotope labeling the Ca^{2+}-binding protein, troponin C. Using the pET expression system and the isopropyl β-D-thiogalactopyranoside (IPTG) induction protocol of Studier et al. ***(5)***, we are able to efficiently produce tens to hundreds of milligrams of the labeled protein from liter-scale growths of host *E. coli*. These labeled proteins made it possible for us to determine the solution structures and dynamics of skeletal and cardiac troponin C in a variety of states ***(6)***. We will summarize the procedures in this chapter. The methods described here should be applicable to other Ca^{2+}-binding proteins with their proper expression system and bacterial host.

2. Materials

1. Expression Medium (Modified M9 Medium), (1 L):
   a. NaH_2PO_4 (6 g)
   b. K_2HPO_4 (3 g)
   c. NaCl (0.5 g)
   d. $(NH_4)_2SO_4$ (1 g) (*see* **Notes 1** and **2**)
   e. D-glucose (2–10 g) (*see* **Notes 3–5**)
   f. 1 *M* $MgSO_4$ (4 mL) (*see* **Note 6**)
   g. 1 m*M* $FeSO_4$ (2 mL) (*see* **Note 7**)
   h. Mineral Mixture (0.5 mL) (optional, *see* **Note 8**)
   i. Vitamins and trace elements mixture (10 mL) (*see* **Note 8**)
   j. Appropriate antibiotics
   k. pH = 7.5

   Dissolve NaH_2PO_4, K_2HPO_4, and NaCl in 1 L of double distilled (dd) H_2O, adjust pH to ~7.5, and divide into two 2-L flasks with 500 mL each. Stop flask with a

pad of folded cheesecloth and cover with a double layer of aluminum foil. Autoclave. After autoclaving, this can be stored a day or two at room temperature or for several days at cold room temperature. The other ingredients should be made up, sterilized separately, and added just prior to inoculation.

2. TY Medium, (1 L):
   a. Bacto Tryptone (16 g)
   b. Bacto Yeast Extract (8 g)
   c. NaCl (5 g)
   d. Appropriate antibiotics
   e. pH = 7.5

   Dissolve all ingredients except antibiotics in 1 L ddH_2O and sterilize by autoclaving. Add antibiotics before inoculation.
3. NZ Medium, (1 L):
   a. NZ amine (10 g)
   b. Bacto Tryptone (5 g)
   c. NaCl (5 g)
   d. $MgSO_4$ (2.5 g) (*see* **Note 6**)
   e. Appropriate antibiotics
   f. pH = 7.5

   Dissolve all ingredients except antibiotics and $MgSO_4$ in 1 L ddH_2O and sterilize by autoclaving. Add antibiotics and $MgSO_4$ before inoculation.
4. IPTG (Isopropyl β-D-Thiogalactopyranoside): Dissolve 120 mg into 8 mL of water and filter-sterilizes. This is enough for a 1-L prep.
5. Antibiotics:
   a. 5% ampicillin: Weigh out and dissolve in water. Filter sterilizes into a sterile container. Store in freezer. This stock can be used for up to ten days but breaks down over time or heating up above 55°C. Use 1 μL per 1 mL of medium.
   b. 2.5% chloramphenicol: Weigh out with a sterile spatula and dissolve into 99% ethanol in a sterile test tube or other sterile container. This solution cannot be sterilized by either autoclave or filter since it breaks down with heat and ethanol breaks down the filter membrane in filter-sterilizers. This stock can be kept frozen up to 6 mo. Use 1 μL per 1 mL of medium.
   c. Other antibiotics: Follow specific protocols for specific antibiotics.
6. Vitamins and Trace Elements Mixture (*see* **Note 8**): Weigh out 10 mg per 1 mL each of biotin, choline chloride, folic acid, niacinamide, D-pantothenic acid, and pyridoxal chloride and 1 mg per 100 mL of riboflavin in H_2O. All above can be mixed and made as a single batch, then filter-sterilized, or made individually and filters sterilized as desired. Store frozen and in the dark. Use 10 mL of above mixture per 1 L of medium

   25% of nicotinc acid in H_2O, filter-sterilizes separately. Use 2.5 mL per 1 L of medium.

   10% of thiamine in H_2O, filer sterilizes separately. Use 0.5 mL per 1 L of medium.

7. Mineral Mixture (*see* **Note 8**): A solution made of 0.2 g/L of any of the following ingredients: $CuSO_4$, $MnSO_4$, $ZnSO_4$, and $CaCl_2$. Filter sterilizes and store at 4°C. Use 0.5 mL for each 1 L of medium.
8. Equipment:
   a. A selection of autoclavable glassware including test tubes, flasks, beakers, Pasteur pipets, pipet tips for pipetmen, autoclave trays, and access to an autoclave.
   b. Equipment for filter sterilization of nonautoclavable solutions: such as Millipore stericups and sterifil filter units with 0.22-micro filters (500 mL and 1 L sizes), Millex GS filter units (0.22 micro) plus 10-mL syringes.
   c. Access to a vacuum line, gas outlet, Bunsen burner, bacterial loops, and Petri dishes. Access to temperature controlled incubators or warm room with shakers, cold room, or refrigerators. Access to a UV-Vis spectrometer.
   d. Expression system: an expression system capable of efficiently expressing the target proteins at high levels. A bacterial cell strain compatible with the expression system. The cell strain should be capable of efficient growth on limited medium. In expressing TnC, we use the pET expression system with the cell strain BL21 (DE3) *pLysS*.
9. Expression medium for ^2H-labeling, 1 L contains:
   a. NaH_2PO_4 (12 g)
   b. K_2HPO_4 (6 g)
   c. $(NH_4)_2SO_4$ (1 g) stock solution in D_2O (*see* **Notes 1** and **2**)
   d. D-glucose (2–10 g) stock solution in D_2O (*see* **Notes 3–5**)
   e. 1 *M* $MgSO_4$ (8 mL) stock solution in D_2O (*see* **Note 6**)
   f. 1 m*M* $FeSO_4$ (6 mL) stock solution in D_2O (*see* **Note 7**)
   g. Appropriate antibiotics: stock solution in D_2O filter-sterilized
   h. pH = 7.5

   Dissolve up NaH_2PO_4 and K_2HPO_4 in 500 mL of D_2O, adjust pH with NaOD and DCl and filter-sterilizes into a sterile 2-L flask (using a 500 mL Millipore filtration unit or equivalent). This should be done the same day or the day before the cells are to be grown. If this solution is stored at room temperature or cold room temperature, the medium must be warmed up to incubation temperature before introducing the cell culture. Other ingredients should be treated the same as aforementioned except in D_2O and added just before use.

   D_2O: 99.9% D_2O available from commercial sources.

   NaOD/KOD: Dissolve some NaOH or KOH in D_2O.

   DCl: Make a 2-*M* solution of HCl in D_2O

   IPTG: 120 mg in 10 mL D_2O, filter-sterilized.

   Vitamins and trace elements mixture: Same ingredients as before except in D_2O, filter-sterilized.

   Activated charcoal: Available from commercial sources. This is used to recycle D_2O.

   TY or NZ amine media: Makeup the required volume as aforementioned except in D_2O. Filter sterilizes.

3. Methods

3.1. Growth of Cells on Limited Media

Transform cells with the appropriate plasmid according to standard protocols (*see* **Note 9**). Plate out overnight at the correct temperature for your cells (usually 37°C). Inoculate five or six colonies from the plate of transformed cells into a 10-mL NZ-amine culture (*see* **Note 10**) containing the proper antibiotics in a 25-mL Erlenmeyer flask and shake at the proper temperature until the cells reach late-log phase, e.g., OD_{600nm} = 1.0. Inoculate 1 L of expression medium with 5 mL of the NZ culture. Incubate at the proper temperature to mid-log phase, OD_{600nm} = approx 0.5–1.0. Add IPTG to induce the cells and incubate for the required induction time (*see* **Note 11**).

3.2. Expression of Uniformly 15N-Labeled or 15N-Depleted Protein

Grow culture and express cells according to above methodology except that 99.9% ^{15}N-$(NH_4)_2SO_4$ or ^{15}N-NH_4Cl (*see* **Note 1**) is used in the expression media as the sole nitrogen source for uniformly ^{15}N-labeled protein (*see* **Note 12**). For ^{15}N-depleted protein, use ^{15}N-depleted [i.e., 99.99% ^{14}N-$(NH_4)_2SO_4$ or NH_4Cl].

3.3. Expression of Uniformly 13C-Labeled or 13C-Depleted Protein

Grow culture and express cells according to above methodology except that 99.9% $^{13}C_6$ glucose is used in the expression media as the sole carbon source for uniformly $^{13}C_6$-labeled protein. For ^{13}C-depleted protein, use $^{13}C_6$-depleted (i.e., 99.99% $^{12}C_6$-glucose).

3.4. Incorporation of Type-Specific Amino Acids

Grow culture and express cells according to above methodology. To the standard expression medium, add 20 common amino acids to the concentration of 0.1 g/L (*see* **Note 13**). These amino acids should be unlabeled except for the amino acid targeted, which should include the desired label. Of this, it is probably most efficient to add 0.033 g when making media, another 0.033 g at induction, and the remaining 0.033 g halfway through the induced period (*see* **Notes 14–17**).

3.5. Expression of 2H-Labeled and 2H,13C,15N-Labeled Proteins

3.5.1. Conditioning of Cells to 80% D_2O (see **Note 18**)

1. Transfer four or five colonies from a plate of freshly transformed cells into a sterile container containing 20 mL of NZ amine medium made in 20% D_2O/80% H_2O with the proper antibiotics. This is incubated at 37°C (*see* **Note 19**).
2. When this culture has reached late-log phase (OD_{600nm} = 1.0), inoculate 1 mL to a similar culture in 45% D_2O/55% H_2O (*see* **Note 20**).

3. When the 45% D_2O/55% H_2O culture reached mid- to late-log phase (OD_{600nm} = 0.5–1.0), use these cells to inoculate a similar culture in 80%D_2O/20%H_2O.
4. When cells from **step 3** have grown to mid- or late-log phase, use these cells to inoculate a flask of M9 or modified M9 expression medium prepared in 80% D_2O/20% H_2O.
5. Grow **step 4** cells until late-log phase (OD_{600nm} = 0.5–1.0), add 30% by volume of glycerol, and flash freeze. Store these cells at –80°C.

3.5.2. Plasmid Retention Test (see **Note 21**)

1. Use one vial (1-mL aliquot) of frozen (at –80°C) conditioned cells prepared as above, to inoculate a sterile flask of 50 mL of NZ amine medium (or similar enriched media, such as TY media) with the required antibiotics.
2. Incubate until mid-log phase (OD_{600nm} = 0.5–1.0).
3. Induce protein expression by IPTG.
4. After the required induction time, harvest cells and run a gel to check protein expression levels.

3.5.3. Protein Expression

1. Use 1 mL (*see* **Note 22**) of frozen (–80°C) conditioned cells to inoculate a sterile container of 30–50 mL of the expression medium (note, it is recommended to use large amounts of glucose, 10 g/L in this step, *see* **Note 23**) in 80%D_2O/H_2O as described in **Subheading 2.**
2. Incubate overnight or until late-log phase (*see* **Note 24**).
3. Use 30–50-mL cells (*see* **Note 24**) from **step 2** to inoculate 500 mL of expression medium in 99.9% D_2O to achieve the highest levels of deuteration.
4. Grow until mid-log phase (*see* **Note 25**).
5. Induce by using IPTG in D_2O and incubate for the appropriate time (*see* **Note 26**).
6. Harvest cells according to usual methods.

3.5.4. A Suitable Method for Recycling D2O (see **Note 27**)

1. Remove visible debris by centrifuging and/or settling and/or filtering.
2. Using a common laboratory distillation apparatus, distill two or three times.
3. Mix with activated charcoal (1 g/100 mL works well) and stir for 20 min.
4. Remove charcoal by filtration. This D_2O is ready for growing cells (*see* **Notes 28** and **29**).

4. Notes

1. NH_4Cl can be used in place of $(NH_4)_2SO_4$, but the latter is slightly better because
   a. The chloride ion is of no use at all to most nonhalophilic bacteria like *E. coli*, although sulfate is essential;
   b. Considering cost vs stoichiometry, $(NH_4)_2SO_4$ will sometimes give a little more ^{15}N per dollar (depending on relative prices).
2. The ^{15}N-NH_4Cl or $(NH_4)_2SO_4$ can be mixed and autoclaved together with the phosphate buffers, but it is better to make it up separately and add it just before

cell growth because in the event that the starter culture does not grow properly, or for some reason, you decide not to go ahead with cell growth, the ^{15}N-stock solution can be conveniently stored frozen for an indefinite period of time and used at a later date.

3. Glucose should not be autoclaved in the presence of salts and buffer because it can break down and form toxic complexes inhibitory to the metabolism of the cells. It is best to make it up separately, 2 g in 10 mL or 10 g in 50 mL ddH_2O, and filter-sterilizes it into a sterile container. This can be stored frozen indefinitely or at cold room temperature for several days.
4. Because glucose is the sole carbon source, it is important to have plenty available for the most quick and efficient cell growth, thus, for unlabeled ^{12}C-glucose, up to 10 g/L or even 12 g/L can be used. However, for expensive $^{13}C_6$-glucose, it is important to use the least amount that gives the most efficient target protein expression. For preps in H_2O, 2 g/L has been found in our lab and others to give the most cost-effective result, i.e., most protein yield per gram of glucose used per liter prep. However, the yield per liter can be increased somewhat by adding extra glucose up to the level of 3 g/L, after which point adding extra is probably wasted. Please note, for deuterated preps, 2.5 g/L seems to give the highest yield per gram of glucose, whereas increasing glucose to 6 g/L gives greater amount of protein per liter prep.
5. When ordering ^{13}C-labeled glucose, 6-^{13}C-glucose refers to glucose with the ^{13}C label in the number 6 position, whereas $^{13}C_6$-glucose refers to uniformly labeled glucose. Unlabeled, primarily ^{12}C-glucose is simply called D-glucose (or dextrose), whereas $^{12}C_6$-glucose refers to ^{13}C-depleted glucose.
6. Make 10–20 mL of $MgSO_4$ in an autoclaveable stoppered container and autoclave. This can be stored indefinitely in cold room or at room temperature, but it is better to make fresh every time.
7. $FeSO_4$ solution should be filter-sterilized. Autoclaving will result in forming $Fe(OH)_3$ and precipitation.
8. For *E. coli* strain BL21, vitamins and minerals are not absolutely essential because the cell has plenty of trace elements from the starter culture used to initiate growth and can make its own vitamins as required. For some other stains, some of these could be essential. Nevertheless, different researchers may favor adding different things to their media. It is recommended to do trial expressions to test if these ingredients help.
9. Freshly transformed cells from frozen stocks of plasmid are the most reliable and commonly used. Frozen stocks of transformed cells may work equally well, based on our experience.
10. TY medium can be used instead of NZ amine medium as an enriched starter culture. However, NZ amine is a less enriched and more strenuous medium so the cells should suffer less shock when introduced into the limited expression media.
11. The best induction time may be the same or may be longer than on enriched medium. It is strongly advised to try out a trial growth to check that the expres-

sion system works efficiently under these conditions and to familiarize the researcher with all the techniques involved. During this trial, it is also recommended to run a time course of target protein production against induction time.

12. If the only source of nitrogen is ^{15}N-labeled, then the cell has to incorporate ^{15}N into any proteins produced because no other source of nitrogen is available. For the techniques described herein, $(NH_4)_2SO_4$ or NH_4Cl will be the primary source of nitrogen, with the only other source being the small amount available from the starter culture. This is why the amount of starter culture used for inoculation should be kept to minimum (1% or less). Mass spectroscopy analysis has shown that our methods result in protein with 96% or greater ^{15}N incorporation. The same consideration applies to the expression of ^{15}N-depleted, ^{13}C-labeled, and ^{13}C-depleted protein.
13. This results in a medium, which is much more enriched than M9, or a similar limited medium. Thus, it may be possible to grow strains that will not normally grow on limited media. Of course, proteins with uniformly ^{15}N or ^{13}C labeling cannot be expressed from this medium.
14. The efficiency of incorporation for any specific amino acid will vary according to amino acids metabolic pathway, particularly its catabolic pathway in a particular cell strain. Therefore, glycine, serine, cysteine, glutamic acid, and aspartic acid, which are used as metabolic precursors for a variety of pathways, tend to incorporate with poor efficiency, whereas threonine, valine, alanine, leucine, and isoleucine are incorporated fairly well. The ^{13}C-labeled methyl group of methionine incorporates very well. For more information on the amino acid metabolism, refer to Muchmore et al. *(7)*.
15. *See* **Table 1**.
16. There are quite a few ways to vary the timing of addition of labels during growth. The major considerations here include:
    a. Adding label at the beginning of cell growth wastes label on cell proteins other than the target protein;
    b. However, without having available any of the targeted amino acid, the cell must induce the necessary enzymes required to make that particular amino acid. Thus, when the labeled amino acid is added, it will be in direct competition with unlabeled endogenous synthesized amino acids, which ends up diluting out the label anyway.
    c. Likewise, whereas adding the unlabeled version of the target amino acid at the beginning of the cell growth may cut off the induction of its synthetic pathway, in the end, the cell will still be making proteins from a mixed pool of labeled and unlabeled amino acid any ways. Thus, for maximal efficiency of label incorporation, it is probably best to add some of the labeled amino acid at the beginning of the cell growth and the remainder during induction, either at the beginning of the induction or at times intervals throughout.
17. The most efficient method to incorporate any given amino acid is to get an auxotrophic strain for that particular amino acid. However, that strain must be compatible with the expression system being used, which may not be readily available.

Table 1
Taking Care of Specific Type of Amino Acids in Type-Specific Labeling Preps

Amino acid	Keep separate	Mix together	Filter sterilize only	Autoclave or filter-sterilize	Store in fridge	Store at room temperature
Trp	√		√		√	
Tyr	√		√		√	
Asp, Glu		√	√		√	
Ala, Leu, Ile, Val		√		√		√
Met	√			√	√	
Phe, Pro, Ser, Thr		√		√	√	
Arg, Lys		√		√	√	
Gly	√			√	√	
His	√		√		√	
*Asn, Gln, Cys						

*Bacteria can convert Asp to Asn, Glu to Gln, and Ser to Cys.

One may often get satisfactory results from the aforementioned methods without having to distract their attentions on a hunt for the appropriate auxotrophs.

18. The method employed here follows a particular strategy, which is:
    a. To prepare a batch of cells conditioned to grow and express protein in ≥80% D_2O;
    b. To flash freeze these cells in 1-mL aliquots and store at –80°C, these conditioned cells can then be used at any later date (up to 18 mo) to grow cells and express proteins in ≥ 80% D_2O.

    Central to this strategy is the fact that cells already conditioned to grow in D_2O will grow and express proteins in higher levels of D_2O much more reliably than cells not previously conditioned to D_2O. The nonconditioned cells may or may not grow at all in D_2O. If they do grow, it will take an exasperatingly long time during which they are very likely to lose their plasmids and therefore their ability to express target proteins. In the process of conditioning, which involves multiple growths under varying concentrations of D_2O, it is possible for the cell to lose its plasmids. However, future damage, such as wasting of expensive labels and time, can be minimized by simply talking an aliquot of frozen cells and running a small scale test in H_2O or D_2O (it takes much less time in H_2O than in D_2O) without using labeled materials to check if the cells can still produce high levels of protein so you know if the cells have maintained their plasmids through the conditioning process. Cells that have not maintained their plasmids must be discarded, but in our experience, conditioned cells that have retained their plasmids will always

reliably produce high levels of target proteins in ≥ 80% D_2O with ^{13}C, and/or ^{15}N labeling. Thus, most of the "risk" has been eliminated from the process so that expensive amounts of D_2O and isotope labeling ingredients need not be wasted.

19. It is better to try two or three flasks of culture at the same time. These should be prewarmed to the proper incubation temperature prior to inoculation.
20. The cells grown up in 20% D_2O culture may grow successfully in 80% D_2O culture. If so, the 45% step can be avoided.
21. This test does not have to be in D_2O or in limited media, because the only object here is to check the frozen conditioned cells still have retained the plasmid required for protein expression. It is possible to have two flasks of cells conditioned side by side, grown from the same cell stock, and frozen at the same time, in which cells from one flask express well while cells from the other show no expression. Thus, this test is critical.
22. For cells grown to OD_{600nm} = 1.0, use 1 mL to inoculate 10 mL. If the cells are only grown to OD_{600nm} = 0.5, use 1.5 mL per 10 mL. There are no constraints here about using a minimal inoculation volume as there is in the case of expressing uniformly labeled proteins in minimal media in H_2O, where the volume used for inoculation is usually 1%.
23. Cells grown on limited media using glucose as a sole carbon source are forced to make their amino acids and all other biosynthetic intermediates from the metabolism of glucose, which involves breaking it down into simple molecules (in particular the various intermediates of the tricarboxylic acid and citric acid cycles) and using these to build up the carbon skeleton of all the cell components. In this process, most of the hydrogen in the carbon skeleton is exchanged with environmental hydrogen, which are deuterons in D_2O. Also, by bypassing these metabolic processes and using media (such as TY), which involve the simple enzymatic degradation of cellular components and their straightforward assimilation into cellular anabolism, the exchange of skeletal carbon protons with deuterons from the environment will be severely inhibited. Therefore, cells grown in NZ amine or similar enriched media do not make a good starter culture for the preparation of deuterated proteins, not only are they not metabolically suited to do the job, but there might still be a considerate amount of enriched media left unconsumed. This consideration is even more important if the deuterated proteins are also ^{13}C, ^{15}N-labeled because the cells will have access to unlabeled carbon and nitrogen sources.
24. With the expression of ^{13}C-labeled protein, for maximal incorporation of ^{13}C into the deuterated proteins being expressed, it is necessary to remove any unlabeled from the overnight culture. This can be accomplished by the following procedures: the cells from the overnight culture should be gently centrifuged out of solution (avoid temperature shock at this stage, i.e., centrifuge at the same temperature as the cells are grown at, with prewarmed, previously autoclaved centrifuge tubes. After centrifuging, pour off the supernatant (save for D_2O recycling), resuspend the pellet gently with a few mL of expression medium containing ^{13}C

and ^{15}N labeled ingredients, then carefully pour this back into the flask containing ^{13}C and ^{15}N medium in 99.9% D_2O.

25. Because of the higher level of Mg^{2+} and Fe^{2+} salts used here and their lower solubility in D_2O than in H_2O, visibly high levels of precipitates may form during incubations. These are mainly metal hydroxides. These precipitates have no detrimental effect on cell growth, but they scatter visible light as cells do. Thus, the precipitates may cause a problem in the measurement of cell growth (typically monitored by measuring OD_{600nm}). However, as the cells approach mid-log phase, the precipitates clear away when the minerals are consumed by bacteria, OD_{600nm} becomes a reliable indicator for cell growth.
26. Because cell metabolism is slower in D_2O, induction will be longer, perhaps 3–4 times longer than in H_2O. Accurate induction times should be determined by time-courses.
27. This method is good to recycle D_2O used previously to grow cells and/or D_2O accumulated over the years from various NMR samples. A good rule of thumb is: distill until visibly clear, then distill one more time. This distillate will still contain trace amounts of volatile organic compounds, which are toxic to biological organizms so will not support life. It can therefore be stored indefinitely in the refrigerator or at room temperature.
28. This step should be done immediately before use. Because this D_2O will now support life, it should be stored at refrigeration temperature otherwise mould, alga, and other exotica will grow in it and it will have to be recycled again.
29. The most convenient way to determine the percent deuteration of the D_2O recycled is gravimetrically.

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