PURIFYING HIS_{6-10}-TAGGED MEMBRANE PROTEINS FROM *E. COLI* AND MAKING NMR SAMPLES

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The goal is to prepare a ca. 0.55 ml sample in a 5 mm NMR tube that contains a membrane protein in detergent micelles, with the protein concentration being at least 0.2 mM (preferably ca. 0.5 mM).

Note, using 3 mm NMR tube you can get the same S/N at 800 or 900 MHz for any given sample as for a 5 mm sample; however only 180 microliters are required. (!)

Regardless of which of the following procedures you follow, always verify sample purity at the end by running SDS-PAGE. However, DO NOT BOIL membrane protein samples before loading on SDS-PAGE.

References:

Oxenoid, K., Kim, H.-K., Jacob, J., Sönnichsen, F. D., and Sanders, C. R. (2004) NMR Assignments for a Helical 40 kDa Membrane Protein in 100 kDa Micelles. *J. American Chemical Society* 126, 5048-5049 (2004). *(see especially supp materials to that paper)*

Oxenoid, K., Sonnichsen, F. D., and Sanders, C. R. (2002) Topology and secondary structure of the N-terminal domain of diacylglycerol kinase. *Biochemistry* 41, 12876-12882.

Paul J. Barrett*, Yuanli Song*, Wade D. Van Horn, Eric J. Hustedt, Johanna M. Schafer, Arina Hadziselimovic, Andrew J. Beel, and Charles R. Sanders (2012) The Amyloid Precursor Protein has a Flexible Transmembrane Domain and Binds Cholesterol. Science, 336, 1168-1171. PMC3528355 *(see especially supplementary materials to that paper)*

Andrew J. Beel, Charles K. Mobley, Hak Jun Kim, Fang Tian, Arina Hadziselimovic, Bing Jap, James H. Prestegard, and Charles R. Sanders (2008) Structural Studies of the Transmembrane C-Terminal Domain of the Amyloid Precursor Protein: Does APP Function as a Cholesterol Sensor? BIOCHEMISTRY, 47, 9428-9446. PMC2572687 *(see also Supporting Information section)*

Kang C, Vanoye CG, Welch RC, Van Horn WD, and Sanders CR (2010) Functional Delivery of a Membrane Protein into Oocyte Membranes Using Bicelles. Biochemistry 49, 653-655. PMC2811756 *(see especially supporting information)*

Changlin Tian, Carlos G. Vanoye, Richard C. Welch, Hak Jun Kim, Congbao Kang, Alfred L. George Jr., and Charles R. Sanders. (2007) Preparation, Functional Characterization, and NMR Studies of Human KCNE1, a Voltage-Gated Potassium Channel Accessory Subunit Associated With Long QT Syndrome Biochemistry, 46, 11459-72. PMC2565491

The methods given here are used in the Sanders Lab to express and purify His-tagged integral membrane proteins, followed by acquisition of a preliminary NMR spectrum. While the protocols given here specify purification into DPC micelles, these protocols can easily be adapted to purification into any of a wide variety of different types of micelles and bicelles. This is a one column purification procedure, which usually generates protein of sufficient purify for NMR provided that the expression level of the protein is 2 mgs of purified protein per liter of original *E. coli* culture or higher.

Expression Strains

For expressing polyHis-tagged mammalian proteins in E. coli, we suggest testing several different E. coli strains, most of which are BL21-based. There are many variations of BL21. Since we are expressing mammalian proteins, a strain which is optimized for using eukaryotic codons may be best. For this we often try: BL21(DE3) or BL21-CodonPlus-RIL/RP(DE3), RosettaBlue(DE3), and C43(DE3). For a new target protein we usually try expressing the protein in both N- and C-terminally His-tagged forms using pET16b and pET21b vectors, respectively (note, both of these requires that the host strain contain the DE3 lysogen)

An important issue is whether you need to start expression cultures using freshly-transformed cells or whether you can start with glycerol stocks of transformed cells stored in the deep freeze. The best strategy is to use only freshly-transformed cells until you document high level expression. At that point, you can then make a glycerol slurry and see if you continue to get good expression in cultures started from it.

Often, initial expression tests are first conducted by growing small scale cultures in LB medium and testing for expression of a desired target by SDS-PAGE/Western Blotting on whole cell extract. It may be worth doing this first before proceeding to the large culture minimal medium methods described below.

UNIFORMLY ^{15}N-LABELED CULTURES USING H2O

Note: Membrane proteins usually express better in E. coli when cells are grown in minimal medium (rather than rich medium) and at reduced temperatures rather than 37C. This is probably because E. coli finds making recombinant membrane proteins to be stressful. E. coli seems to be able to manage this stress best at reduced temperatures and poor medium where everything gets slowed down.

We first grow a small liquid culture (3-6 ml of LB media in 15 ml sterile Falcon tube) from one plate colony. Then this small liquid colony is used to inoculate a 0.5 liter of minimal medium.

Small tube (3-6ml) culture: LB medium with appropriate antibiotic

Large scale culture: Minimal medium (as described below) with appropriate antibiotic

1. Make minimal medium and put 0.5 L into 2 L flasks
   (To maintain high O_2 aeration of the large cultures it is important to either use Fernbach (wide bottom with baffles) flasks or (at least) to make sure you don't add too much culture to standard flasks… i.e., not more than 500 ml in a standard 2 L flask. Cultures must also be shaken vigorously)

   A. Add the following amounts per liter of medium:

Na_2HPO_4	6 g (12.8 g if the 7-H2O hydrate form is used)
KH_2PO_4	3 g
NaCl	0.5 g
$^{15}NH_4Cl$	1.0 g

   B. Adjust pH to 7.0
   C. Autoclave
   D. Let medium cool till you can touch the flask without feeling you are getting burned.
      Then add:

0.1 M $CaCl_2$	1 ml/L
1 M $MgSO_4$	1 ml/L
40% Glucose (dextrose)	10 ml/l
Ampicillin	100 mg/l
Vitamins (see below).	2 ml/liter

   Stock solution of $CaCl_2$ is prepared by dissolving 1.47g of $CaCl_2$ in 100 ml of ddH_2O. Filter sterilize.

   Stock solution of $MgSO_4/7H_2O$ is prepared by dissolving 24.65 g of $MgSO_4/7H_2O$ in 100 ml of ddH_2O. Filter sterilize.

   20 or 40% sterilized Glucose (prepared autoclaving for 10-15 minutes)

   Ampicillin stock (100 mg/ml, 1000x) is filter-sterilized.

   Vitamin prep: smash 1 vitamin (Spectravite, multivitamin/multimineral from CVS Pharmacy) *and*

mix with 20 ml of H2O. Mix, bath sonicate, and remove insoluble junk by low speed centrifugation. Filter with steriflip. (There are a number of variations within the lab of how to do the vitamins and which ones to use. It probably doesn't matter very much). You also can use commercially available MEM vitamin solutions (supplied as 100x)

2. Grow 2-5 ml LB/amp cultures overnight with shaking at 37C.

3. Use 1 tube of LB to inoculate each 2L Fernbach flask with 1 L of minimal medium. (You may get a higher yield if you do 500 ml of medium in the same 2L flask).

4. Grow with vigorous shaking at room temperature until OD600 = 0.6-1.0.

5. Induce with 0.2 g/L IPTG (or whatever inducing agent is appropriate).

6. Continue shaking at room temperature for 12-24 hours

7. In morning, harvest cells.

Variations of the above: Depending the strain of E. coli, additional antibiotics (such as kanamycin or chloramphenicol) may be required. Some proteins only are well expressed when the cells are grown and induced at higher or lower temperatures (37, 30, or 12 degrees). For 37C, induce the cell when OD600 reaches 0.6-1.0 and then continue to culture the cells (with shaking) at 37C for 4 hours before harvesting. When cell are cultured at 30C, the induced cells are usually cultured with shaking overnight before harvesting. For 12C cultures, it is usually OK to grow cells at 37 degrees until OD600 is 0.6-1.0. At this point switch to 12C (with shaking) for two hours and then induce and continue to culture with shaking for 24 hours.

Some proteins are proteolyzed with time in E. coli, so that the amount of time between induction and harvesting must be reduced relative to what is suggested above. For a new protein, it is recommended that you test varying the length of post-induction culturing prior to harvesting to see what works best.

HARVESTING CELLS

Harvest cells by centrifuging at 4 deg. C for 15 minutes on any preparative centrifuge. Generally spin at 90% of the maximum allowed RPM for the rotor being used (the limit is usually stamped on top of the rotor). Some centrifuge tubes also have an RPM or g limit—do not exceed this. Cell pellet should be very firm and stick to bottom of tube even after supernatant is poured out. Discard supernatant and save the cell pellet. Store by transferring to 50 ml polypropylene Falcon tubes and then freezing (do not use clear polystyrene Falcon tubes because they can't withstand freezing). You should get several grams of wet cells from 1 liter of culture. **Note: weigh Falcon tube before and after adding cell pellet to it**. **This will give you the weight of cells- always record this.**

LYSIS PROCEDURE

1. Take *E. Coli* cell paste and dilute 20X with lysis buffer in a sealable bottle (i.e. use 20 mL lysis buffer per gram of wet cells). Disperse cells in the solution (mild mixing).
   Lysis Buffer:
   75 mM Tris-HCL (Tris is the buffer component, make this starting with Trizma base)
   0.3 M NaCl (salt to mimic physiological ionic strength)
   0.2 mM EDTA (chelating agent to bind multivalent cations which might inactivate DAGK)
   pH = 7.7 (adjust pH by adding HCl to the basic trizma solution)
2. Add PMSF (phenylmethylsulfonyl fluoride- a poison!) from a commercially prepare 0.1 M solution in

ethanol to a concentration of 1 mM). PMSF is a protease inhibitor which will help keep protein from getting chewed up.

3. Add the following:

lysozyme powder	0.2 mg/ml
powdered DNase and RNase	0.02 mg/ml each

The RNA and DNA in E. coli tend to form thick suspensions which are hard to handle. RNase and DNase will break up this goop.

4. Seal the container and incubate for ½ hour at room temperature with tumbling (But not stir-bar stirring). Then add MgAcetate to 5 mM from a 500 mM stock (stock: 11 g/100 ml)
5. Tip sonicate at amplitude 40, 50% duty cycle for 5 minutes (5 sec on, 5 sec off, repetitive). Place your sample in an ice water bath during sonication. *This sonication step can be regarded as optional… for some delicate proteins it may be better to skip sonication.*
6. If indicated for your future analyses of pure protein, add dithiothreitol (DTT) to a concentration of 0.5 mM (10 mgs per 100 ml). DTT is a reducing agent which will help keep the Cys thiol groups from getting oxidized. *If DTT is added, new nickel resin, not regenerated, must be used.*
7. If you wish to stop at this point, lysate can now be divided up into ≤40 ml portions in 50 ml Falcon tubes. These can then be frozen in liquid nitrogen and stored in the -80° freezer. *Note: it can be frozen and stored at this point (before adding detergent), but not after adding detergent.*

Note About DTT

Dithiothreitol is a reducing agent which is typically used in concentrations of 0.1 to 1 mM as a way of keeping cysteines in their reduced form (no disulfide bonds). DTT is preferred to the use of mercaptoethanol for several reasons and is also generally superior to TCEP (a non-thiol-based reducing agent). The only problem with DTT is that it does have a high affinity for certain metal ions and can also reduce some metal ions. Supposedly, Ni(II)-NTA agarose can be used in the presence of modest (1 mM or less) DTT concentrations, but in our experience there are sometimes problems with regenerated Ni-NTA resin is used.

For cases where things must remain reduced in the presence of low or no concentrations of DTT, it is advisable to use buffers which have been depleted of oxygen by saturation with Argon gas (bubble it into the solution, preferably through a frit aerator). If detergents are to be used, de-oxygenize the final detergent-containing solution (but watch for excess bubbling when saturating with argon!).

Note that DTT has a finite lifetime in aqueous solutions (on the order of 1 day). So, DTT should always be added to solutions from a *freshly-prepared* stock solution. (15 mgs of dry DTT per 1 ml to make a 100 mM stock solution).

Finally, it is sometime seen that when Ni(II)-metal ion affinity chromatography is carried out with DTT-containing elution buffers that DTT will cause the resin to turn brown and cause the eluate also to turn brown. We have never figured out what exactly is going on and this only seems to be observed when using regenerated resin, not new resin. If it happens, don't panic. Continue on with the protocol and analyze your results (with SDS-PAGE) as usual. Hopefully, you will find that you still isolate your protein.

Testing Where the Protein is Expressed

For a new membrane protein being expressed for the first time, note the protein could be expressed into **inclusion bodies**, into the **plasma membrane**, or into the cytosol (perhaps in the form of small vesicles that cannot be spun down even with an ultracentrifuge). If the protein is expressed primarily into inclusion bodies, then you will definitely want to isolate the inclusion bodies as the first step in purification. If the protein is expressed primarily into the plasma membrane, then you will want to isolate the membranes as the first step in purification. However, the latter instance seems to be rare. Most often, membrane proteins express mostly either into inclusion bodies or into the cytosol (again, perhaps in the form of small vesicles). Here, we describe how to isolate inclusion bodies, the plasma membrane, and the cytosol.

First, isolate the inclusion bodies. Take the cell lysate prepared as described above (this is before any detergent is added) and centrifuge to spin down unlysed cells and inclusion bodies. Centrifuge extracted lysate at 10,000 g or higher for 20 minutes. Save the "supernatant A" (containing both cytosol and plasma

membrane). The pellet will be include the inclusion bodies. These can be "cleaned up" by suspending in lysis buffer and re-centrifuging once or twice. A small portion can then be dissolved by SDS in an Eppendorf tube. You can then spin the Eppendorf tube (to pellet insoluble material) and run the sup on SDS-PAGE/Western Blot to see if your protein is **expressed into inclusion bodies**.

Then, **separate the plasma membrane from the cytosol**. Take "supernatant A" from above and spin the in an ultracentrifuge for 2 hours at 60000 g. Save the supernatant as "supernatant B". The pellet will contain the plasma membrane. These membrane should be re-suspended in lysis buffer and re-centrifuged once or twice to clean them up. A sample of this can be dissolved by SDS in an Eppendorf tube. You can then spin the Eppendorf tube (to pellet any insoluble material) and run the sup on SDS-PAGE/Western Blot to **see if your protein is expressed into the plasma membrane**. A small aliquot of supernatant B can also be mixed with SDS and then run on SDS-PAGE/Western Blot.

Note, when you run SDS-PAGE, do NOT boil or heat your sample before loading on gel. This tends to induce irreversible aggregation of membrane proteins.

All Western Blots should be run using an anti-His-tag antibody. (See Sanders lab protocols for details).

When assessing the expression levels in the inclusion bodies, plasma membrane, and sup, keep in mind that the volumes of these three fractions are very different, so that some degree of normalization is needed when using Western Blot band intensities to assess the total amount of protein in each fraction.

It is good to know where your protein is expressed. If your protein is expressed into inclusion bodies then you will definitely want to purify the protein starting with isolated inclusion bodies (see below). If your protein is expressed into the cytosol only, or into both the cytosol and the plasma membranes, then you may be able to skip isolating the inclusion bodies and there is also no need to isolate the plasma membranes. This was the case for diacylglycerol kinase and some other membrane proteins we have worked with

Below, we present methods for purification of membrane proteins from whole cell lysate and from isolated inclusion bodies. The whole cell lysate procedure can be easily adjusted to be applicable to the plasma membrane fraction or to the cytosol-only fraction.

The Western Blot procedure used for His-tagged membrane proteins is found in the general Sanders Lab "Protocols for Working With Membrane Proteins" found at the Sanders Lab web site.

PURIFICATION PROTOCOL 1. EXTRACTION OF MEMBRANE PROTEIN FROM TOTAL *E. COLI* CELL LYSATE

THIS PROTOCOL IS FOR THE FAVORABLE CASES WHERE THERE IS NO NEED TO ISOLATE INCLUSION BODIES AND/OR THE PLASMA MEMBRANES PRIOR TO DETERGENT EXTRACTION OF THE MEMBRANE PROTEINS.

1. Cool lysate solution on ice.
2. For every 10 ml portion of lysate (either fresh or thawed after storage in the freezer) add 1 ml of Empigen detergent (comes as a 30% solution from Calbiochem) to make the lysate 3% by volume Empigen. Mix thoroughly (but do not stir with stir bar) and allow to incubate for an hour with tumbling. *Do not freeze the lysed solution at this point.*
   {Empigen is a fairly harsh zwitterionic detergent which is fully compatible with the use of Ni(II)-agarose resin. (This is unlike SDS, which can be used with Ni(II)-agarose, but only VERY carefully). It may not be as harsh a detergent as SDS and our whether it can dissolve all inclusion bodies or not is still not clear—there are some IBs for which only SDS will work}.
3. Centrifuge extracted lysate. Save supernatiant and discard any "goop" pellet (this is important!). If use the 50 mL rotor, spin at 10,000g or higher for 20 minutes.

PURIFICATION SOLUTIONS

If a reducing agent is required, 0.25 mM dithiothreitol (DTT) should be added to all the buffers below *on the day the buffer will be used*, and ONLY if you will NOT be doing SH-modification, SH-determination, or -S-S- mapping experiments following purification.

Always adjust the pH of your buffer BEFORE adding detergent, since detergent can confuse pH meters.

DPC = dodecylphosphocholine = Anatrace Fos-Choline-12

-Lysis Buffer:
75 mM Tris-HCL (Tris is the buffer component, make this starting with Trizma base)
0.3 M NaCl (salt to mimic physiological ionic strength)
0.3 mM EDTA (chelating agent to bind multivalent cations which might inactivate DAGK)
pH = 7.7 (adjust pH by adding HCl to the basic trizma solution)

-Buffer A:
40 mM HEPES (HEPES is a zwitterionic buffer)
300 mM NaCl
pH = 7.5

-Emp/A:
buffer A plus 3% Empigen

-Sanders Wash Buffer:
Buffer A + 1.5% empigen + 0.04 M imidazole, pH 7.8

-Rinse buffer:
0.5% DPC in water

-Elution buffer
0.5% DPC
0.25 M imidazole (make sure a high grade of imidazole is used, see below) in water
pH 7.8 (adjust the pH *then* add the DPC)

Note: Use only high quality imidazole (Sigma I-0250 or better). Lower grades contain impurities which absorb strongly at 280 nm and interfere with quantitation of protein.

ELUTION NOTES

Empigen and dodecylphosphocholine (DPC) are detergents. Both have critical micellar concentrations (CMC) near 2 mM. Empigen is a harsh detergent and probably denatures most proteins. It is our belief that DPC is somewhere in between harsh and mild—it definitely destabilizes some membrane proteins. For proteins where stability is an issue, another detergent may be better. But just to see if you can purify you protein DPC is a good starting choice. *The following procedure can be adapted for final elution in the detergent or choice by simply replacing DPC with that detergent (although for detergents with higher CMCs, you may have to use higher than 0.5% to make sure the concentration is well above the relevant CMC).*

Ni^{2+} RESIN INCUBATION AND ELUTION

1. "Superflow" Nickel-Agarose resin should be equilibrated by rinsing with buffer A. Use about 1.2 ml of packed resin for every gram of wet cells represented by the lysate. Pack resin into a column and rinse twice with 2 bed volumes of buffer A.
2. Transfer the resin into a tube containing the empigen-extracted lysed cells (on ice).

3. Tightly close the lid and rotate the tube for ½ hour (no longer) in the cold room. During this time the detergent-solubilized protein will bind to the nickel resin.
4. Following incubation, isolate the resin by centrifugation of the solution. 70-100% speed for any tabletop centrifuge for 15 minutes should be fine.
5. Pour off the supernatant (try not to lose any of the resin) and either freeze the resin in liquid N2 and store until later use or transfer to an appropriately-sized column. The height of the packed bed should be more than 4 times the bed diameter, and the total column volume should be about 3-5 times the bed volume. Wash the resin with 3 X 1 bed volume of ice-cold Emp/A. Freezing protein-on-resin and storing at -80 usually does no harm to the protein of interest, at least when storage is only for a matter of days. *Note: throughout this protocol buffers should be added to elute the column in 1 column bed volume aliquots and allows to drain down to reach the top of the bed before adding the next aliquot. "3 X 1" means you do this three times.*
6. Turn on chart recorder and start monitoring A280.
7. Wash column with cold Sanders wash buffer until the "junk" peak has finished eluting (as monitored by the chart recorder). The wash buffer contains enough imidazole to knock proteins off the column which have a weak affinity for the nickel ions but which do not have the His_6 tail. After this step, the target protein will be just about the only protein left sticking to the resin. After A280 returned to baseline add 2 X 1 more column volumes of wash buffer before going to next step.
8. Rinse column with **12 X 1** bed volume portions of cold rinse buffer. This does not mean a single pulse of 12 bed volumes. It means to do 12 portions ("pulses"), 1 at a time, allowing rinse to enter top of column before adding next portion. The purpose of this buffer is to switch from empigen to DPC as the only detergent in contact with the protein on the column.
9. Elute the protein with elution buffer. The target protein will elute as a sharp A280 peak (as monitored using the chart recorder). Collect this peak. Remember to tare your collection vial while it is empty so you can measure the weight of the of protein solution eluted. Weigh the eluted protein solution.
10. Zero a 1 cm cuvette containing elution buffer on a spectrophotometer at 280 nm. Clean and dry the QUARTZ cuvette and blank the spectrophotometer using protein-free elution buffer. Clean and dry the cuvette and then measure A280 of your eluted protein solution. If A280 is greater than 1.6 dilute 200 microliters of your eluted protein solution 1:5 into elution buffer and measure A280 again. The extinction coefficient for your protein can be calculated from its sequence using programs found at the ProtParam web site: http://us.expasy.org/tools/protparam.html.

You can now calculate both the mg/ml of your protein solution and the total mgs of protein. Note that the concentration of DPC in the final solution is not just 0.5%. It will be 0.5% plus the DPC that is bound to your protein (for example, DAGK binds about 1.5 its own weight in detergent).

Run SDS-PAGE on your lysate, the flow through when the lysate resin is added to the column, the junk peak that elutes with wash buffer, the eluate at the rinse/equilibration step, and for the final pure protein pool.

When you run SDS-PAGE, do NOT boil or heat your sample before loading on gel. This tends to induce irreversible aggregation of membrane proteins.

PURIFICATION PROTOCOL 2. EXTRACTION OF MEMBRANE PROTEIN FROM INCLUSION BODIES

THIS PROTOCOL IS FOR THE CASE WHERE THE PROTEIN EXPRESSES INTO INCLUSION BODIES.

Following cell lysis and prior to adding any detergent spin down the inclusion bodies using a 20 minute spin on a preparative centrifuge run at 90% of the maximum allowed rpm of the rotor. Save the pellet. Suspend the pellet in lysis buffer and spin down again. Repeat this pellet-washing experiment twice.

NOW, YOU COME TO A CROSSROAD: SOME INCLUSION BODIES CAN BE SOLUBILIZED USING 3% EMPIGEN. BUT SOME REQUIRE THE USE OF EVEN MORE HARSH SDS MICELLES PLUS UREA.

TEST TO SEE IF YOUR INCLUSION BODIES CAN BE SOLUBILIZED BY 3% EMPIGEN IN LYSIS BUFFER (USE A VOLUME EQUAL TO ½ OF THE ORIGINAL VOLUME OF THE LYSED CELLS FROM WHICH THE INCLUSION BODIES WERE ISOLATED). IF THE PELLET CAN BE SOLUBILIZED BY THE EMPIGIN SOLUTION THEN PURIFICATION IS MUCH EASIER THAN IF SDS/UREA MUST BE USED. IN THE FORMER CASE YOU JUST USE "PURIFICATION PROTOCOL 1" GIVEN ABOVE, STARTING AT THE POINT WHERE THE EMPIGEN EXTRACT IS MIXED WITH NICKEL RESIN AND LOADED TO A COLUMN (EXCEPT IN THIS CASE IT IS THE EMPIGEN-SOLUBILIZED IB PELLET THAT IS LOADED OT THE COLUMN- CENTRIFUGE OUT ANY REMAIN PARTICULATE FIRST).

If the inclusion bodies CANNOT be solubilized using Empigen, then the following SDS/Urea solution should be used to solubilize the pellet:

Solubilize with a volume of the following solution equal to ½ of the volume of lysis buffer used for the cells that the pellet was derived from):

20mM Tris (pH 7.8)
8M Urea (high quality grade)
150mM NaCl
0.2% SDS (2 mgs/ml) (DO NOT USE HIGHER SDS CONCENTRATIONS!)

2/2015 note: Instead of 0.2% SDS, 0.1% lauryl sarcosine can be used. While LS is a harsh anionic detergent like SDS, it appears that it is not as prone to interfere with His-tagged protein binding to nickel-resin as SDS.

Tumble at room temperature until the inclusion bodies are completely solubilized or for no longer than four hours. (Make sure to seal the outside of whatever container you are using with parafilm, Urea is prone to making bottles leak and is very messy to clean up.)

Centrifuge at 18C (SDS precipitates at cold temperatures) for 45 min at >10,000g. Solution should be clear after centrifugation, but if there is debris present, use 0.2uM filter and syringe to transfer solution to a clean bottle

Transfer supernatant to a clean bottle. If you needed you can pause by freezing in liquid nitrogen and store at -80C until ready for purification

Purification from SDS/Urea into DPC micelles.

This is a tricky protocol. Even when the directions are followed closely, there can be problems that seem to stem from the fact that the presence of SDS seems to reduce the affinity of His-tags on recombinant proteins for Ni(II)-resin. When you try this with a new protein, you should monitor the eluted solutions at all steps with SDS-PAGE to see if your protein is failing to stick and/or eluting prematurely.

Solutions (filter all solutions using a 0.2uM filter, add detergents after filtration)

TBS (tris-buffered saline) (1x)
20mM Tris (pH 7.8)
200mM NaCl

SDS Rinse buffer
0.2% SDS in 1x TBS

DPC rinse buffer (1x)

10 mM Imidazole, pH 7.8
0.1% DPC
In 1x TBS

DPC wash buffer (1x)

30mM Imidazole, pH 7.8
0.5% DPC
1x TBS

Elution Buffer

250mM Imidazole, pH 7.8
0.5.% DPC
In 1x TBS

(the above buffers can also contain fresh DTT at 0.25 mM, if needed)

Note that SDS will crystallize out of aqueous solutions that are cooled to 4C. SDS-containing solutions need to be stored and worked with near room temperature.

Procedure

1. Equilibrate Ni resin (transfer 2ml Ni resin for each wet gram of original wet cells) with the following buffers:
    a. 1X TBS (2 column volumes (CV))
    b. SDS-Urea (2CV)
2. Transfer resin from column to same bottle as solubilized inclusion bodies
3. Tumble at RT for 30-60mins
4. Allow Ni Resin to settle to bottom of bottle
5. Gently remove supernatant and transfer resin pellet back to column
6. Rinse bottle with SDS-urea and add rinses to column
7. Affinity column chromatography
    a. Set up chart recorder and attach to column (allow 30 min warm up time)
    b. Add solutions to column in the following order
        i. SDS-urea (4 X 1cv)
        ii. 0.2% SDS rinse (4 X 1 cv)
        iii. 0.1% DPC rinse (8 X 1 cv)
        iv. 0.5% DPC wash (30mM Imidazole) (6 X 1 cv, more if needed for A280 to return to a constant baseline)
        v. Elution buffer (typically 2-3cv, but as much as needed to collect 95% of protein peak)
8. Quantify protein concentration
    a. Zero a spectrophotometer using elution buffere and then measure A_{280} for your purified protein pool and determine the concentration of your protein from this.
9. Run polyacrylamide gel electrophoresis (SDS-PAGE, no heating of sample prior to loading) to determine protein purity
    a. Add approximately 10-15ug of protein per lane

If your protein has cysteines in it that should be kept in reduced form you should now add additional fresh DTT to the solution (1 mM). It is best to add DTT *AFTER* measuring A280, because DTT (probably its oxidized form) does tend to absorb light at 280 nm.

Variations of the Above Procedures

If you would like to purify your protein in a different detergent (or bicelles) than DPC, just swap in the detergent of interest (or bicelles) for DPC at the final stages of purification. It is key that the detergent concentration be above CMC, although just above is usually sufficient.

For example, we have found that a number of membrane proteins give optimal solution NMR spectrum when purified into LMPG micelles (LMPG = lyso-myristoylphosphatidylglycerol). To purify in LMPG, just replace DPC at all steps of the above procedures with 0.05% LMPG and elute using 0.05% LMPG.

For a truly difficult new membrane protein we have a list of many different detergent and micelle conditions to screen (see separate protocol).

Also, for membrane proteins that seem to express into E. coli membranes and retain native-like structure, we also have a protocol for extracting the membranes and purifying the protein using a mild detergent all steps. We have used this to purify E. coli-expressed human GPCRs, for example. Just ask CS for this protocol.

Occasionally some proteins will not elute from Ni(II)-resin even at 250 mM imidazole. In such cases you may have to use 500 mM or 1 M imidazole to get the protein to elute.

Freezing and Thawing of Membrane Protein Solutions.

When purified in DPC we have found that some membrane proteins will get denatured by a round of freeze-thaw (unlike some other detergents). So, you may or may not be able to freeze your solution for storage before NMR. The only way to know for sure for a given protein is to test by freezing in liquid nitrogen and then comparing protein functionality, its NMR spectrum, or some other informative property for that protein before and after freeze-thawing.

Final Stage of Preparing Membrane Protein Samples for HSQC or TROSY NMR

Protein needs to be 15N-labeled.

Purify protein off of Ni(II)-agarose as described above, resulting in DPC solution.

For routine screening of samples, we just use fully protonated DPC.

Measure weight of solution and A280.

Add fresh DTT as a thiol reducing agent, if needed. (0.5-5 mM)

Add D2O to sample to a concentration of 8% (80 microliters per ml).

Add 100 mM pH 7 EDTA stock solution to the protein solution to make it 0.5 mM in EDTA to sequester any metal ions which may have eluted with your protein. Alternately, add a little Chelex resin to your solution and then filter or centrifuge it out. The presence of EDTA will also suppress microbial growth.

Lower the pH to 6.5 using perdeuterated acetic acid and a 20 microliter pipettor. If you undershoot the pH, raise pH using ammonium hydroxide. The use of the weak acid (acetic acid) and weak base (ammonia) allows you to avoid transient exposure of your protein to extremely high or low pH (as would occur if a strong acid and/or base is used to adjust pH). pH meters don't seem to mind the presence of DPC.

Concentrate your solution using either an Amicon Ultra-4 or Ultra-15 Centrifugal Filter Unit (depending on the volume you need to concentrate). In old protocols we used Centricon YM-50 or Centricon PLUS-20 PL-10 filter units. (From Millipore).

Wrap the cartridge with 1 or more Kimwipes when inserting into centrifuge to help avoid junk from the centrifuge from contaminating sample (and always cap sample).

The best way to monitor the centrifugal concentration process is to tare the weight of both the receiver and filter cartridges before and after putting your sample in. You can then monitor how much the solution has been concentrated just by weighing the filter cartridge and the receiver. At this point carefully "swirl" the cartridge to disperse concentration gradients which have developed near the filter during the process of centrifugation. I generally stop when the solution is concentrated to about 0.7 ml. If you go too far (volume is lower than needed) just dilute to the correct level using the filtrate.

Note that most of the detergent is retained with the protein rather than passing through the filter… concentrating the protein means concentrating the detergent also. Only the population of soluble detergent (concentration equal to the CMC) will be lost.

Transfer the desired volume (550 microliters for a 5 mm tube, 180 microliters for a 3 mm tube) to an NMR tube. Save the rest in an eppendorf tube for non-NMR analysis (it is usually good to have at least 50 microliters for this purpose).

The final protein concentration in the NMR sample should be >0.1 mM (ideally ca. 0.5 mM) in order to get a decent 2-D spectrum in a <12 hour run.

For routine screening of new proteins, we usually take TROSY spectra at 25 and 45 degrees. Note that it matters a lot exactly which TROSY sequence you use. Some are ineffective at filtering out the huge residual proton peaks from imidazole and the detergent. On both Varian and Bruker machines we have gotten good results when we run the Weigelt sequence (JACS 120, 10778-10779, 1998).

Buffer Exchange to Switch From 250 mM Imidazole to a Different Buffer

If you follow the procedure immediately above, your protein will end up being purified in a 250 mM imidazole solution. While imidazole is an excellent buffer for pH 6.5, this is a very high concentration of imidazole. You can use the spin column to switch buffers. When you initially concentrate (as described above) re-dilute the solution using the desired buffer (which should contain D2O), mix the contents of the cartridge well, and reconcentrate. Repeat these steps. Note that the buffer you add (twice) should NOT contain any detergent, as most of the detergent present in the initial sample will be retained with the protein during the centrifugation step. Addition notes on optimal buffer composition are found at the end of this document in a separate section.

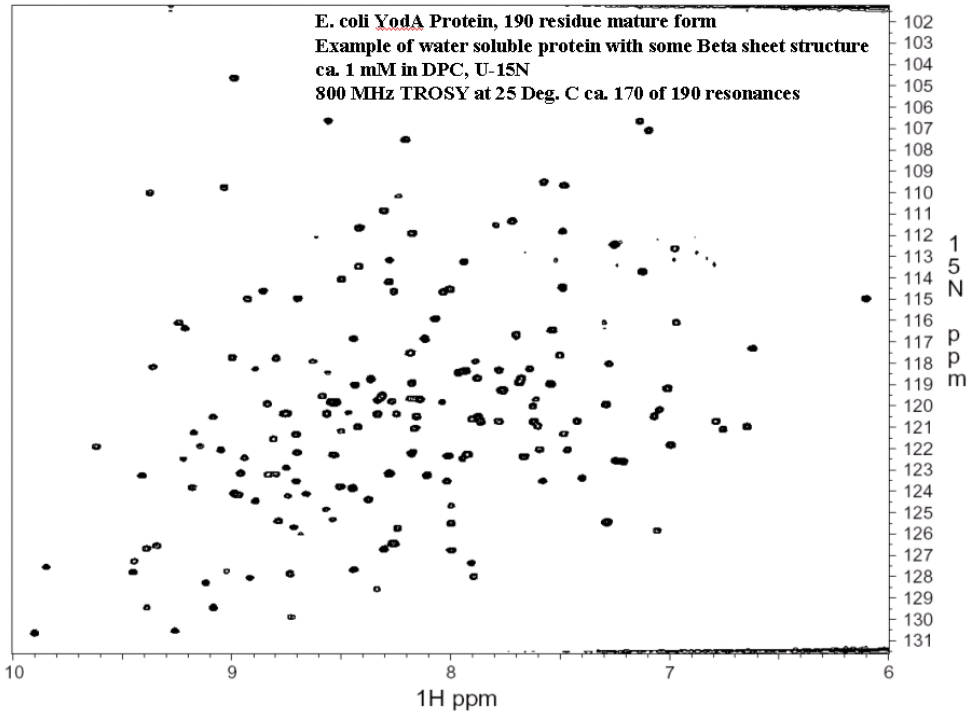
What is the Detergent Concentration in the Final Membrane Protein NMR Sample?

When the protein is eluted from the column in a 0.5% detergent solution, the final detergent concentration will not be 0.5%, but rather will be 0.5% detergent PLUS the amount of detergent that is complexed with the membrane protein (which can be 2X the weight of the protein… a significant amount if you have a 5-10 mg/ml protein solution!). The only way to determine the final total detergent concentration is to measure it. There are various ways of doing this. A 1-D NMR-based method for this is used by the Sanders lab… just e-mail CS if you want the details.

Beware of the YodA protein.

There is a recurring problem we often experience involving an endogenous E. coli protein which often co-purifies with both MTB and E. coli membrane proteins. The protein is the mature form of a periplasmic E. coli protein called YodA (or a protein closely related to it). The mature form of this protein has about 190 residues, migrates in the 27 kDa range on SDS-PAGE, and has (get this!) an N-terminal sequence of HGHHSH-- not only will it co-purify with polyHis-tagged proteins from MIAC, but it may also be recognized by antibodies which recognize polyHis tags. YodA is well established to be a stress response protein which avidly binds soft divalent cations. Moreover, it is also established that one of the stress signals which can lead to its massive overexpression is the overexpression of other proteins. See: David, G., Blondeau, K., Shiltz, M., Penel, S.,

and Lewit-Bentley, A. (2003) J. Biol. Chem. 278, 43728-43735. David, G., Blondeau, K., Renouard, M., and Lewit-Bentley, A. (2002) Acta Crystallogr. Sect. D Biol. Cryst. 58, 1243-1245. We have found that YodA often is co-overexpressed with other recombinant membrane proteins in *E. coli*. It has a characteristic (and beautiful) NMR spectrum which is shown below.



Notes on Buffer Composition for Protein NMR Samples *Chuck Sanders 12/2014*

Choice of pH and Buffer

As always, the buffer needs to have a pKa within 0.7 units of the desired pH.

For the Sanders lab pH 6.5 is the default pH value if there are no reasons to make your sample at a different pH.
6.5 is close to physiological pH, but just slightly acidic to keep the amide-H exchange rate reasonably low level.

Good buffers for use at pH 6.5 (based having pKa values near 6.5) are PIPES and imidazole.

pH values above 7 are generally avoided, because some amide proton peaks may disappear due to rapid or intermediate time scale exchange between amide protons and water protons. If you do need to go to a higher pH (7.5 -8.5) HEPES is a good buffer to use.

Some proteins give much better spectra at lower pH values. If you find that you need to work at lower pH values, acetate is often a good choice because its pKa is 4.76 (so it is a good buffer in the pH 4 to 5.5 range). MES is a good buffer to use for pH 6.0.

50 mM buffer is usually about right.

Salt

You will almost always include a little. You don't want excess salt because salt induces what is known as "lossiness" in NMR samples: a reduction in the NMR S/N and the potential for sample heating via electrolyte/radiowave interactions.

50 mM NaCl is usually about right.

Chelating Agent

Trace amounts of divalent/trivalent metal ions can catalyze hydrolysis (a big problem for some samples, such as those that contain lipids) and can also lead to unwanted NMR linebroadening.

To sequester any metal ion contaminants always include some EDTA in your buffer. 0.5 mM is the default concentration.

Reducing Agent

If your protein do not have any cysteines there is no need to include a reducing agent such as beta-mercaptoethanol, dithiothreitol, or TCEP.

If your protein contains one or more disulfide bonds and no free thiols then you should not include a reducing agent.

If your protein contains free cysteines then you will need to include a reducing agent to suppress disulfide bond formation. I am not a fan of TCEP or mercaptoethanol. Instead, I suggest using dithiothreitol (DTT). Usually a concentration of 1 mM DTT is about right. Keep in mind that DTT (and other reducing agents) do not remain in the reduced state indefinitely. Unless care is taken to exclude oxygen from samples, DTT will slowly oxidize, with higher pH values increasing the oxidation rate.

If your protein contains both disulfide bonds and free thiols (some membrane proteins are like this, GPCRs for example), then you may to use a redox buffer. This is usually a mixture of oxidized and reduced forms of glutathione. The higher the oxidized-to-reduced glutathione ratio, the higher the oxidation potential. At "just the right" ratios only native disulfide bonds will form and the native free Cys will remain reduced.

Deuterium Oxide

NMR samples should contain 5-10% D_2O (v/v). The purpose of the D_2O is to introduce the NMR-active deuterium atom into your sample so that the NMR spectrometer can "lock" on the NMR signal of this isotope to allow for correction during the course of an NMR sample of magnetic field drive. Spectrometers also use the deuterium lock signal as the basis for the critical magnetic field shimming to correct for magnetic field inhomogeneity.

Detergent (or other model membrane)

If the protein under study is a membrane protein then detergent micelles or some other isotropic model membrane (such as small bicelles or nanodiscs) will need to be present to solubilize your protein. Advice on detergent types and concentrations are given in other samples lab protocols, but a

key think to keep in mind is that the detergent concentration needs to be above its “critical micelle concentration” (CMC) .

A Default NMR Buffer

A decent NMR buffer for a water soluble (non-membrane) protein that contains free cysteine thiols in its native state is:

50 mM imidazole, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, pH 6.5, plus 7% D_2O

This will be fine for many membrane proteins also, as long as there is also detergent or some other type of model membrane present.