Cell-free protein synthesis for analysis by NMR spectroscopy

Margit A. Apponyi, Kiyoshi Ozawa, Nicholas E. Dixon and Gottfried Otting

Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia

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Abstract

Cell-free protein synthesis offers fast and inexpensive access to selectively isotope labeled proteins which can be measured by NMR spectroscopy in the presence of all the unlabeled proteins in the reaction mixture. No chromatographic purification is required. Using an extract from *Escherichia coli* in a simple dialysis system, the target protein can be prepared at a typical concentration of about 1 mg/mL which is sufficient for subsequent analysis by NMR. The present report describes in detail the protocol which we use in our laboratory.

Key Words

Cell-free protein synthesis, dialysis system, high-throughput protein production, isotope labeling, NMR spectroscopy

1. Introduction

Cell-free protein synthesis has won great interest in structural biology, since Kigawa *et al*. demonstrated the possibility of obtaining milligram-per-mL yields in a coupled cell-free transcription-translation system based on *E. coli* cell extract ***(1)***. These concentrations are sufficient for analysis by nuclear magnetic resonance (NMR) spectroscopy on modern high-field NMR spectrometers, providing a convenient and rapid way to analyze proteins, which would be difficult or costly to make otherwise, *e.g*., proteins which are toxic or sensitive to proteolysis *in vivo* ***(2)***, proteins enriched with non-natural amino acids ***(3,4)*** and proteins requiring binding partners for expression in soluble form ***(5)***, including membrane proteins ***(6)***. A number of recent reviews can be found in ***(5,7-11)***.

Using NMR spectroscopy for the analysis of proteins made by cell-free protein synthesis is attractive, because of the efficiency with which selectively isotope-labelled amino acids can be incorporated into proteins ***(5,8,12,13)***. Cell-free protein synthesis uses isotope-labelled amino acids much more economically than *in vivo* expression systems and is much less affected by isotope-scrambling by metabolic enzymes ***(14)***. In addition, since the target protein is the only protein synthesized during the reaction, isotope-filtered NMR experiments allow selective observation of the target protein directly in the reaction mixture without prior purification of the protein by chromatography or concentration of the sample ***(15)***. Cell-free protein synthesis coupled with selective isotope labelling and NMR analysis thus presents a powerful combination by which proteins can be made from DNA and structurally characterized within 24 hours ***(13,15,16)***.

The multitude of potential applications of cell-free protein synthesis has been widely recognized and commercial kits for high-yield cell-free protein expression have become available (*e.g.* the RTS system by Roche and the Expressway system by Invitrogen). Yet, the costs of these systems tend to be prohibitive for routine applications. In the following we describe the *E. coli* based cell-free protein synthesis protocol used in our laboratory at the Australian National University. The process is very efficient once the cell extract and the stock solutions have been prepared, and well over 1000 NMR-size reactions can be performed with a single 20 L fermenter preparation of cell extract.

Cellular extracts prepared in-house tend to be more active than commercial ribosomal extracts which are prepared in view of shipping requirements. The methods used by different groups to obtain the cellular extract vary in details ***(6,14,17,18)***. Our method has been evolved from the protocol by Kigawa *et al.* ***(1,18,19)*** in view of NMR applications with minimal sample handling. The preparation of S30 extract uses the protocol by Pratt ***(20)*** and the heat treatment described follows the protocol described by Klammt *et al.* ***(6)***.

2. Materials

1. Spectrapor #2 (10 mm flat width corresponding to 6.4 mm diameter) and #4 dialysis tubing, 12-14,000 MWCO (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA)
2. French press
3. Centrifuge with rotors suitable for 50 mL, 500 mL and 1 L tubes
4. Optical densitometer

5. 10 mL ultrafiltration centrifugal filtration units, 10,000 MWCO, *e.g.* Amicon Ultra-4

6. 10 mL and 50 mL disposable centrifuge tubes

7. Shaking tray

8. SDS-PAGE equipment

9. Vortex mixer

10. 20 L capacity fermenter

11. Tris(hydroxymethyl)-aminomethane (Tris)

12. Potassium acetate

13. Magnesium acetate

14. Acetic acid

15. Potassium dihydrogen phosphate

16. Dipotassium hydrogen phosphate

17. Yeast extract

18. 1-Hydroxyethyl-piperazineethane sulfonic acid (HEPES)

19. 1,4-Dithiothreitol (DTT)

20. Adenosine triphosphate (ATP)

21. Cytidine triphosphate (CTP)

22. Guanosine triphosphate (GTP)

23. Uridine triphosphate (UTP)

24. Cyclic adenosine monophosphate (cAMP)

25. Folinic acid

26. Ammonium acetate

27. tRNA (from *E. coli* MRE 600; Roche)

28. Creatine kinase

29. Ribonuclease inhibitor (RNasin; Roche or Promega) [Comment: unnecessary]
30. Potassium hydroxide
31. Potassium glutamate
32. Magnesium acetate
33. Creatine phosphate
34. All amino acids, optionally ^{15}N labelled
35. Phenylmethanesulfonyl fluoride (PMSF)
36. Antifoam 289 (Sigma)
37. Milli-Q (MQ) water
38. 1 mg/mL thiamine
39. 2 M glucose
40. 1 M Tris acetate pH 8.2
41. 50% PEG 8000 w/w: 1000 g PEG 8000 and 1L of 1X S30 buffer (V_{total} ~ 2L)
42. LBT: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride, 2.5 mL/L 1 M sodium hydroxide, 0.5 mg/L thymine
43. 4X Z-medium: 165 mM potassium dihydrogen phosphate, 66**4** mM potassium phosphate dibasic, 40 g/L yeast extract ***(20)***
44. 10X S30 buffer: 100 mM Tris acetate, pH 8.2, 160 mM potassium acetate, 140 mM magnesium acetate, pH 8.3 with potassium hydroxide
45. S30 buffer α: 1X S30 buffer containing 0.5 mM PMSF, 1 mM DTT and 7.2 mM β-mercaptoethanol
46. S30 buffer β: 1X S30 buffer containing 1 mM DTT
47. S30 buffer γ: 1X S30 buffer containing 1 mM DTT and 400 mM sodium chloride
48. Vector containing the gene of interest under the T7 promoter

49. T7 RNA polymerase or T7 RNA polymerase vector pKO1166 ***(21)***

50. *E. coli* strain BL21 DE3 *star* or BL21 DE3 Rosetta pRARE

3. Methods

The methods outlined below describe (1) the preparation of the S30 extract, (2) the stock solutions required for cell-free protein production, (3) the preparation of the amino-acid mixtures for cell-free protein expression, (4) the protein synthesis reaction, and (5) the preparation of the samples for NMR analysis.

3.1. Preparation of the S30 extract

S30 extracts can be prepared from *E. coli* grown in a flask or a fermenter. Fermenter preparations have the advantage of high ribosome concentrations. The protocol described here is for a fermenter preparation.

The stock solutions required for S30 extract preparation are listed in **Section 2**. The lifespan of the S30 extract when stored at -80 °C is greater than one year, and a 20 L fermentation yields approximately 100 mL of concentrated extract. This volume of extract is sufficient to prepare over 1000 NMR samples.

The extract can be heat-treated at 42 °C to precipitate mRNA and some unnecessary proteins. This process acts to speed up initial expression of the desired proteins when the extract is used. It does not necessarily improve the final yield ***(6)***.

Concentration of the S30 fraction by dialysis against PEG 8000 ***(1)*** decreases the volume required to add to each reaction. This allows more room in the reaction mixture for other components.

3.1.1. Day One: Buffer preparation and overnight culture

1. Prepare and autoclave stock solutions of glucose (2 M, 224 mL), thiamine (1 mg/mL, 200 mL), 10X S30 buffer (2 L) and 50% PEG 8000 (2 L) as listed in the materials section. Stir the mixture of 50% PEG 8000 at room temperature overnight. Autoclave the stock solutions of glucose, thiamine and 10X S30 buffer, 1 L of LBT medium and 20 L of MQ water for diluting the 10X S30 buffer stock. Autoclave a large glass funnel and two 250 mL measuring cylinders required for measuring the glucose and thiamine solutions to be added to the medium on day three.
2. Grow a 10 mL culture of BL21 DE3 star cells in LBT overnight at 37 °C. If the BL21 DE3 Rosetta pRARE strain is used, include 33 μg/mL of chloramphenicol.
3. After autoclaving, store MQ water and 10X S30 buffer at 4 °C.

3.1.2. Day Two: Z-medium preparation and overnight 1 L culture

1. Prepare 5 L of 4X Z-medium buffer.
2. Add 5 L of Z-medium concentrate, **4.5** mL of antifoam and 14 L of MQ water into the fermenter. This gives a total volume of 19 L. When 1 L of overnight culture is added to this the following day, 20 L of 1X Z-medium will result.
3. Sterilise the medium at 121 °C using the sterilisation function of the fermenter.
4. Calibrate the pO_2 electrode of the fermenter to 0% after 25 min at 121 °C.

5. Inoculate 1 L of LBT medium prepared the previous day with the 10 mL overnight culture, and incubate overnight at 37 ^{o}C.
6. Autoclave 2 L of 50% PEG 8000 and store at 4 ^{o}C.

3.1.3. Day Three: 20 L culture and processing of cell pellets

1. Equilibrate the fermenter at 37 ^{o}C, with 100% air and mixing rate at 395 rpm.
2. Prior to addition of the overnight culture, remove a sample from the outlet at the base as a blank for later measurement of the absorbance of the culture at 595 nm (OD).
3. Add the 1 L overnight culture, 224 mL of 2 M glucose and 200 mL of 1 mg/mL thiamine to the Z-medium with sterile glass funnel. .
4. Allow these to mix before removing a time zero sample for OD measurement.
5. Remove samples for OD measurements every 30 minutes until the culture reaches an OD of 3. This density should be achieved after 3-4 hours.
6. Fill a large sink or tub with a large amount of crushed ice in preparation for the next steps.
7. Drain the culture into 5 L conical flasks and immediately place them on ice. Weigh empty 1 L tubes.
8. Harvest the cells by centrifugation at 4 ^{o}C for 12 min at 10,000 *g* in 1 L tubes. Use pre-weighed tubes so that the pellet mass can be determined later.
9. Resuspend the cell pellets in 400 mL of 1X S30 buffer α. Centrifuge the resuspended cells for 10 min at 10,000 *g* and 4 ^{o}C.
10. Remove the supernatant and pack the pellet by centrifugation for a further 5 minutes. Remove the remaining supernatant with a pipette.

11. Snap freeze the pellets in liquid nitrogen, then store them at –80 ^{o}C.

3.1.4. Day Four: Preparation of the S30 extract

1. Remove the pellets from the freezer and defrost slowly on ice for at least one hour prior to resuspension.
2. Resuspend the pellets in 400 mL of S30 buffer α, and centrifuge for 12 min at 10,000 *g*.
3. Remove the supernatant and pack the pellet by centrifugation for a further 5 minutes at 10,000 *g*.
4. Resuspend the pellets in 1.3 mL of S30 buffer α per gram of cells.
5. Subject the suspension to a single pass in a French press at a pressure of 6,000 psi. If the lysate is very viscous pass it through the French press again.
6. Centrifuge the lysed cells at 5,200 *g* at 4 ^{o}C for 15 min to remove any cell debris in 1 L tubes. Transfer the supernatant to clean tubes.
7. Centrifuge the supernatant at 30,000 *g* at 4 ^{o}C for 1 h to produce the S30 fraction.
8. Prepare 3 batches of 3 L each of S30 buffer β. Dialyse the S30 fraction in Spectrapor # 4 dialysis tubing against each 3 L batch of S30 buffer β for one hour at 4 ^{o}C, *i.e.* for three hours in total.

8a. Optional: Take 1/3 of S30 extract before the following concentration step. Snap-freeze and store it at -80 ^{o}C.

9. Transfer the dialysis tubes containing the S30 fraction to a large evaporating dish and dialyse against two batches of 1L of 50% PEG 8000. Place the dish on a shaking tray to speed dialysis, and leave for 2 hours at 4 ^{o}C.

10. Change the 50% PEG buffer and repeat until the volume of the extract has been reduced to approximately half volume of the S30 extract (about 2h). It is measured by visual comparison with dialysis tubing containing 60 mL of water.

11. The concentrated S30 fraction is then dialysed against a further 3 L of 1X S30 buffer β for 15 minutes to remove the residual PEG 8000 buffer.

If the extract is to be heat-treated, continue with ***steps 13a*** *onwards. If the extract is not to be treated, continue with* ***steps 12-14.***

12. Measure the volume of the extract. Centrifuge the S30 extract if the level of precipitation is high.

13. Dispense the extract into 1 mL aliquots in 1.5 mL Eppendorf tubes.

14. Snap-freeze the aliquots in liquid nitrogen and store at -80 °C.

12a. Place the dialysis bag containing the S30 extract into a further 2 L of S30 buffer γ for dialysis overnight at 4 °C.

13a. Place the dialysis bag containing the S30 extract into a 250 mL Pyrex glass bottle, filled with the S30 buffer γ from the overnight dialysis. Prewarm that S30 buffer γ to 42 °C.

14a. Heat the bottle in a 42 °C water-bath with gentle shaking for 45 minutes exactly.

15a. Dialyse the sample against 1 L of 1X S30 buffer β overnight. (Dialysis for 4 h is sufficient.)

16a. Centrifuge the dialysate at 30,000*g* for 45 min at 4 °C.

17a. Divide the extract into 1 mL aliquots and snap freeze in liquid nitrogen, then store at -80 °C.

For each new batch of S30 extract, the concentrations of magnesium acetate and the extract itself must be optimized. A series of reactions using between 9-25 μL S30 extract per 100 μL reaction mixture and between 15-25 mM magnesium acetate should be sufficient to find the optimal concentrations of each.

3.2. Stock solutions for cell-free protein production

The stock solutions for cell-free protein production are prepared as listed in **Table 1**. The dry weight of each solute is specified for the corresponding stock volumes. The stock solutions stored at -80 °C are removed from the freezer in the afternoon preceding the reaction preparation, and placed on ice in a cold room overnight to defrost. To avoid degradation of creatine kinase it has to be removed from the freezer just before sample preparation. Folinic acid is slower to defrost and should be left at 4 °C overnight. Stocks stored at –20 °C can be removed from the freezer at the commencement of sample preparation. The rNTP solution contains CTP, UTP and GTP at 25 mM each in one stock.

Table 1: Stock solutions for cell-free protein synthesis

Reagent	Stock conc.	MW	Dry weight (g)	Stock volume (mL)	Final conc. in reaction mixture	Supplier	Storage (°C)
HEPES	2.0 M pH 7.5	238.3	95.35	200	55 mM	Sigma	RT
DTT	0.5 M	154.25	3.85	50	1.7 mM	Sigma	**–20**
ATP	96 mM	551.14	0.529	10	1.2 mM	Sigma	–80
rNTP	25 mM each	527.1	0.132		0.8 mM	Sigma	

		523.18	0.131		0.8 mM	Sigma	
		484.14	0.121		0.8 mM	Sigma	
Total				10	0.8 mM		–80
Cyclic AMP	100 mM	351.2	0.351	10	0.64 mM	Sigma	–80
Folinic acid	10 mM	511	0.0511	10	68 μM	Sigma	–80
Ammonium acetate (monohydrate)	9.2 M	77.08	35.5	50	27.5 mM	Ajax Finechem	–80
tRNA	17.5 mg/mL		0.0175	1	0.175 mg/mL	Roche	–20
Creatine kinase	10 mg/mL	18 kDa	0.1	10	250 μg/mL	Roche	–80
T7 RNA polymerase					93 μg/mL		–20
pKO1166					32 μg/mL		–20
Plasmid					16 μg/mL		–20
S30 extract					~225 μL/mL		–80
Potasisum glutamate	4 M	203.23	40.65	50	208 mM	Sigma	–80
Magnesium acetate	1.07 M	214.45	11.47	50	~19.3 mM	Ajax Finechem	RT
Creatine phosphate	1 M	327.14	16.36	50	80 mM	Sigma	–80
L-Alanine	100 mM	89.09	0.089	10	2 mM	Sigma	–80

3.3. Preparation of the amino-acid stock solutions

The amino-acid stock solutions are prepared in three groups, water-soluble, acid-soluble, and base-soluble. Each amino acid is present in the stock solutions at a 50 mM concentration. The acid-soluble and base-soluble combinations are dissolved in 1 M hydrochloric acid and 1 M potassium hydroxide, respectively, such that when they are mixed, the pH of the solutions is close to neutral. The stocks can be kept on ice but the complete mixture must be mixed at room temperature to avoid precipitation at the final near-neutral pH.

For preparations with individual amino acids provided in isotope labelled form, the stocks are prepared minus the amino acid of interest. The labelled amino acid is added later to the final reaction mixture from a stock of a concentration adjusted for economical usage of the labelled amino acids ***(14)***. The concentrations of the individual 100X ^{15}N stock solutions are also listed in **Table 2**.

For combinatorial ^{15}N-labelling ***(13)*** each amino acid stock must be prepared in five sets, with different ^{15}N amino acids included in each set.

To same procedure is used to prepare each of the three types of amino acid stock solutions.

1. Weigh out the individual amino acids in the quantities listed in **Table 2**.
2. Place all of the amino acids into a 10 mL tube.
3. Add 10 mL of the appropriate solvent to the mixture and vortex until dissolved.
4. Store the amino-acid stock at -80 °C.

Table 2: Amino acid stock solutions

Water soluble mixture - dissolved in 10 mL water

Amino acid	Weight (mg)	^{15}N stock (mM)
Ala	44.5	100
Arg	105.0	35
Gly	37.5	100
His	105.0	15
Lys	91.3	100
Pro	57.6	n/a
Ser	52.6	100
Thr	59.6	15
Val	58.6	100

Acid soluble mixture - dissolved in 10 mL 1 M HCl

Amino acid	Weight (mg)	^{15}N stock (mM)
Asn	66.0	35
Asp	66.5	100
Cys	60.1	35
Glu	73.5	100
Gln	73.1	100
Leu	65.6	100
Met	74.6	100
Trp	102.0	5
Tyr	90.6	5

Base soluble mixture - dissolved in 10 mL 1 M KOH

Amino acid	Weight (mg)	^{15}N stock (mM)
Ile	65.5	15
Phe	82.5	35

3.4. Carrying out the cell-free reaction

3.4.1. Preparation of the reaction chamber

1. Soak one 10 cm length of Spectrapor #2 dialysis tubing per reaction in MQ water for 5 min, then tie a knot as close as possible to one end. Trim the knot to have minimal overhang.
2. Using clean scissors, prepare one 500 μL Eppendorf tubes per reaction by cutting off the lid and hinge sections. The lid is retained and the hinge is discarded. Removal of the hinge allows the tube to fit inside a 10 mL tube. The bottom 0.5 cm of the tube is then sliced off using a scalpel.
3. Insert each cut Eppendorf tube into the top of a pre-tied length of dialysis tubing, as shown in **Fig. 1**. The Eppendorf tube ensures the contents of the dialysis tube are readily accessible. Remove the dialysis tubing from the MQ water and insert the Eppendorf tube immediately prior to use, so that the tubing does not dry out before assembly of the complete reaction.

Fig. 1: Schematic drawing of the assembly of the cut Eppendorf tube in the knotted dialysis bag to provide a readily accessible container for the reaction mixture.

3.4.2. Preparation of the reaction mixtures

Working on ice at all times, mix the stock solutions in sequence according to **Subheading 3.3** The amino-acid mixture is prepared first, followed by the 10X B solution that contains nucleotides and other factors required in both the inner and outer chambers (**Table 3**). Finally, the reaction mixture and outside buffer are prepared (**Table 4**).

Table 3: 10X B solution for a 500 μL reaction with 5 mL outside buffer

Stock solution	μL
rNTP	179.5
HEPES	162.5
ATP	70.0
Water	38.5
Folinic acid	38.0
Cyclic AMP	35.0
DTT	19.0
Ammonium acetate	17.0
Total	559.5

Table 4: Components of a 500 μL reaction mixture and 5 mL outside buffer

Stock solution	Reaction mixture (μL)	Outside buffer (μL)
10X B	50.0	500
Creatine phosphate	40.0	400
Potassium glutamate	26.0	260
Magnesium acetate	7.0	90
L-Alanine	5.0	50
Amino acid mixture	35.0	335
Creatine kinase	12.5	
T7 polymerase vector	8.0	
S30 extract	112.5	
Vector of interest	8.0	
tRNA	5.0	
Water (adjust)	190.0	3365
total	500.0	5000

The quantities listed in this section are for one 500 μL reaction mixture with 5 mL outside buffer. An Excel spreadsheet is available online (http://rsc.anu.edu.au/~go) that facilitates scaling to different volumes, number of reactions or different vector concentrations. The quantities of vectors listed assume stock solutions with a concentration of 1 mg plasmid per mL to achieve final concentrations in the reaction mixture of 16 μg/mL, as per **Table 1**. If different volumes of vector solutions are to be added, adjust the quantity of water so that the final volume of the reaction is 500 μL. The pH of the outside buffer must be adjusted to 7.5 using 1 M KOH, and the volume of water added adjusted accordingly.

T7 RNA polymerase must be supplied if the expression of the target gene is under control of the T7 promoter. The pKO1166 vector is used in this example rather than purified T7 RNA polymerase so that fresh polymerase is continuously generated during the reaction ***(22)***. In most cases, pKO1166 delivers higher protein yields than purified T7 RNA polymerase, whereas experiments with incorporation of non-natural amino acids may depend on provision of the purified polymerase, which should be supplied at a final concentration of 32 μg/mL. When using the purified polymerase enzyme there is no need for additional L-alanine to be added to the reaction mixture. The water volume to be added must be adjusted accordingly ***(21)***. The synthesis and purification of T7 RNA polymerase from a strain containing pKO1166 has been described in ***(14)***.

3.4.2.1. Mixing the amino-acid stock solutions for use in a 500 μL reaction mixture with 5 mL outside buffer

1. Pipette 37.5 μL water into a 1.5 mL Eppendorf tube.
2. Add 112.5 μL water soluble stock and pipette up and down to mix well.
3. Add 112.5 μL acid soluble stock and pipette up and down to mix well.
4. Add 112.5 μL base soluble stock and pipette up and down to mix well.

3.4.2.2. Preparation of the 10X B mixture

Working on ice, add the stock solutions (described in **Table 1**) in the quantities listed in **Table 3** in a 1.5 mL Eppendorf tube.

3.4.2.3. Preparation of the reaction mixture and outside buffer

Working on ice, mix the components listed in **Table 4** for the reaction mixture in a 1.5 mL Eppendorf tube. Leave the reaction mixture on ice whilst the outside buffer is prepared in a 10 mL tube. Adjust the pH of the outside buffer using **1** M KOH prior to making up the final volume with water.

3.4.2.4. Assembly of the reaction into the reaction chamber

1. Pipette the reaction mixture into the dialysis tubing and seal the Eppendorf tube with the pre-cut lid.
2. Place the dialysis tubing inside the 10 mL tube containing the outside buffer.
3. Place the entire assembly into a 37 °C water bath, shaking at 200 rpm, for 6-8 hours or overnight.

At the end of the incubation period the reaction mixture may be placed on ice overnight or processed immediately. The sample is ready for initial SDS-PAGE electrophoresis after steps 1 and 2 of **Section 3.5** which remove precipitated reaction components. 5 μL of the supernatant is generally sufficient to give clear bands with Coomassie Brilliant Blue stain. **Fig. 2** shows a typical example from the expression of the protein τ_C16 ***(13)***.

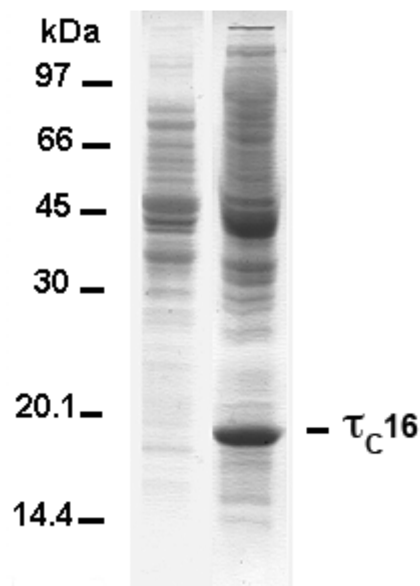

Fig. 2: Typical SDS-PAGE gel of S30 extract (left lane) and reaction mixture after expression of the protein τ_C16 (right lane). Mobilities of molecular mass markers were as indicated.

3.5. Preparation of the samples for NMR analysis

1. Pipette the reaction out of the dialysis tubing with a 200 μL pipette.
2. Place the mixture in an Eppendorf tube and centrifuge for 1 hour at 30,000 *g* at **4** °C.
3. Prepare 2 L of the desired NMR buffer.
4. Remove the supernatant from the tube and place it into # 2 dialysis tubing.
5. Dialyse the sample against the NMR buffer overnight.
6. Remove a 5-10 μL aliquot of the dialysed sample for SDS-PAGE analysis.
7. Concentrate the dialysed sample by centrifuging in an Amicon 10 K MWCO Ultra-4 tube for 10-15 min according to the manufacturer's instructions.
8. Adjust the concentrated sample to a total volume of 500 μL by adding 50 μL of D_2O and the NMR buffer.
9. Transfer the sample to an NMR tube for measurement.

The volume of the reaction can be scaled up or down. 200 μL reactions with 2 mL outside buffer are appropriate for optimization of the extract and magnesium acetate concentrations and also when a sample is only required for analysis by SDS PAGE.

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