Sanders Lab Protocols

(2014 version)

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Many of the following protocols are written specifically for working with *E. coli* diacylglycerol kinase, an integral membrane protein (homotrimer of 14 kDa subunits, each with 3 transmembrane helices). However, the protocols can often be adapted for applications to other proteins, especially membrane proteins.

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WORKING WITH E. COLI CELLS

MAKING GLYCEROL STOCKS OF E. COLI STRAINS

Glycerol stocks are the best way to store bacterial strains at -80 °C. Glycerol is a cryoprotectant which will help the culture to survive under frozen conditions. These frozen cultures are stored at -80 °C and are used for "plating out" colonies. Everyone in the lab should have their own set of strains as glycerol stocks. Don't use other people's stocks.

Preparing glycerol stocks

Things you will need:

- *Sterile (autoclaved) 50% glycerol solution in water*
- *Sterile cryo vials with caps*
- *LB media, antibiotics, fresh cells on a LB plate*

1. Plate bacteria on LB plate (+antibiotic) and grow at 37 °C overnight.
2. Use single colony to inoculate 3-5 mL of LB (+antibiotic). Grow this culture at 37 °C with shaking for 6-8 hours.

3. Aliquot 300 µL 50% sterile glycerol into each cryo vial.
4. Add 900 µL cell culture.
5. Vortex. Place at -80 °C (no need to freeze in liquid N_2).
6. Store glycerol stocks at -80°C.

PLATING E. COLI CELLS

1. Place plates on bench top to let them come to room temperature.
2. Using an inoculating loop (either metal flame-sterilized or plastic disposable), lightly scratch the surface of the frozen glycerol stock. **DO NOT ALLOW STOCKS TO THAW**.
3. Streak the loop across the plate.
4. **Return the stock to the freezer immediately**.
5. Incubate plates at 37 °C overnight.
6. Store plates in refrigerator for no more than one week. Wrap plates in Parafilm to keep them from drying out.

POURING PLATES

(Recipe for LB agar is also posted in the chemical weighing area in MRBIII 5120)

1. To LB medium, add 15 g/L agar. The agar will not dissolve before autoclaving.
2. Autoclave at 121 °C for 10-15 minutes.
3. When media is cool enough to handle, add antibiotics.
4. Using sterile technique, pour ~ 25-30 mL LB agar into sterile Petri dishes.
5. Allow plates to sit at room temp for up to several hours, with the lids slightly opened to allow moisture to evaporate.
6. Store plates inverted in plastic bags at 4 °C. Kanamycin and ampicillin plates can be kept for about a month. For plates without antibiotics, use your own judgment.

USING THE AUTOCLAVE

NEVER AUTOCLAVE ANY PLASTIC THING UNLESS YOU ARE SURE IT WON'T MELT AT 120 °C. IF YOU AREN'T SURE, MAKE SURE THE PLASTIC THING IS INSIDE A CONTAINER WHICH YOU *ARE* SURE WON'T MELT.

Liquids are autoclaved separately from solid things (like pipette tips). Place flasks containing liquids into an autoclavable plastic pan to catch any boil-over. Containers containing liquids should never be more than 2/3 full and should never be completely closed to the atmosphere.

Autoclaving liquids: set the sterilizing time for 15-20 minutes . Do not open autoclave until cycle is done and pressure is 0.8 or less.

Autoclaving solids: set the "gravity" sterilizing time for 10-15 minutes. Do not open until cycle is done and pressure is 0.8 or less. If pipette tips are still wet, they can be dried by placing in 37°C incubator for a while.

PREPARING LB MEDIUM

1. Add the following per liter of LB desired:
   - Tryptone 10g
   - Yeast Extract 5g
   - NaCl 10g
   - adjust pH=7.0
2. Pour into flasks or bottles. If using flasks, wrap mouth of flask with foil. If preparing

small bottles of LB, remember to place the caps loosely on the bottles. Place a small piece of indicator tape on top.

3. Autoclave.
4. Add antibiotics after LB medium is cool to touch.

COMMONLY USED SANDERS LAB ANTIBIOTICS

Ampicillin, 1000X (100 mg/mL)
Dissolve 5 g ampicillin (sodium salt) in 50 mL MQH_2O.
Filter sterilize using syringe filters into 15 ml conical tubes. Label and keep at -20 °C.
Leave one tube at 4 °C for immediate use.

Kanamycin, 1000X (20 mg/mL)
Dissolve 1 g kanamycin in 50 mL MQH_2O.
Filter sterilize using syringe filters into 15 ml conical tubes. Label and keep at -20 °C.
Leave one tube at 4 °C for immediate use.

(CS note: *Do not add to LB in big flasks in which large-scale protein expression is desired.* Kanamycin inhibits protein synthesis.)

Chloramphenicol, 1000X (50 mg/mL)
Dissolve 500 mg in 10 mL 95% ethanol in a 15 mL tube. Label and keep at -20 °C.

Tetracycline, 1000X (20 mg/mL)
Dissolve 0.2g tetracycline hydrocloride in 5 mL absolute ethanol and 5 mL MQH_2O. No need to filter. Label and keep at -20 °C.

TO TRANSFORM OR NOT TO TRANSFORM

In the case of the DAGK overexpression system, we have found that once cells are transformed with a DAGK-encoding plasmid, the cells retain the plasmid rather well and can be stored as glycerol slurries indefinitely without concern that the cells will lose their ability to express DAGK. This does not seem to be the case for all protein/plasmid/cell combinations. Some combinations can be rather unstable such that cells either modify or spit out the plasmid under conditions of culture storage (even at -80 °C). In such cases, aliquots of the plasmid should be stored at -20 °C and used to transform competent cells which are then immediately grown and induced when protein is desired.

Purification of Plasmid DNA

Qiagen's QIAprep plasmid DNA purification kits are the best way to quickly isolate high-quality, pure plasmid DNA for routine molecular biology applications. The kits use silica gel-based spin columns to bind plasmid DNA. Binding of DNA is followed by ethanol wash step and elution of DNA in small volume of Tris buffer (included in the kit) or water.

QIAprep Spin Miniprep Kit

Each spin column from this kit can bind up to 20 µg of double stranded DNA and 85-95% of the bound DNA is typically recovered in the elution step. If you are purifying a high-copy plasmid, use not more than 5 mL of overnight cell culture per spin column. If you are purifying a low-copy plasmid, use 5-10 mL of overnight cell culture per spin column. Bacterial cultures for isolation of plasmid DNA should always be grown from a single colony picked from a freshly streaked selective plate. A single colony should be inoculated into 1-5 mL of LB containing the appropriate antibiotic, and grown with shaking for 12-16 hours. Cultures should not be allowed to grow for longer than 16 hours because the cells will begin to lyse, resulting in decrease of plasmid yield.

A detailed QIAprep Spin Miniprep Protocol can be found in the QIAprep Miniprep Handbook, a manual which is provided with each kit.

Please consider the following before starting the QIAprep protocol:

- Read the Qiagen protocol, including the important notes for QIAprep procedures as instructed by QIAprep Handbook.
- Sterile pipette tips and microcentrifuge tubes should be used.
- *If you are opening a new kit box*, add the provided RNase A solution to buffer P1 and add 100% ethanol to buffer PE, as instructed by the QIAprep Handbook. If you are using an already opened kit box, the bottles should be marked indicating that these components have already been added.
- Buffer P1 should always be kept at 4ºC, so please return in to this storage temperature when done.
- Final elution should be done in elution buffer provided in the kit (EB). However, if the plasmid DNA will be used for sequencing, DNA should be eluted in sterile water because EB interferes with the chemistry of sequencing reaction.
- After 12-16 hours of growth, harvest cells by centrifugation. Depending on the plasmid copy number, there will be between 5 and 10 mL of cell culture. Ideally, to follow the Qiagen protocol and obtain optimal yield, the pellet should be in one tube. Culture tubes can be placed in a refrigerated benchtop swinging bucket centrifuge (Sorvall Legend) and centrifuged at 3700 *x g* for 15 minutes. The cells can also be transferred into 1.5 mL tubes and centrifuged at higher speeds in a microcentrifuge.
- When the cells are harvested, continue by following the QIAprep Spin Miniprep Protocol which begins with resuspension of cell pellet in buffer P1.

The plasmid DNA should be eluted into a sterile (autoclaved) tube. The solutions used in the kit are initially sterile of course, but one kit is usually intended for at least 50 plasmid preparations, so the solutions are not sterile once they are opened. Everyone using these kits should therefore be extremely careful not to cross contaminate solutions or introduce any cells or plasmid DNA into the buffer bottles. **Pipette tips should be sterile and must be changed after each pipetting step and buffer bottles closed as soon as they are no longer needed.**

Once purified, plasmid DNA should be stored at -20 °C. A single Qiagen prep typically yields 50µL of plasmid DNA solution. The concentrations of eluted plasmid DNA can vary depending on the plasmid copy number. For high copy plasmids, the concentrations of eluted DNA are usually between 150 ng/µL and 600 ng/µL (from 5 mL cell culture). For low

copy plasmids, the concentrations of eluted DNA are usually between 50 ng/μL and 150 ng/μL (from ~6 mL cell culture).

Since the volume of eluted DNA is already relatively small (~50 μL), I usually don't divide it into smaller aliquots before storage. The plasmid DNA is very stable and, in my experience, it is not negatively affected by repeated freezing and thawing.

The concentration of plasmid DNA can be estimated by measuring A_{260} and using the following conversions:

A_{260} of 1 = 50 μg/ ml dsDNA
A_{260} of 1 = 40 μg/ ml ssDNA
A_{260} of 1 = 30 μg/ ml ssDNA (oligonucleotide)

Estimate DNA concentration from A_{260}:
Concentration (μg/ml) = A_{260} x dilution factor x 50 μg/ml

Remove 5 μL from the tube containing the eluted plasmid DNA (always use a sterile pipette tips when working with DNA) and mix it with 145 μL water for a 1:30 dilution, and then use an ultra-micro quartz cuvette. After concentration measurements I discard the contents of the cuvette. The possibility of the contamination is just too great to keep the solution used to measure.

Concentration (μg/ml) = A_{260} x 30 x 50 μg/ml → this will give you ng/μL DNA.

PCR Amplification of Genes from Plasmid DNA (Arina's protocol)

Stocks needed:

1) Primers, 10 µM each oligonucleotide, in TE buffer
2) dNTPs, 2.5 mM each (10 mM total), in MQH_2O
3) template DNA, 10 ng/µL, in MQH_2O

I make my own dNPTs from individual dATP, dCTP, dTTP, and dGTP. They are also available as a PCR mix, with or without a polymerase. (We may not always have the commercial mix in Sanders lab freezer, so please check this when planning your PCR)

Thaw buffer, primers, dNTPs, and template DNA at room temperature, then keep them on ice while setting up. Polymerase should always be on ice. Add polymerase enzyme last and start the thermal cycler program immediately.
Use thin-walled 0.2 mL PCR tubes to prepare the reactions as follows:

MQH_2O	24.5 µL
10 X Vent Polymerase Buffer	5 µL
FW primer (10 µM)	5 µL
REV primer (10 µM)	5 µL
dNTPs (2.5 mM each)	5 µL
Template (10 ng/µL)	5 µL
Vent Polymerase	0.5 µL

Use the following program in a thermal cycler with a heated lid (like CSB's Eppendorf MasterCycler):

1. 95 °C 1 min
2. 94 °C 45 sec
   54 °C 45 sec
   72 °C 1 min (adjust based on the length of fragment to be amplified, approx. 1 min per 1 kb)
   Go to step 2. x 29
3. 72 °C 2 min
4. 4 °C hold

Preparation of chemically competent C43 (DE3)

Can be used for preparation of other strains of chemically competent E. coli (based on Qiagen protocol)

Day 1.

1. Start with a glycerol stock of C43 (DE3) strain. Streak out *E. coli* on LB plate and grow overnight at 37 °C. C43(DE3) strain has no antibiotic resistance, so no antibiotic should be included at this point. Once cells are made competent and are used in transformation, the choice of antibiotic will depend on the selective marker present on the plasmid that is being introduced into the C43(DE3) cells.
2. Prepare the following buffers and store them at 4 °C until they are needed. Also, prepare and sterilize LB medium, centrifuge tubes, pipette tips and microcentrifuge tubes.

100 ml TfbI buffer:

30 mM CH_3COOK 3 ml 1 M stock (9.8 g/100ml)
50 mM $MnCl_2$ 5 ml 1 M stock (19.8 g/100 ml)
100 mM KCl 10 ml 1 M stock (7.5 g/100 ml)
KCl can be substituted with RbCl
10 mM $CaCl_2$ 1 ml 1M stock
150 g/L glycerol 15 g

Carefully pH to 5.8 with 0.1 M acetic acid (0.575 ml of glacial CH_3COOH/100 ml of H_2O). Discard if you overshoot the pH, or a precipitate will form which does not redissolve and the buffer does not work.

Mix all components together, adjust pH, and filter sterilize the buffer using a 0.2 μm syringe filter.

10 ml TfbII buffer:

10 mM MOPS-Na 1 mL 100 mM stock (2.09 g/100 mL, pH 7.0 with NaOH)
75 mM $CaCl_2$ 0.75 mL 1 M stock
10 mM KCl 0.1 mL 1 M stock
150 g/L glycerol 1.5 g

Mix all components together and filter sterilize the buffers using a 0.2 μm syringe filter.

Day 2.

1. Grow a single colony from the plate for 2 hr in 5 mL of LB (or TY broth, 2x YT, etc – any rich medium will do), at 37 °C, shaking at 250 rpm. Don't include any antibiotics. Strain C43(DE3) has no antibiotic resistance genes.
2. Transfer to a flask with 100 mL of LB medium and grow until OD_{550}= 0.5-0.6 (~2-3 hrs)
3. Spin cells in sterile centrifuge tubes at 2000 x *g* for 5 min, at 4 °C.
   a) The most convenient way to do this is to use the refrigerated benchtop centrifuge, Sorvall Legend RT, and spin cells in 50 mL conical tubes (they are already in a sterile package).
   b) Another option is to use JA 25.50 rotor in Beckman Coulter Avanti J-25 centrifuge and spin cells in Beckman 50 mL centrifuge tubes (polycarbonate or polyallomer). These tubes must be sterilized prior to use either by autoclaving or some other suitable method. Depending on the material, some tubes may not be able to withstand autoclaving (please consult

Beckman product information sheets provided with tubes for sterilization instructions).

4. Carefully pour off the supernatant and gently resuspend cells by slowly pipetting up an down the side of the tube. Resuspend cells in total of 40 mL of cold TfbI. NEVER VORTEX when making competent cells or working with competent cells. Keep cells on ice while resuspending.
6. Leave on ice for 10 min.
7. Spin cells as gently as possible, at 2000 x *g*, at 4 °C.
8. Remove supernatant and gently resuspend the pellet in total of 4 mL of sterile TfbII. Keep cells on ice while resuspending.
9. Aliquot 25-50 µL cells into sterile microcentrifuge tubes. Freeze in liquid nitrogen and store at -80 °C.

   The cells are now chemically competent and ready for transformation.

METHOD FOR MAKING Competent C43(DE3) SUGGESTED BY COMMERCIAL SUPPLIER (Avidis)

http://www.avidis.fr/htm/purchase/index.htm

Preparation of $CaCl_2$ competent cells of C43(DE3)

- Streak the strain from a LB agarose stab or a seed stock vial stored at -80°C onto LB plate.
- Inoculate a single, well-isolated colony into 5 ml LB medium in a 50 ml tube.
- Incubate 24 hours at 37°C, shaking at 250 rpm.
- Inoculate 100 ml of LB medium, in a 500 ml Erlenmeyer flask, with 1 ml of the saturated overnight culture.
- Grow this flask at 37°C, shaking at 250 rpm, until the OD600 reaches 0.6-0.8.
- Transfer the culture to two 50 ml tubes, and place the tubes on ice for 10 minutes.
- Centrifuge the tubes at 4,000 rpm for 10 minutes at 4°C.
- Discard ALL the supernatant, and resuspend the pellet GENTLY in 10 ml of ICE-COLD 0.1 M CaCl2 / 10% glycerol. Put the tubes back on ice for 15 minutes.
- Centrifuge the tubes again at 4,000 rpm for 10 minutes at 4°C.
- Again, discard ALL the supernatant, and resuspend the pellet GENTLY in 1 ml of ICE-COLD 0.1 M CaCl2 / 10% glycerol.
- Prepare aliquots 50 µl in individual Eppendorf tubes, on ice. Freeze immediately the aliquots and store them in a -80°C freezer.

E. coli transformation protocol

(This is a general protocol that works with most competent cells. If using commercially prepared competent cells, it's always good to check the product insert and protocol.)

1. Thaw an aliquot of cells on ice.
2. Mix ~ 1 µL DNA (in H_2O or elution buffer) and 25 µL cells.
3. Leave on ice for 10-15 min.
4. Heat shock at 42 °C for 45 seconds.
5. Place back on ice for 2 minutes.
6. Add 250 µL of SOC and incubate at 37 °C for 1 hr.
7. Plate 100 µL on LB plate (with appropriate antibiotic) and grow overnight at 37 °C.

S.O.C Medium (adapted from Invitrogen)

For 100 mL:

2 g tryptone (2% tryptone)

0.5 g yeast extract (0.5% yeast extract)

10 mM sodium chloride (200 µL 5 M NaCl)

2.5 mM potassium chloride (250 µL 1 M KCl)

10 mM magnesium chloride (1 mL 1 M $MgCl_2$)

10 mM magnesium sulfate (1 mL 1 M $MgSO_4$)

20 mM glucose (1.8 mL 20% glucose)

PROTEIN EXPRESSION

GROWING CULTURES FOR PROTEIN EXPRESSION

The goal is to grow large cultures. However, you cannot simply add a colony from a plate to 1 L of media. Instead, we first grow a small liquid culture (5 ml or more in LB media) from one plate colony. This small liquid colony is then used to inoculate 1L of media. The 1L culture is grown until cells reach a certain optical density (determined by A_{600}). At this point, IPTG is added to the culture to turn on the lac operon and induce protein synthesis. Cells will normally stop growing at this point and put all their energy into making protein, which is allowed to continue for at least a few hours, after which cultures are harvested.

GROWTH PROCEDURE A: *37 °C GROWTH in LB*

Day 1
Plate cells from a glycerol stock, or transform competent cells. Incubate LB plate at 37 °C overnight.

Day 2, evening:

1. Using sterile serological pipet, add 5 mL LB plus appropriate antibiotics to a loose-capped culture tubes (we currently use 17 X 100 mm sterile culture tube)
2. Inoculate the tubes with a single colony using a disposable loop, sterile toothpick, or pipette tip.
3. Incubate with 250 rpm shaking overnight at 37 °C. Do not tighten the lid of the tube; bacteria need oxygen to grow.

Day 3, morning:

1. The tubes should be very cloudy as a result of the growth of the microbial culture. Carefully transfer the contents of each tube into 1L (each) of freshly autoclaved LB medium with antibiotics. Put foil covering back on top of flask.
2. Shake at 250 rpm, at 37 °C and monitor cell growth by measuring A_{600}.
3. When A_{600} reaches 0.7-1 (typically about 5-6 hours), add 0.2 g/ml stock IPTG to a concentration of 0.2 g/liter. IPTG is a substance which activates the promoter which controls transcription on the plasmid. By adding IPTG, you are telling the *E. coli* culture to start making your protein.
4. Shake culture at 37 °C for another 3 hours to overnight.
5. Harvest cells by centrifugation (see following section for details).

GROWTH PROCEDURE B:
Growth at 37 °C, induction at 12 °C

This procedure is used for proteins that do not express well at 37 °C. We have found this to be effective for a number of difficult-to-express membrane proteins. The detailed protocol is tucked away in the literature as **on-line supporting infomation** to a Correction in *JACS:* Tian, C., Breyer, R. M., Kim, H. K., Karra, M.D., Friedman, D. B., and Sanders, C. R. (2005) Correction to: Solution NMR spectroscopy of the human vasopressin V2 receptor, a G protein-coupled receptor. *J. American Chemical Society* **128**, 5300 (2006). Contact CS if you have trouble tracking this down.

GROWTH PROCEDURE C:
Minimal medium for uniformly ^{15}N labeled proteins using H_2O

Reference: Oxenoid, K., Kim, H.-K., Jacob, J., Sonnichsen, 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.

As with growing cells in LB medium, we first grow a small liquid culture (3-6 ml of LB) from a single colony. This small starter culture is then used to inoculate a 0.5-1L of minimal medium.

1. Make minimal medium

- Add the following amounts per liter of medium:

Na_2HPO_4	6 g (12.8 g if the $7H_2O$ hydrate form is used)
KH_2PO_4	3 g
NaCl	0.5 g
$^{15}NH_4Cl$	1.0 g

  1) B. Adjust pH to 7.0.
- C. Autoclave at 121 °C, 15-20 minutes.
- D. Let medium cool, then add:

  1 mL 0.1 M $CaCl_2$
  1 mL 1 M $MgSO_4 \cdot 7\ H_2O$
  10 mL 40% glucose (or 20 mL 20% glucose)
  + vitamin solution (see below)
  + antibiotics

  - 0.1 M $CaCl_2$
    (MW=111 g/mol, 0.111 g $CaCl_2$ in 10 mL MQH_2O, filter sterilize)
  - 1 M $MgSO_4 \cdot 7\ H_2O$
    (MW=246 g/mol, 2.46 g $MgSO_4 \cdot 7\ H_2O$ in 10 mL MQH_2O, filter sterilize)
  - 40% Glucose (or 20% glucose – can be autoclaved)
  - Vitamins:

    <u>Option 1 (CVS vitamins)</u>
    Vitamin prep: smash 1 vitamin and mix with 20 ml of H2O. Mix, bath sonicate, and remove insoluble junk by low speed centrifugation. Filter with steriflip. Store at -20 °C. Use 2 mL per L minimal media.

    <u>Option 2.(MEM vitamins)</u>
    Use commercially available MEM vitamin solution, 10 mL to 1L minimal medium.

2. ***Grow 5 ml LB/amp cultures overnight with shaking at 37 °C.***
3. ***Use one 5 ml tube of LB to inoculate each large flask of minimal medium.***
4. ***Grow with shaking at 37 °C until A_{600} reaches 0.7-1.0 (about 6-10 hours).***
5. ***Induce with 0.2 g/L IPTG.***
6. ***Continue shaking at 37 °C overnight.***
7. ***Harvest cells the following morning.***

GROWTH PROCEDURE D:

Minimal medium for uniformly *^{15}N,^{13}C labeled cultures using D_2O*

Reference: Oxenoid, K., Kim, H.-K., Jacob, J., Sonnichsen, 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.

Cells to use when expressing DAGK: BL21 with pSD005 plasmid containing an inducible synthetic gene for the I53C/I70L/V107D triple mutant of *E. coli* diacylglycerol kinase. Be careful not to get confused and grow this same mutant in WH1061 (which is Leu auxotroph). BL21 is amp resistant but not Kan resistant.

Note: if WH1061 is used, then you also need to include leucine in the medium. Use 0.25 g/L. Leucine is autoclavable.

Small tube (3-6ml) culture:
LB medium with ampicillin 100 ug/ml and NO kanamycin.

Medium scale (50 ml in 250 ml flask) cultures with 100 ug/ml amp:
(1) unlabeled,
(2) 70% D2O as only label,
(3) 99% D2O plus labeled glucose and $^{15}NH_4Cl$.

Large scale (0.5 L per 2 L flask) culture:
Minimal medium with 100 ug/ml amp in 99% D2O plus labeled glucose and $^{15}NH_4Cl$.

1. Make the appropriate amounts of the 3 different types of minimal medium (above) and place into correct flasks for autoclaving.

A. Add the following amounts per liter of medium:

Use the appropriate amounts of H2O and/or 99% D2O

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=7.0

C. Autoclave

D. Let medium cool, then add:

$CaCl_2$	1 ml/L
$MgSO_4/7H_2O$	1 ml/L
40% Glucose (possibly 13C and 2H-labeled)	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 dd H_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 dd H_2O. Filter-sterilize.

40% sterilized Glucose is ordered from molecular biology prep lab or can be prepared by large scale sterile filtration.

Ampicillin solutions are prepared and filter sterilized as described above in LB medium section.

Vitamin prep: smash 1 vitamin and mix with 20 ml of H2O. Mix, bath sonicate, and remove insoluble junk by low speed centrifugation. Filter with steriflip.

2. Grow 2-5 ml LB/amp cultures overnight with shaking at 37 °C (NO Kanamycin).
3. Use 1 tube of LB to inoculate a 50 ml culture of unlabeled minimal medium.
4. Grow with shaking at 37 °C to OD_{600}=0.5 (about 5 hours). Only a single 50 ml culture is required.
5. Take 0.5 ml aliquot of culture and inoculate 50 ml of minimal medium which is 70% D2O (no other labels need to be included). Only a single 50 ml culture is required.
6. Grow with shaking at 37 °C to OD_{600}=0.5 (about 16 hours).
7. Take 0.5 ml aliquot and inoculate 50 ml of 99% D_2O minimal medium which also includes ^{15}N-NH_4Cl and sometimes $^{13}C_6$-glucose (which may also be perdeuterated). Only a single 50 ml culture is required.
8. Grow with shaking at 37 °C to OD_{600}=0.5 (about 20 hours).
9. Take 5 ml aliquot and inoculate 500 ml of 99% D_2O minimal medium which also includes ^{15}N-NH_4Cl and sometimes $^{13}C_6$-glucose (which may also be perdeuterated). The same 50 ml culture can be used to inoculate multiple 500 ml cultures.
10. Grow with shaking at 37 °C to OD_{600}=1.0 (about 20 hours using either deuterated or non-deuterated glucose).
11. Induce with 0.2 g/L IPTG and continue shaking at 37 °C for 8 hours to overnight.
12. Harvest cells.

ALTERNATE GROWTH PROCEDURE E:
Labeling with ^{15}N-Cys

Reference: L. Czerski, O. Vinogradova, and C. R. Sanders. NMR-based amide hydrogen-deuterium exchange measurements for complex membrane proteins: development and critical evaluation. J. of Magnetic Resonance 142, 111-119 (2000)

Preparing Minimal Medium for Selective ^{15}N Cys-labeled Liquid Cultures of WH1061

This medium is designed for selective incorporation of ^{15}N-labeled cysteine into DAGK. It can be adapted to labeling using other 15N-amino acids, although problems with loss of labeling due to amino acid metabolism can be problematic for some amino acids.

Preparation is modified from: *Cheng H. et al. Protein expression, selective isotopic labeling . Arch of Biochem and Biophys 316: 619-634 , 1995. See also McIntosh and Dalquist, Q. Rev. Biophysics 23, 1-38 (1990).*

Preparation of this minimal medium is very similar to described above for uniform labeling with a main difference of adding a mixture of unlabeled L-aminoacids.

Per 1 l of minimal media we have to add following amount of amino acids:

Ala	0.50 g	Autoclave	
Arg	0.40 g	Autoclave	
Asp	0.40 g	Filter	dissolve by titrating NaOH
Asn	0.40 g	Filter	
Gln	0.40 g	Filter	dissolve by titrating HCl
Glu	0.65 g	Filter	
Gly	0.55 g	Autoclave	
His	0.15 g	Autoclave	
Ile	0.23 g	Autoclave	
Leu	0.23 g	Autoclave	
Lys	0.42 g	Autoclave	
Met	0.25 g	Autoclave	
Phe	0.13 g	Autoclave	
Pro	0.10 g	Autoclave	
Ser	2.00 g	Autoclave	
Thr	0.23 g	Autoclave	
Tyr	0.17 g	Filter	dissolve by titrating NaOH
Val	0.23 g	Autoclave	
Trp	0.05 g	Filter	
^{15}N-Cys	0.05 g	Filter	

Amino acids marked "autoclave" can be added directly to minimal medium and autoclaved. Amino acids marked "filter" have to be dissolved in ddH_2O and filtered and added later when medium is cooled to room T.

Minimal medium is prepared according to this recipe:

Na_2HPO_4	6 g/l
KH_2PO_4	3 g/l
NaCl	0.5 g/l
NH_4Cl	0.5 g/l
Succinic Acid	0.5 g/l
adjust pH=7.0	

Bring volume to 1l with ddH_20

After autoclaving, the solution is cooled to room temperature and we add :

$CaCl_2$	1 ml/l
$MgSO_4/7H_2O$	1 ml/l
40% Glucose	10 ml/l
Thiamin+ Nicotinic acid	1 ml/l
Ampicillin	100 mg/l
kanamycin (IF APPROPRIATE)	10 mg/l

For amino Acids marked “filter”, prepare solutions as described in the previous section.

When cells are induced another 50 mg/L of ^{15}N-Cys should be added to the culture.

Protocol for Labeling DAGK with U-$^{15}N/^{2}H$ except ($^{13}CH_3$ (δ□) Ile; $^{13}CH_3$,$^{12}CD_3$ Val; $^{13}CH_3$,$^{12}CD_3$ Leu)

Notes:

All temperatures are at 37°C unless stated explicitly

1) "100D": Prepare 500mL of minimal media with all the usual chemicals except CaCl2 in D_20. Use glucose (**deuterated but NOT 13C labelled**)
"100H": Also prepare 500mL minimal media (unlabelled).

2) Precursor A: Incubate a 2.7 mM soln. in 99.9% D2O at pH=10.5. 45°C for 20 hrs.

Precursor B: Incubate a 25 mM soln in 99.9% D2O at pH=12.4, 45°C for 3hrs.

(The pH values are uncorrected. You will have to calculate how much volume to add to the 100mL cultures later to get the correct amounts. Use 40% NaOD to adjust pH and bring the pH back to 7.0 with HCl after incubation at 45°C)

4) Autoclave 5x500mL culture flasks **(empty).** Filter sterilize the minimal media and store at 4°C in an airtight container. Also add the requisite amount of sterile $CaCl_2$ soln.(in D_2O) *after* filter sterilization. Use parafilm to minimize any exchange of D_2O with H_2O (from atmosphere).

5) Day 1: Plate out DAGK from glycerol stocks onto LB+Amp Plates. Incubate overnight.

6) Day 2 (morning): Inoculate 5mL of LB+Amp soln. with a single colony of E.coli and grow shaking for 6-7 hrs
Day 2: (evening): Inoculate 5mL of "100H" media with 50□L of E.coli from LB+Amp culture (100:1) and grow overnight, shaking.

7) Day3: Inoculate 2mL of 70% D_2O minimal media with 20□L of "100H" minimal media overnight culture and grow overnight (100:1) again shaking. The 70% soln. can be made by mixing 1.4mL of "100D" media and 0.6mL of "100H" media.

8) Day 4: Inoculate 2mL of "100D" media with 20□L of 70% overnight culture. Grow shaking overnight.

9) Day 5: In a 500mL sterile culture flask add 100mL of "100D" solution and inoculate with 1mL of previous culture grown with "100D" media. Grow shaking at **25oC**for 24 hrs. At inoculation add

7.5 mgs of Precursor A and
7.5 mgs of Precursor B.

Note: It is not necessary to start growing at 25°C but when growing at 37°C I have noticed that sometimes the cells grow well but express no protein. I have got good results for growth at 25°C but that was only once. If there seems to be a problem with growing at 25°C go back to 37°C.

10) Day 6: The culture OD should be very low. It takes a long time for the E.Coli to get used to the precursors. Transfer the 500mL flask to the shaker at 37°C and grow till the O.D is ~ 1.0. Induce with IPTG to a final concentration of 1mM.

Add along with the IPTG the following:

7.5 mgs of Precursor A and
7.5 mgs of Precursor B.

At this point additional ampicillin can be added (50 mg/L). After induction it is very important that the culture grows for **at least 16 hrs** before the cells can be harvested.

11) For the next 100mL culture use fresh E. Coli. starting all the way from fresh plates. This is very important to ensure the overall sucess of your overexpression.

Purification of specially-I,L,V-labeled DAGK

The purification procedure for DAGK is exactly the same as the standard protocols (below). Extra care must be taken to make sure that the cell lysis proceeds properly and always err on the side of caution. In the final chromatography steps, use DPC-d_{38} instead of regular DPC. Also for a 100mL culture use 1mL of resin and 20 mL of lysis buffer

AVOIDING YodA

YodA is a stress-induced E. coli protein that has a native N-terminal His tag (HHHCHH or something like that) that is sometimes strongly induced when grow E. coli cells on minimal medium. It will co-elute with other His-tagged proteins from Ni(II)-based metal ion affinity chromatography columns. It runs at 28 kDa on SDS-PAGE. It gives rise to a beautiful TROSY/HSQC NMR spectrum, which is shown in the following reference:

Changlin Tian, Murthy D. Karra, Charles D. Ellis, Jaison Jacob, Frank D. Sönnichsen, and Charles R. Sanders. (2005) Membrane protein sample preparation for TROSY NMR Screening. *Methods in Enzymology* **394**, 321-334.

Yoana Dmitrova, then of the Chazin lab, uses the following protocol to avoid YodA expression:

Adding 5uM ZnCl2 (final concentration) to the 1L Minimal media and then before inducing with IPTG, add another 10uM ZnCl2. If your target protein is expressing in very large amounts you can add up to ~20uM ZnCl2. You can add these aliquots from a sterile 50 mM ZnCl2 solution.

Working with Lysophospholipids

If you are working with lysolipid-containing solution, never add base (NaOH, KOH, or ammonium hyroxide). If you do, you will hydrolyze the labile ester linkage during the time after base addition before mixing is complete (the pockets of solution that have locally-high base concentrations will trash lysolipids).

If you think you MUST raise the pH of a lysolipid-containing solution do it with trizma base (use a 1 M stock).

Not only do lysolipids have the base-labile ester linkage, the free hydroxylgroup at the sn-2 positions is ideally placed to serve as a nucleophile...

Also, when working with lysolipids: make just enough for the experiments at hand. Solutions will go bad with time unless they are stored in frozen form at -80 °C.

HARVESTING CELLS

Harvest cells by centrifuging at 4 °C at for 15 minutes on any preparative Beckman or Sorvall centrifuge. Generally spin at 80% 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.**

NOTES ON CENTRIFUGING

Centrifuge bottles should never be more than about 3/4 full unless they have sealed caps. Check that O-rings are in place. Otherwise, liquid will come out during centrifugation. Be sure to clean up after yourself when using centrifuges. They get very smelly if spilled bacteria are not cleaned up.

!! DO NOT use the max RPM for the rotor.

Make sure you determine the correct RPM and RCF (relative centrifugal force, x *g*) for each rotor and centrifuge you are using.

Rotor calculation tools can be found here:
https://www.beckmancoulter.com
Click on Research & Discovery
Go to Tools > Rotor Calculations (right side of webpage)
Under "Rotor calculations" tab, find your rotor in the "Select a Rotor" pull-down menu.
You can then convert RPMs to G-force (RCF) for the rotor you are using.

Preparative Rotors in CSB Megalab (July 2011):
JA-25.50 (25,000 RPM max)
JLA-10.500 (10,000 RPM max)
JLA-16.250 (16,000 RPM max)
JLA-8.1000 (8,000 RPM max)

Ultracentrifuge Rotors in CSB Megalab (July 2011):
Type 45-Ti (for L8-60M ultracentrifuge)
Type 75-Ti (for L8-60M ultracentrifuge)
TLA-120.2 (for Optima TLX benchtop ultracentrifuge)

Keeping Bacteria From Growing in Buffers and Detergent Solutions

Some solutions (including those containing DM and maybe those containing DPC) are excellent media for growing bacteria. Accordingly, you will notice that solutions become cloudy or develop filamentous clumps after sitting for many days at room temperature. This means bugs are growing in the solution and it must be discarded. An easy way to prevent bacteria growth in buffers is to include a low concentration of EDTA (0.2 mM is fine) in the buffer. By scavenging di- and tri-valent metal ions EDTA keeps bacteria from growing. 0.2 mM EDTA is low enough so that it will not interfere with Ni-NTA purification.

PURIFYING MEMBRANE PROTEINS FROM E. COLI CELLS

(WHEN INCLUSION BODIES ARE NOT AN ISSUE)

References: Lau FW, Bowie JU. A method for assessing the stability of a membrane protein.Biochemistry. 1997 36:5884-92. O. Vinogradova, P. Badola, L. Czerski, F. Sonnichsen, and C. R. Sanders. E. Coli diacylglycerol Kinase: A Case Study in the Application of Solution NMR Methods to An Integral Membrane Protein. Biophysical Journal 72, 2688-2701 (1997) Oxenoid, K., Kim, H.-K., Jacob, J., Sonnichsen, 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.

This method is primarily used in the Sanders Lab to purify the membrane protein DAGK, but it can be used for some other proteins as well. PMSF is a protease inhibitor designed to help protect your recombinant protein from protease. Lysozyme degrades the bacterial cell wall. DNase and RNase chew up the RNA and DNA. Mg(II) complexes with certain lipids and also help to break up the cell wall. Membranes are disrupted by osmotic forces in the absence of a cell wall, by sonication, and are ultimately dissolved by detergent.

LYSIS PROCEDURE

1. Take *E. coli* cell pellet 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).
2. Add PMSF (phenylmethylsulfonyl fluoride- a poison!) from a 20 mg/ml stock solution in isopropanol (this can be stored indefinitely in the freezer) to a concentration of 20 mgs per 100 ml of sup (1.1 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
   MgAcetate to 5 mM from a 500 mM stock (stock: 11 g/100 ml)

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

   Seal the container and incubate for ½ hour at room temperature with tumbling (do not mix with stir bar).

4. Tip sonicate at 50% power, 50% duty cycle for 5 minutes (5 sec on, 5 sec off). Place your sample in an ice water bath during sonication.
5. 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 more than 0.5 mM DTT is added, new nickel resin, not regenerated, must be used.*
6. 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 °C 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 wall of keep 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 is 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 Ar!).

EXTRACTION OF PROTEIN FROM E. COLI MEMBRANES

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) to make the lysate 3% by volume Empigen. Mix thoroughly by tumbling in the cold room for ½ hour (do not stir with stir bar). *Never freeze the lysed solution at this point.*
3. Centrifuge extracted lysate at 30,000 x *g* for 20 minutes and discard any "goop" pellet.

PURIFICATION SOLUTIONS

If a reducing agent is required, 0.5 mM dithiothreitol (DTT) should be added to all solutions 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. **Regenerated resin cannot be used in the presence of DTT above 0.5 mM, new resin must be used.**

-Lysis Buffer:
75 mM Tris-HCl (Tris is the buffer component, make this starting with Tris/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 Tris/Trizma solution)

-Buffer A:
40 mM HEPES (HEPES is a zwitterionic buffer)
300 mM NaCl
10 μM BHT
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:
0.2% DPC (**OR** 0.2% decylmaltoside) in 25 mM Na-Phosphate, pH 7.2

-Elution buffer
0.5% DPC (**OR** 0.5% decylmaltoside)
0.25 M imidazole in H_2O (make sure a high grade of imidazole is used, see below)
pH 7.8 (adjust the pH *then* add the DPC or decyl maltoside)

Note: for many studies you have to worry about protein misfolding. In these cases you will use DPC (dodecylphosphocholine, "FOS-Choline-12") in your elution buffer rather than DM. See the section on "Reconstitutive Refolding"

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 DAGK.

ELUTION NOTES

Empigen, maltoside (DM), and dodecylphosphocholine (DPC) are all detergents. All three have critical micellar concentrations (CMC) near 2 mM. DAGK is more stable in DM than in DPC. However, DM cannot be used in reconstitutive refolding to correct misfolded DAGK- only DPC can be used for this.

0.5% Formic acid with 0.5% DPC or DM can be also used for elution of DAGK from the nickel column, although this is generally not preferable (DAGK is not real stable at acidic pH and DAGK/DPC/formic acid solutions cannot be lyophilized because it kills DAGK).

Ni^{2+} (Ni-NTA) 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 in 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 N_2 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. Do not keep the DAGK on resin sitting around at room temperature any longer than you have to; keep on ice and purify immediately following incubation or thawing.
   FROZEN DAGK-on-resin can be safely stored at -80 °C for about 3 months. However, even at that very low temperature, Ni(II)-catalyzed protein oxidation should be a concern and very old DAGK/resin should be suspect as a source of high quality protein.
6. Wash the resin with about 5 X 1 bed volume of ice-cold Emp/A.
7. Turn on chart recorder and start monitoring A280.
8. 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.

9. Rinse column with **12 X 1** bed volume portions of cold rinse buffer.
   This does not mean a single rinse 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 or DM.
10. Elute the protein with elution buffer. Target protein will elute as a sharp band which can be monitored using the chart recorder. Only this band need be collected. If you are using an eluting buffer containing deuterated imidazole, use only the amount actually needed to elute. Remember to tare your collection vial while it is empty so you can measure the volume of protein solution eluted.

11. The protein-containing pool can now be stored **if the elution buffer contained DM**. In this case, you can now freeze the solution in liquid nitrogen you can store at -80 °C and later thaw (for DAGK, loss of activity from freeze-thawing is typically <10%).

    If the elution buffer contained DPC, do NOT freeze the solution. Go straight to reconstitutive refolding or store at 4 °C for up to a few days. (Super DAGK in DPC can be stored for weeks at 4 °C with no loss of activity). When subjected to freeze-thaw DAGK in DPC often loses considerable activity.

DAGK Elution Notes: DAGK has an extinction coefficient at 280 of 1.8 O.D. units per mg/ml. Use elution buffer to zero the spectrophotometer. The real absorbance of the DAGK solution at this point may be 15 O.D. units. However, the spectrophotometer only can accurately measure O.D.s of solution with real absorbencies of 2.5 or less. Thus, normally you will add 50-100 microliters of your solution to 1 ml of elution buffer and measure the absorption of that and then multiply by the dilution factor in order to determine the actual O.D. of the DAGK pool. If solution is cloudy it can normally be clarified by adding formic acid to 0.5 % (5 microliters of acid per ml solution).

PURIFYING DAGK INTO SDS SOLUTION

To purify DAGK in to SDS use the exact same procedure as above, but with the following variations:

(1) after washing the column with Sanders Wash Buffer (step # 8), change the detergent from empigen to DM by rinsing with 2 bed volumes of 0.5% DM in 25 mM sodium phosphate buffer, pH 7.2

(2) change the detergent to SDS by rinsing the column with 8 bed volumes of 0.05% SDS in 25 mM sodium phosphate buffer, pH 7.2 (this is equivalent to the old step # 9 except for the choice of detergent) *The concentration of SDS MUST be 0.05% (0.5 mg/ml), not higher!*

(3) elute the protein using 1% SDS in 250 mM imidazole, pH 7.8 (this is equivalent to the old step # 10 except for the choice of detergent)

REGENERATING USED QIAGEN Ni-NTA RESIN

more detailed protocol is posted here:
https://structbio.vanderbilt.edu/twiki/bin/view/SandersLab/LabProtocols

1. Pour used resin into filter funnel. May connect to vacuum line, but only if necessary.

2. Wash twice with 6M guanidine HCl + 2% formic acid. Stir well after EACH wash, and allow to drain completely between washes.
3. Wash twice with 100mM EDTA + 3% Empigen at neutral or basic pH.
4. Wash twice with water.
5. Incubate resin twice with batches of 200 mM $NiSO_4$ (**hazardous** – must collect all NiSO4 as hazardous waste).
6. Wash 3-4 times with water.
7. Store in 20% ethanol in water, at 4 °C.

BOWIE PROCEDURE FOR PURIFYING DAGK (RARELY USED IN SANDERS LAB)

Reference: Lau FW, Bowie JU. A method for assessing the stability of a membrane protein.
Biochemistry. 1997 36:5884-92.

This procedure is rarely used since the development of reconstitutive refolding. It provides a route to avoiding the use of the harsh detergent empigen.

1. Lyse cells as described for the usual Sanders procedure.
2. To extract, add β-octyl glucoside to 5% (5g/100 mL).
3. Tumble 30 min. (only) at 4°C (**do NOT use a stir bar, instead gently rotate a sealed container**).
   The lysate cannot be frozen at this point!!!
4. Remove insoluble material by spinning 20 min., 4°C, at 15000 RPM in 50 mL tube Beckman rotor.
5. Discard pellet.
6. Equilibrate nickel resin with 4 x 1 bed volumes Buffer A solution. Use 1 mL packed resin per gram wet cells.
7. Add resin to supernatant.
8. Tumble 30 min., 4°C.
9. Spin 15 min. in a tabletop centrifuge.
10. Decant sup.
11. Disperse resin in BOG/A buffer (see below). Pour resin into column. (Alternately, resin can be frozen in a Falcon tube at this point.)
12. Wash resin with Bowie Wash until A280 goes down and stays at a minimum.
13. Rinse resin with 8 X 1 bed volumes of Bowie Rinse to re-equlibrate with decylmaltoside as the detergent.
14. Elute DAGK with Bowie Elute.

After making all detergent-containing buffers, carefully bubble Argon through the solutions for half an hour.
DTT should not be used with regenerated resin; use new resin.

-Buffer A:
40 mM HEPES
300 mM NaCl
10 uM BHT
0.5 mM DTT. Add to buffer A only on day of purification, and only if will not interfere with further experiments.
pH = 7.5

-BOG/A: Buffer A + 1.5% BOG

-Bowie Wash:
Buffer A + 1.5% BOG + 0.04 M imidazole, pH 7.8

-Bowie Rinse: 1% decyl maltoside in 25 mM Na-Phosphate, pH 7.2: on day of use add DTT to 0.5 mM unless you are going to be doing experiments with DAGK (such as SH-modification) that require that no reducing agent be present)... only to volume you will actually use

-Bowie Elute: 1% decyl maltoside + 0.25 M imidazole, pH 7.8 (adjust the pH *then* add the decyl maltoside) (on day of use add DTT to 0.5 mM unless you are going to be doing experiments with DAGK—such as SH-modification or -S-S- formation—that require that no reducing agent be present... only to volume you will actually use): .

B-octyl glucoside and B-decyl maltoside (DM) are both detergents. BOG has a critical micellar concentration (CMC) near 25 mM, while for DM the CMC is 2 mM. DAGK is more stable in DM which is why it is used in the final steps of the purification and to store DAGK.

The DAGK containing pool can now be stored. If you now freeze the DAGK solution in liquid nitrogen you can store at -80 and later thaw with only an about 10% loss of activity. Alternately, you can freeze-dry (lyophilize) the solution, although lyophilizing DM/formic acid solutions of DAGK does not work well- the enzyme denatures. Also, you cannot lyophilize DAGK solutions containing high salt (e.g., 300 mM NaCl or 25 mM MgAcetate).

Freezing and Thawing of DAGK Solutions.

I (CS) have frozen and thawed a variety of micellar **LOW SALT ONLY!** DAGK solutions and have never observed a loss of activity for DAGK in DM. **This not true for DPC solutions, where freeze-thaw should generally be completely avoided.** Even for DM solutions, repetitive freeze-thawing of a single DAGK solution should be generally avoided. Thus, when DAGK is to be stored, it should be generally be stored as a series of aliquots (which can be removed, used, and then discarded one at a time) rather than as a pool which is thawed, a little removed, and the rest re-frozen until the next experiment.

UV-Vis Spectrophotometry

UV-Vis Spectrophotometry is based on the fact that many molecules can absorb light in the UV/Visible range (200-700 nm wavelength). For a given molecule and a given wavelength, the absorption of light by that compound is described by “Beer’s Law”, which is expressed as an equation:

Light absorption at wavelength $\lambda = \varepsilon \cdot L \cdot c$

where ε is the “extinction coefficient” that describes how much absorption there is by the molecule of interest at that wavelength at a standard concentration of that compound (usually either 1 mg/ml or 1 M) and when the light passes through a solution that is “L” in thickness. Since the sample holders for spectrophotometry are usually 1 cm in thickness, L is usually 1 cm. For small molecules values for ε are typically given in molar concentration units. A typical example would be for ATP at 259 nm: 15,400 AU per molar per cm. For proteins, values for ε are often given in mg/ml concentration units (also describe as 0.1% units). A typical example would be for diacylglycerol kinase at 280 nm: 2.1 AU per mg/ml per cm.

“AU” stands for “absorbance units”, which is often also referred to as “OD” (for “optical density”).

Note that most spectrometers get saturated when solution absorbance is >2.5. This means that “in practice, Beer’s law only applies for 1 cm solutions where OD is 2.5 or less”. If a solution has a higher absorbance, it must first be diluted to get it with a range of concentrations where Beer’s law applies.

Note that for a given size of cuvette and a give spectrophotometer there will be a minimum allowed sample volume. We usually use “semi-micro” cuvettes that hold up to 1.5 ml. Using these cuvettes on our old HP spectrophotometer, the minimal sample volume was 0.7 ml. For the new Varian machine, 0.7 ml should be safe, but do not go below that! If the sample beam is not going through solution, but is going through only air or the meniscus, you obvious aren’t going to get good results.

Measuring A_{280} To Determine Protein Concentration

This method works only for purified proteins.

Note that for a given size of cuvette, and a given spectrophotometer, there will be a minimum allowed sample volume. We usually use “semi-micro” cuvettes that hold 1 mL. On our old HP spectrophotometer, the minimal sample volume was 0.7 mL. For the new Varian machine, 0.7 mL should be safe, but do not go below that! If the sample beam is not going through solution, but is going through only air or the meniscus, you obvious aren’t going to get good results.

For purified protein solutions where you have 0.7 mL or more solution, and for which you have protein concentrations of roughly 0.1 mg/mL or higher, the most accurate way to determine protein concentration is to measure A280.

To measure A280 accurately, there are several things to keep in mind:

(1) You must use a quartz cuvette, which has a low background absorbance at 280 nm (unlike regular glass or most plastics).

(2) We use semi-micro cuvettes that have a path length of 1cm and 1 mL. The range of volumes to use in these is 0.7 mL to 1.0 mL.   If you use less than 0.7 ml then you have to worry that the top of the sample may go through the light beam of the spectrophotometer.

(3) It is important to first "zero" the spectrophotometer using a "blank" solution that contains everything in your protein sample except for the protein. Often, this solution will simply be the elution buffer used to elute the protein solution whose A280 you want to measure (so always save a little of this buffer).   Keep in mind that the buffer cannot have a large "background" A280 or you will saturate the spectrophotometer. This is why we use only ultrapure imidazole (cheap imidazole has impurities that absorb strongly at 280 nm). Other compounds that have strong A280 are thiol reducing agents at concentrations higher than 1 mM and any compound that contains an aromatic ring, such as Triton X-100.

When you zero the spectrophotometer using a blank, it is important to use the same cuvette as the one you will use to measure the A280 of the protein solution.

To get an accurate A280, the measured A280 of the solution (after first zeroing the spectrophotometer) should be in the range of 0.1 to 1. Less than this and you are approaching the noise level. More than this you are approaching the limit of "saturation" where Beer's lab no longer applies.   If you need to dilute your protein solution, use the "blank" buffer to do so. (For example, In a 1.5 mL eppendorf tube, mix 25-200 microliters of concentrated protein solution with buffer/detergent to bring the volume up to exactly 1000 microliters. Using a syringe for both solutions will give the most accurate volumes).

If there are visible particles in your solution, the A280 will not be accurate, but will include a component due to light scattering (the amount that is due to light scattering will be proportional to A600, where only light scattering leads to apparent "absorbance"). In this case, centrifuge your sample to remove the particle prior to adding the supernatant to cuvette. For protein samples, aggregated protein can also sometimes (but not always) be disaggregated by adding 5 microliters of formic acid to clarify a 1 ml solution to be subjected to A280 measurement. Of course, this protein in this 1 ml sample should then be discarded afterwards rather than being added back to the parent protein solution.

The extinction coefficient for any protein of known sequence can be calculated based on primary sequence. A good on-line site for this is http://expasy.org/cgi-bin/protparam.

A great website for information on any given protein is UNIPROT.
See: http://www.uniprot.org/

To get an extinction coefficient for your protein, you can just enter your protein's amino acid sequence at this site:

http://web.expasy.org/protparam/

Don't forget to include the sequence of any added tag.

If you grab the sequence from a UNIPROT file, scroll down to the "Sequence" section. There, hit "FASTA". The FASTA format will appear. Copy and paste. FASTA is an easier format to cut and paste into Proteinparam than the default UNIPROT format.

Detergent Concentrations in Membrane Protein Samples

Under Conditions of Chromatography

When a membrane protein is eluted from a chromatography column, it will carry detergent that is associated with it. This means that the total detergent present in the elution pool is:

detergent concentration in the buffer + concentration of detergent bound to the protein

For an integral membrane protein, a rough rule of thumb is that the concentration of bound detergent can be estimated to be equal to twice the protein concentration in weight/volume units.

For example, if you elute KCNE1 from either a IMAC resin or from an SEC column using a 0.1% LMPG (detergent) solution and find that the KCNE1 concentration in the pool is 1 mg/ml, then you can assume that the bound detergent concentration is 2 mg/ml, such that the total detergent concentration is:

0.1% (from buffer) +0.2% (bound to protein) = 0.3%

Detergent Content of Samples Subjected to Centrifugal Ultrafiltration

When you concentrate a detergent-containing solution, keep in mind that the detergent will also be concentrated because micelle size is usually larger than the molecular weight cut-off of the concentrator. While free detergent can indeed escape, for most of the detergent we work with (that have CMC or 2 mM or less) the amount that escapes as free detergent is usually negligible.

Some of our protocols call for repeated rounds of concentrate, then redilute with buffer, and then concentrate again. *It is very important to realize that you should NOT redilute with a detergent-containing buffer.* If you do, you will increase the total amount of detergent in your sample each time you redilute. Usually you will not want to do this.

Purifying Membrane Proteins from Inclusion Bodies for NMR

See:

Changlin Tian, Murthy D. Karra, Charles D. Ellis, Jaison Jacob, Frank D. Sönnichsen, and Charles R. Sanders. (2005) Membrane protein sample preparation for TROSY NMR Screening. *Methods in Enzymology* 394, 321-334.

Page, R.C., Moore, J.D., Nguyen, H.B., Sharma, M., Chase, R., Gao,F.P., Mobley, C.K., Sanders, C.R., Ma, L., Sonnichsen, F.D., Lee,S., Howell,S.C., Opella, S.J., and Cross,T.A. (2006) Comprehensive evaluation of solution nuclear magnetic resonance spectroscopy sample preparation for helical integral membrane proteins. *J. Structural and Functional Genomics* 7, 51-64

Final Stages of Making NMR Samples for HSQC or TROSY from Ni(II)-Metal Ion Chromatography Purified Protein

- Protein needs to be 15N-labeled.
- Purify protein off of Ni(II)-agarose as usual.
- Measure weight of solution and A280.
- Add D2O to sample to a concentration of 10%.
- Add 100 mM pH 7 EDTA stock solution to the protein solution to make it 0.5 mM in EDTA. EDTA will sequester any metal ions which may have eluted with your protein.
- Lower the pH to 6.5 using acetic acid. 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).
- Concentrate your solution using Millipore Amicon Ultra centrifugal concentrator units. Use the appropriate capacity and MWCO, depending on your protein elution volume and protein size.

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.

- Transfer the desired volume (normally 550 microliters, unless Shigemi tube will be used—see below) 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).

Notes on Buffer Composition for Protein NMR Samples

Choice of pH and Buffer

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, imidazole, and phosphate.

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 mercapoetehanol. 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.

The Usage of Shigemi Tubes for NMR. by Murthy Karra/Jaison Jacob

(11/06 Note from CS: I think that it is possible that for most applications involving small sample volumes that the use of standard 3 mm tubes may be preferred over Shigemi tubes(3 mm tubes require 180 microliters of sample). This is especially the case because radiation damping in cryo-probes is much less of a problem with 3 mm samples than for 5 mm samples (Shigemi or not). Note that if 3 mm tubes are used that it is important to re-calibrate 90 degree pulse widths, which will be significantly shorter than for 5 mm samples).

This is an attempt to clarify the advantages of using a Shigemi tube instead of regular NMR tubes in multi-dimensional NMR spectroscopy. It has long been recognized that in regular NMR tubes, since the sample length is usually longer than the coil, there is a loss of signal associated with B1 field inhomogenity. This means that when a 90 or 180 degree pulse is applied, sample molecules which are spatially distant (in the z-direction) from the edges of the coil experience rotation angles less than what is nominally expected.

The detected signal which is a summation of the magnetization vectors in the x-y plane is then reduced. An estimate of the B1 inhomogenity can be achieved by performing the *nutation* experiment where the signal is acquired after applying pulses of increasing nominal rotation lengths (eg. □/2, 3□□ /2 etc.). In the ideal case the signal intensity after a □ /2 and a 3□□ /2 should be the same but that is usually never the case. A more advanced method of determining the spatial inhomogenity of the coil can be performed using field-gradient pulses (Jerschow, A and Bodenhausen, G, J. Mag. Res., 137, 108, 1999). In this

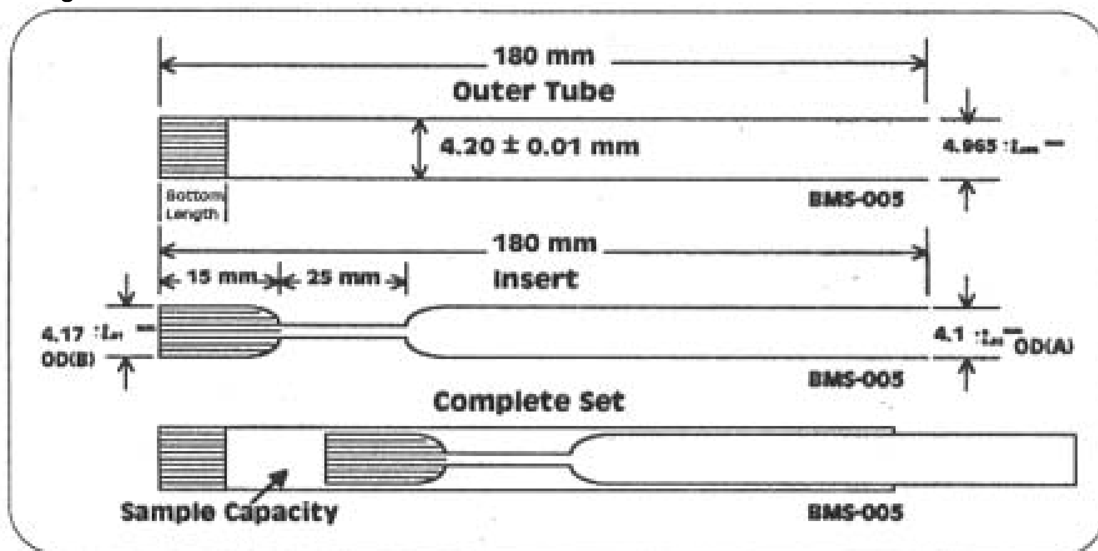
paper, the authors conclude that the usage of susceptibility matched plugs could greatly improve the performance of NMR experiments both with respect to S/N and water-suppression techniques.

Susceptibility matched plugs restrict the sample volume to the “homogenous” region of the coil and hence the volume of the sample is reduced. If the concentration of the solute (protein) can be *safely increased*, this leads to an increase in S/N for the same total amount of solute.

In Bruker 5mm probes the recommended solvent length is 36mm which corresponds to a sample volume of 500 microliters (we typically use 600 microliters). The usage of a Shigemi tube with susceptibility matched plugs (for D_2O) can reduce this volume to 300 microL (or even 200 microL !!!). In the case of expensive samples ($^{15}N/^{13}C/^{2}H$ labeled) this translates to a reduction of the sample amount by factor or 1.7 to 2.5 (assuming that concentration is kept a constant).

In the case of poorly expressed proteins (many membrane proteins) this becomes a very important factor.

The price paid for the increase in S/N is increased cost of the tube (~80$/tube) and slightly more complicated experimental preparation. A schematic diagram of a Shigemi tube is given below



I have personally used ***Shigemi tube BMS-3*** with a sample volume of 300□□L. After the sample is placed into the outer tube (using a NMR pipet), the insert is pushed in all the way (slowly) till it rests on the liquid surface. There is always the likelihood of bubble formation and this can be taken care of as follows (also talk to Kirill, Hak-Jun or Murthy):

The best way to deal with the bubble is to give a quick, light but strong tap on the plunger. Just make sure you have only one bubble close to surface i.e. touching the plunger and give a brisk tap. For us, it works all the time, and that is what shigemi people suggested during last ENC.
-anwer mujeeb
UCSF NMR group
San Franscisco.

After the sample is satisfactory, use teflon tape to hold the outer tube and insert at the top. The tape is also useful in preventing excessive evaporation. Shimming of Shigemi tubes should not be much more difficult but a careful watch on the line-width of a standard peak (in our case imidazole) must be kept. If there are problems in shimming, let some one know (and save shim files when satisfactory).

Shegemi tubes should be used only for expensive samples (13C and/or 2H labeled, for example) or in cases where the amount of available protein is limited.

Jaison's additions regarding use of Shigemis
I have found that in a cryoprobe, the sample outside the coil is a major problem. However radiation damping masks this problem and is noticed rarely. One major problem is expansion of dissolved gases leading to the formation of air bubbles over the course of an experiment. I have found the following two ways to be helpful:

1) Preheat the sample in the Shigemi tube over night. This obviously will not work with sensitive samples. I recall when dealing with protease we just had time to get the sample in the tube.

2) Construct a simple device (I will be glad to help your lab with this, like I have told Murthy before) to degas the sample under low pressure. Samples degassed this way have remained bubble free for over 1 month of data acquisition (over a three month period) at 37 C in my hands.

I also recall reading recently someone using shrink-wrap tubes to make the tubes airtight. Have not tried it on a serious sample yet.

Cleaning NMR Tubes

Remove Sample From NMR Tube.

Rinse tube with water if sample was aqueous.
If sample was not aqueous rinse tube with organic solvent (ethanol or chloroform). If chloroform was used, always follow with a rinse with ethanol and finally with water. *Always do last rinse with water before adding aqua regia: certain organic solvents may reaction violently if mixed with regia.*

Place tubes in beaker. Place beaker on paper towel in fume hood. *Make sure the barrel of the tubes are pointed away from you and that you are wearing eye protection and gloves.*

Add aqua regia to the NMR tubes with a pasteur pipette (fill tubes). Rinse pipette with water before discarding.

Let NMR tubes sit overnight with aqua regia.

Fill a beaker about 1/2 full with water. Empty the aqua regia from the NMR tubes into the water in the beaker. Discard the acidic solution by emptying beaker into the acids waste container found under the sink. (Open and add in the fume hood. Make sure you are using eye protection).

Rinse NMR tubes 5X with H_2O, 2X with ethanol and 1 X (again) with water.

Shake as much water from tubes as possible. Then turn upside down and allow additional draining on a clean paper towel. Shake again.

Allow tubes to dry at room temperature. Store with a cap on tube to keep dust out.

About Aqua Regia

Aqua regia is a 4:1 mixture of conc. HCl and nitric acid. This should be mixed in the fume hood in a bottle which is sitting on a couple of layers of paper towels. After mixing, allow this mixture to sit for a few days in the fume hood with the bottle cap loose: let sit like this until bubbling has stopped and solution has a deep peach/amber color. The cap can then be tighted and the mixture can be stored in the acids cabinet. **Always wear gloves and eye protection when working with this and never look down the barrel of an NMR tube containing it.**

Preparing Protein Saturated Gels for NMR Alignment
(CS updated 6/02):

Make a 35% acrylamide stock solution which includes bis-acrylamide at a 1:50 bis:mono ratio in buffer:

1.75 grams of acrylamide (71 g/mol)
76 mgs of bis-acrylamide (154 g/mol)

dilute the above solid to 5 ml with buffer (add 3.155 ml or 4X789 microliters):
250 mM imidazole, pH 6.5
Do not use detergent at his point.

This solution should be prepared fresh for each set of gels.
Remember that acrylamide is a neurotoxin!

Note: the 1:50 Bis:acrylamide ratio gives a nice "plastic" gel. 1:20 gels are brittle. 1:80 are REALLY plastic but also swell a lot upon prolonged incubation in solution.

Make a 10% stock solution of ammonium persulfate in water. This can be stored at room temperature for many days.

To make up a 1 ml solution which will polymerize to form a certain % gel:

Pipette out the appropriate amount of 35% stock acrylamide solution (114 microliters if a 4% gel is desired).

Add (1000 – 16 - #of microliters of stock 35% used) microliters of the same buffer as used above.

Add 8.3 microliters of 10% ammonium persulfate.

Start polymerization by adding 7.2 microliters of TEMED.

Mix thoroughly.

Use a positive displacement measuring device (e.g. a syringe) to measure out the gelling mixture into the casting mold.

I take 300 microliters of the gelling mixture and add it to the barrel of a 0.5 cm i.d. plastic syringe (with its plunger serving as the bottom of the cylinder). The gel polymerizes pretty rapidly, so you only have maybe half a minute transfer the solution to the syringe barrel. Put Parafilm over the top of the tube while the gel sets. Syringe: B-D 1ml Tuberculin Slip Tip #309602 (Becton Dickinson).

Allow the get to set for 24 hours at room temperature.

Then, soak the gel in 5 ml of whatever buffer your protein is going to be in (which normally will include 15% D2O and which may now include detergent. Do this 2X for at least a few hours each (to remove any unreacted materials and/or byproducts).

The gel may now be added and equilibrated with X.S. protein solution (typically 1 gel and 1 ml of solution). This should be for at least 2 days to reach the point of near equilibration. Gentle rocking or rotating of the sample is probably important. Gels tend to swell during this equilibration period. Swelling is greatest with low percentage gels and in gels with low Bis content (1:20 Bis:acrylamide swells only a little, 1:80 swells a lot). Gels appear to become brittle after sitting around for long periods of time. Thus, it is recommended that "fresh" gels be used and transferred immediately to NMR tubes.

Submitting Proteins for Identification by Tandem Mass Spectroscopy

(1) Only one band on each gel should be the target for mass spec analysis. If you have multiple samples on one gel, the gel should be sliced (in the same direction as the lanes) before drying so that there is only 1 band.

(2) Gels should be not be dried before submission.

(3) Provide a hard copy photograph of the gel, with the bands targeted for analysis labeled. (Keep a copy for your own records)

(4) Label the gel with an identifier: SAND_yourinitials_Date_A (or B, or C, or D if you are submitting multiple samples).

(5) Provide both a hardcopy and a file with a document that provides information about this sample in terms of what you think the protein is, what its possible sequence is (including any tags), what organism the protein is from, and any other information. Indicate which part of the sequence is the native sequence and which parts have been added (His tag, etc.). If part of the native sequence has been deliberately removed, indicate that also. If you can provide the Genbank identifier number for the protein it may be helpful. Put on the paper information which links it to a page in your lab notebook.

H→D Back-Exchange of Perdeuterated True Wild Type DAGK
(Kirill Oxenoid, With Minor Updates By Chuck Ellis and Hak Jun Kim)

This protocol is also used to generate mixed subunit forms of DAGK.
Reference: Oxenoid, K., Kim, H.-K., Jacob, J., Sonnichsen, 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.

0.5 mM DTT is used for the empigen steps of purification, but we switch to 0.1 mM for the final steps of equilibration and elution.

(1) After washing the column with empigen-containing Sanders Wash Buffer, change the detergent from empigen to DM by rinsing with 2 bed volumes of 0.5% DM in 25 mM sodium phosphate buffer, pH 7.2

(2) Change the detergent to SDS by rinsing the column with 8 bed volumes of 0.05% SDS in 25 mM sodium phosphate buffer, pH 7.2 (this is equivalent to the old step # 9 except for the choice of detergent) *The concentration of SDS MUST be 0.05% (0.5 mg/ml), not higher!*

(3) Elute the protein using 1% SDS in 250 mM imidazole, pH 7.8

(4) If different mutant forms of DAGK are to be mixed, this is the point at which the appropriate ratios of mutants in SDS are mixed

(5) To the DAGK/SDS/imidazole mixture, add DPC/POPC stock to make DAGK:POPC=1:120.

(5) Dialysis buffer is 3.5L of 10mM imidazole, plus 0.5 mM EDTA, plus 100mg DTT (to 0.2 mM), pH 6.5. Use Spectra/Por 2.1 (NOT 1 or 2!) tubes (0.32-0.45 ml/cm) and add protein so that the tube is half full (not half empty:-). NOTE, DEPENDING ON WHAT THE RELATED EXPERIMENT THE DAGK IS FOR, MORE OR LESS DTT MAY BE APPROPRIATE.

(6) Dialyze for 3 days and change dialysis buffer at the beginning and end of each day. Last buffer has no DTT (sometimes, depends on the specific goals for the sample). Dialysis is carried out one cycle past the point out which the discarded buffer exhibits no foaming due to the presence of significant amounts of detergent.

(7) Break vesicles with DPC. Add DPC to the point where the solution clarifies from a 0.5-5% DPC stock.

(8) Add 1 ml resin for every 2.5 mgs of DAGK and tumble for 30 min at 4C; load resin into column; rinse at least 12 X 1 column volumes of 0.5% DPC (to get rid of all POPC) and elute with 0.5% DPC, 250mM imidazole, pH 7.8. Again, depending on the final experiments to be conducted, you may or may not want to include 0.1 mM DTT at this stage.

(9) If you will run NMR, add D2O and EDTA to 10% and 1mM, respectively. Adjust pH to 6.5 using d-Acetic Acid and d-ammonium hydroxide.

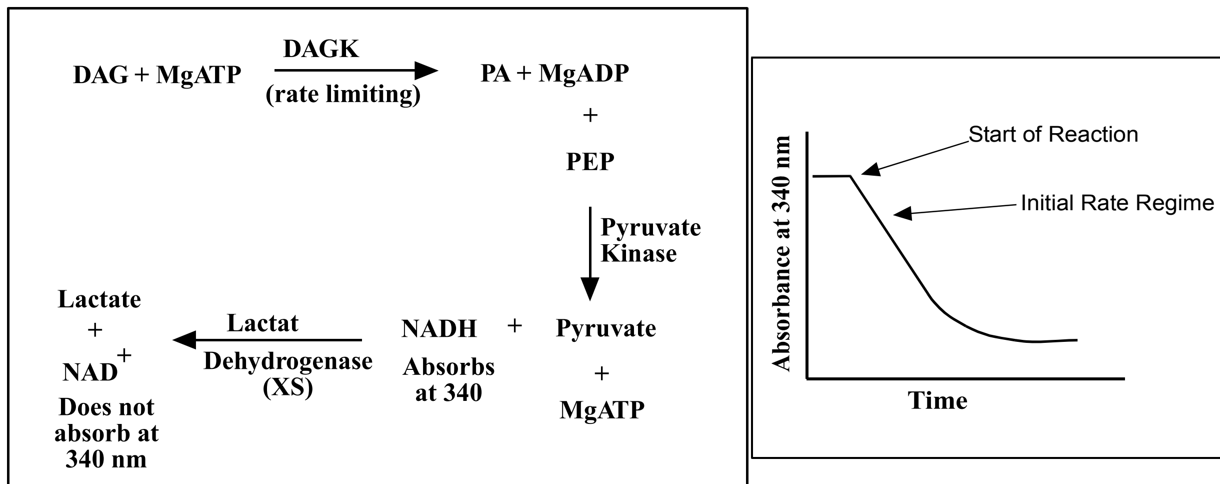
(10) Concentrate your solution using either a 2 ml capacity Centricon YM-50 centrifugal concentrator or a ca. 15 ml capacity Centricon PLUS-20 PL-10 (10 kDa MWCO) centrifugal

concentrator (depending on the volume of solution you want to concentrate. Do NOT use Centricon PLUS-20 Biomax-100 (100 kDa MWCO) cartridge.

DAGK ASSAYS

References: *Czerski, L. and C. R. Sanders. Functionality of a membrane protein in bicelles. Analytical Biochemistry 284, 327-333 (2000). P. Badola and C. R. Sanders. E. Coli Diacylglycerol Kinase is and Evolutionarily-Optimized Membrane Enzyme and Catalyzes Direct Phosphoryl Transfer. J. Biological Chemistry 272, 24176-24182 (1997).*

Our DAGK assays are based on a standard "coupling" system for kinases and provides a convenient alternative to the radioisotopic assay (Walsh and Bell, *Meth. Enzym.* 209, 153-163, 1992). All kinases produce ADP. It is possible to follow ADP production by including several compounds and two enzymes in the assay mixture. When ADP is formed, pyruvate kinase phosphorylates it using phosphoenolpyruvate (PEP) as the phosphate donor. As a result PEP gets converted into pyruvate which then undergoes a reaction with NADH which is catalyzed by the second enzyme, lactic dehydrogenase to produce NAD^+ and lactate. Thus, for every molecule of ADP produced by the kinase of interest, 1 molecule of NADH gets converted into NAD^+. Since NADH strongly absorbs light at 340 nm and NAD^+ does not, it is possible to follow ADP production by the kinase of interest by simply measuring the decrease in absorption of light at 340 nm, provided that the substrates and enzymes for the coupling reaction are present in high enough amounts to insure that when ADP is produced by your enzyme, NADH is "instantly" oxidized to NAD^+ in response (in other words, the initial kinase reaction is rate limiting for the entire reaction series).



Based on this basic reaction coupling scheme we have developed several variations on the basic theme. The **standard mixed micellar assay** involves the use of detergent/lipid mixed micelles to solubilize both DAGK and its DAG substrate and is used for routine assays of purified DAGK and also for more advanced steady-state kinetics. The **vesicular assay** is used for assaying purified DAGK in lipid bilayers (vesicles/liposomes) in the absence of detergent. For this assay a water soluble form of DAG is used since more lipophilic forms of DAG often significantly perturb lipid bilayers. For **assaying DAGK under conditions where it is not pure** (e.g., in cell lysate or isolated cell membranes) we have developed an assay system which allows the DAGK activity to be measured even if there are other molecules present which can hydrolyze ATP and/or oxidize NADH. These assays can all be run using either a spectrophotometer or using a microplate reader.

DAGK MIXED MICELLAR ASSAY

The final reaction volume prior to starting the reaction by adding DAGK will be 1.02 ml and the final reaction mixture will contain:

75 mM PIPES, pH 6.9
50 mM LiCl
0.1 mM EGTA
0.1 mM EDTA
0.2 mM DTT
21 mM DM (1% w/v) (19 mM of which is micellar)
0.66 mM CL (3 mol%)
0.95 mM DHG (4.5 mol%)
20 units each of LDH and PK (we found the reaction rate is independent of LDH and PK conc. at > 10 units each)
1 mM PEP
3 mM ATP
0.25 mM NADH
15 mM Mg^{2+}

To construct this final solution, many steps are involved.

MAKING ASSAY MIX

1. First, make the basic assay buffer. Do not bring it up to final volume yet.
   Assay Buffer:
   75 mM PIPES (1.5 Na form... 335 g/mol) 2.5 g/100 ml)
   50 mM LiCl (42 g/mol): 0.21 g/100 ml
   0.1 mM EGTA (disodium form is 380 g/mol): 3.8 mgs/100 ml
   0.1 mM EDTA (disodium form is 372 g/mol): 3.7 mgs/100 ml
   adjust pH to 6.9 using 2 N LiOH (pH 6.9 was chosen instead of 6.6 because the coupling enzymes (LDH/PK) have higher pH_{opt} than DAGK)
2. Continue adding ingredients to Assay Buffer to make Assay **Mix** . . .
3. For every 100 ml of solution add 1 g of DM (1% DM).
4. For every 100 ml of solution add 0.13 g of cardiolipin (3 mol% CL). It is very difficult to get the CL to dissolve. Turn on the heater in the sonicator and alternate between stirring and sonicating. (Heating moderately with sonication will get it to dissolve). The solution will get completely clear.
5. Cool this solution to room temperature in the fridge or freezer.
6. Dump out the hot water in the sonicator, turn off the heater and refill with RT water.
7. Add 29 mg DHG (288 g/mol) per 100 ml solution. Dissolve by repetitive cycles of stirring with a magnetic stir bar and mild bath sonication at room temperature.
8. Add
   3 mM ATP (disodium, MW 551: 0.165 g/100 ml)
   15 mM MgAcetate (MW 214: 0.32 g/100 ml)
   1 mM Phosphoenolpyruvate (sodium salt, MW 190, 19 mg/100 ml)
   0.2 mM DTT (a reducing agent, 154 g/mol): 3 mg/100 ml (add fresh)
9. Bring the solution to its final volume.
10. Divide into aliquots for ease of use.
11. Freeze in liquid N_2.

12. Store at -80°C.

MAKING NADH SOLUTION

1. Add 1 mL of Assay Mix to a pre-weighed vial containing 2 mg NADH. This results in 2.5 mM NADH. Keep this solution on ice.
2. Leftover NADH solution may be frozen in liquid N2, stored at -80°C, and reused **once** more. Do not reuse more than once. Jim Bowie's folks have reported that older NADH solutions actually inhibit DAGK.

PERFORMING DAGK ASSAYS

1. Add to a clean, dry cuvette:
   900 uL Assay Mix (use syringe)
   100 uL NADH solution (use syringe)
   20 uL PK/LDH enzymes (use Pipetman)
2. Cover the cuvette with a plastic cuvette cover, or a clean section of Parafilm.
3. Invert 5 times to mix.
4. Incubate cuvette in spectrophotometer which should be at 30°C, for a minute.
5. Remove cuvette and use a syringe to add DAGK to it.
6. Replace cuvette in spec and observe reaction at 340 nm. If all is well, ΔA_{340} vs time should be linear (sometimes a lag phase which may be related to refolding is observed: *any time you see a significant lag phase, you should make a note of this in your notebook: this may be a sign that the protein is partially unfolded at the start of the assay and that you are watching it refold*).
7. Record the slope and the volume of DAGK added to the cuvette. If the slope is higher than 0.01 AU/sec then it is too fast to be reliably measured. Use a lower volume or dilute the stock DAGK solution.
8. The specific rate in units/mg for a 1 ml, 1 cm cuvette reaction can be calculated:

$$\frac{\text{change in } A_{340}}{\text{sec}} \cdot 60\ \text{sec/min} \cdot [6110\ \text{AU/cm-M}]^{-1} \cdot 10^{6}\ \text{microM/M} \cdot 0.00102\ \text{L} \cdot [\text{quantity of stock DAGK}]^{-1}$$

$= \Delta A_{340}/\text{sec} \cdot 10{,}000 \cdot [\text{quantity of stock DAGK in mgs}]^{-1}$

= DAGK activity (micromoles ADP produced/[min·quantity of DAGK (vol. or mgs)] = units/mg

To put it simply, multiply the slope you obtained by 10,000. Then divide by the number of microliters of DAGK added, and then by the concentration of DAGK (in mg/mL).

WHAT IS THE ACTIVITY OF "FULLY ACTIVE" DAGK IN THE MIXED MICELLAR ASSAY?

One of the problems with the Sanders lab DM/CL/DHG mixed micellar assay is that we sometimes seem to get different activities (in U/mg) for a single DAGK sample from assay batch to assay batch. For C46, WT, or Cysless DAGK the range observed for "fully active"

DAGK is from about 50 to 120 U/mg. We are not certain what the source of this variation is although there are two likely sources: (1) either isomerization of DHG from the correct 1,2-diacyl isomer to the 1,3-diacyl isomer with time or (2) failure to completely solubilize the DHG in a given assay batch mix.

The recommended way of handling this problem is to keep frozen a series of aliquots of C46, WT, or Cysless DAGK (robust forms which can generally be assumed to be "fully active" following standard purifications). When a new assay batch is prepared, all vials containing it should be dated. At the same time, one of the standard aliquots of DAGK should be thawed and the activity of DAGK in the new mix should be recorded. The observed activity can be viewed as the "100%" activity for that assay batch and all subsequent measurements should be normalized with respect to this. *Thus, it is important to record the date written on the assay mix you are using and that you are sure someone has recorded the "100%" activity for that batch with a standard.*

EXPRESS MIXED MICELLAR DAGK ASSAY

When you are going to run a lot of assays on a single day, you can run things much faster by following the following procedure:

1. Add 10 mL of standard Assay Mix to a vial containing 2 mg NADH.
2. Add 200 uL PK/LDH enzymes to the vial.
3. Mix well.
4. To perform the assay, add 1 mL of this mix to cuvette and proceed as usual.
5. Express assay solution can be frozen in liquid N2, stored at -80°C, and reused **1 time only**.

ASSAY OF VESICULAR DAGK

Reference: L Czerski, L. and C. R. Sanders. *Analytical Biochemistry* 284, 327-333 (2000)

Goal: To assay DAGK activity in lipid vesicles (following reconstitution) without disrupting the vesicles.
The old vesicular DAGK assay system was based upon the use of dihexanoylglycerol (DHG). Since DHG is not water soluble, it is very difficult to add to pre-formed DAGK/lipid vesicles. In the new assay system, we use a form of diacylglycerol which is more or less water soluble: dibutyrylglycerol (DBG).

1. Make Assay Mix just as described in the micellar assay section, BUT—do not add any detergent or lipid (i.e., leave out the DM, DHG and cardiolipin). This solution is called "Vesicle Assay Mix", or "V.A.M." It can be stored at -- -80°C.
2. Add 10 mL of V.A.M. to a vial containing 2 mg of NADH.
3. Add 20 uL of dibutyrylglycerol (DBG). DBG has a molecular weight of 232, so its concentration in the mix will be ca. 7.7 mM.

DO NOT ADD LDH/PK TO THIS STOCK MIXTURE AT THIS POINT. DBG TENDS TO DENATURE LDH/PK WITH TIME.

4. Bath sonicate this mixture with occasional vortexing for 30 minutes. Be very thorough in dispersing the DBG.
5. Allow to sit at room temperature for 30 minutes.

THIS SOLUTION SHOULD NOT BE KEPT ON ICE.... KEEP AT RT AS COOLING TENDS TO MAKE THE DBG FORM OIL DROPLETS.

6. Add 1 mL of the prepared mix to a cuvette.
7. Add 20 uL PK/LDH.

8. Allow temperature to equilibrate to 30°C and take A340 baseline.
9. Start assay by adding DAGK vesicles and remixing. You expect a DAGK specific activity in the range of 2-30 units per mg. Thus, if you have a 0.3 mg/ml DAGK stock, using about 2-20 microliters per assay should give you a reasonable slope (I suggest using the 25 microliter syringe). *Make sure the vesicle stock solution is well-mixed before removing a sample for assay- there should be no pellet sitting on the bottom of the vial containing the vesicles.*
10. To calculate activity:
    U/mg = (10,000 X ∆OD340/sec) / (microliters DAGK stock X mg/ml of DAGK stock)

ASSAY OF DAGK IN NATIVE MEMBRANES

The following assay was developed for measuring DAGK activity in isolated/washed E. coli membranes (see other protocol for how to prepare them). This assay does not appear to work in cell lysate because there are too many water soluble kinases and other proteins that lead to a high baseline activity.

This assay is based on DAGK the standard detergent-free DBG assay used for measuring DAGK activity in vesicles (see “Assay of Vesicular DAGK” protocol). However, because native membranes are being used it is critical to take into account the possible presence of other activities which can also lead to NADH oxidation and a “false positive” reaction curve. An analogous assay using DM/CL/DHG is also possible, but the membranes will, of course, be dissolved by the detergent in this assay mix.

Here are the two non-DAGK activities which can lead to NADH oxidation:

1) NADH oxidase: this is an activity which leads decreases in A340 that does not require the presence of DAG, LDH/PK, MgATP, or PEP… just NADH. NADH oxidase is a protein found in the cytoplasmic membrane of E. coli.

2) “DAGK-like” activity which requires LDH and PK (and possibly MgATP and PEP), but which does not require DAG.

The steps to the determining of DAGK activity in a native membrane sample.

1. Determine whether NADH Oxidase Activity is a Problem using the assay below. If there is oxidase activity, then it will be necessary to run “Measurement of Apparent DAGK Activity” and “Measurement of DAGK-Like Activity” assays in the presence of 5 mM KCN. (You will need to verify this is method of inhibition does work… see assay details below). If activity remains even after adding KCN, record this activity.

2. Measure the “Apparent DAGK Activity.” using the assay below (use KCN if needed based on results from “1” above. This gives the true DAGK activity plus false DAGK-like activity (measured in “3” below), plus any residual oxidase activity (measured in “1” above)

3. Measure the DAGK-like activity using the assay described below (use KCN if needed based on the results from “1” above”.

4. Calculate true DAGK activity based the results from the three assays.

<u>Measurement of NADH Oxidase Activity</u>

1. Run a standard vesicular DBG assay **WITHOUT ANY PK/LDH**. Start the assay by adding an aliquot of native membranes. There will be no lipid or detergent present except for the native membranes.

   The only reaction which can occur will be the NADH oxidase activity.

If A340 changes little with time, then it may not be necessary to use KCN to inhibit this activity. Just record the slope for use in correcting the final "Apparent DAGK Activity" (below).

If the slope is significant, then you need to test whether KCN will inhibit this activity. Add KCN to your native membranes to a concentration of 5 mM. Run this assay again to verify that KCN inhibits the oxidase activity. Assuming it does, wait 15 minutes and repeat using the same membrane/KCN stock. This is to test whether the oxidase activity stays inhibited or whether KCN gets converted into something else so that inhibition is lost with time. If inhibition is lost with time, then it will be important to run all assays shortly after adding KCN to the native membranes. (A 100 mM KCN stock solution in H2O can be stored at room temperature and used for this). *REMEMBER, CYANIDE IS A POISON: AVOID BREATHING VAPOR, SKIN CONTACT, OR ANY INGESTION- ESPECIALLY DO NOT LET KCN COME INTO CONTACT WITH ACID SINCE THIS WILL RELEASE HCN GAS.*

Measurement of Apparent DAGK Activity (True DAGK activity + DAGK-like Activity + oxidase activity, if present)

1. To the DAGK-containing native membranes, add potassium cyanide (KCN) to 5 mM, if necessary.
2. Run a standard vesicular DBG activity assay (no added detergent or lipid present except for the native membranes). Be sure to add the PK/LDH enzymes directly to the cuvette rather than to the assay mix so you can use the same batch of mix for different membrane assays.

Measurement of "DAGK-Like" Activity

1. To the DAGK-containing native membranes, add potassium cyanide (KCN) to 5 mM, if necessary.
2. Run a vesicular DBG assay (*including* LDH/PK), except that no DBG or DHG is present.

Calculation of True DAGK Activity

1. Calculate "apparent", NADH oxidase and DAGK-like activities in Units per ml of stock.

True DAGK activity =
(Apparent Activity) - (NADH Oxidase activity, if any) - (DAGK-like Activity)

2. We have not found any very reliable method of converting from U/mL to U/mg. There are a couple of ways to estimate the mg/mL concentration of DAGK present in the crude preparation. You could make this estimation based on the quantity of protein that was ultimately purified from a known volume of the crude preparation. Alternately, one could do SDS-PAGE on the native membrane stock solutions followed by coomassie/siliver staining or quantitative Western Blots. One could then compare band intensity with the intensity of standard DAGK bands representing a known quantity of DAGK.

NOTES ON VOLUMETRIC MEASUREMENTS

Pippettemans are a highly precise way of measuring the volumes *provided that the solutions do not contain detergent, protein, or organic solvents.* When accurate volumes are required (as for enzyme assays or A280 determination), syringes should be used for solutions which contain detergent, protein, or organic solvent. When using a syringe with a new solution for the first time, care should be taken to flush out the syringe with the new solution and to purge bubbles before measurements are made. Also, wipe needle with Kimwipe after uptaking solution to remove any droplets clinging to outside of needle.

MICROPLATE ASSAYS

Note: Jie-Pan determined that a 200 microliter sample in a round bottom well which gives an O.D. of 1.0 on the reader will give an O.D. of 1.6 when examined in a 1 cm cuvette in the spectrophotomer.
Note also: plate reader gives a linear response only up to an OD of 2.0. Don't trust anything higher than this.

Program Setup

1. Turn on machine
2. Open SOFTmax Pro Software
3. If there's a red X over the picture of the machine in upper left corner, click on it. From the window that appears, choose:
   Serial Port: COM1
   Reader: THERMOmax
   Filters:
   1: 405
   2: 570
   3: 490
   4: 690
   5: (none)
   6: 340

   (if there's no red X over the picture of the machine go on to the next step)
4. Control→Incubator→ON (choose your temp)
5. Check the Setup-> Kinetic options to make sure wavelength is set to 340 nm, run time is set to 1200 sec, and sampling interval is set to 6 sec.
6. Reduction→choose Kinetic Reduction: Slope
   min OD: 0
   lag time:0
   max OD:2
   end time:(leave as it is)
   √ **absolute values**

Assay

7. Add 5.5 mL DHG assay mix + 110uL PK/LDH to a vial containing 2mg NADH (0.36 mg/ml, this is roughly twice as much NADH as in the cuvette assay).
   Note: if you are doing DBG assay, do not add LDH/PK at this stage. Add it instead to the final reaction mixture in an eppendorf tube.

8. Take 500 microliters of assay mix and add to an eppendorf tube. Then, add 10 microliters of LDH/PK at this point (if doing the DBG assay).
9. Add DAGK stock into cap of vial and "rinse" syringe tip by dipping into the assay mixture. You should add between 2 and 20 microliters. Less than 2 cannot be accurately measured out. When ready, start the DAGK reaction by carefully closing the cap and mixing the vial contents by inverting the tube 10 X.
10. The ideal amount of DAGK to add is enough so that the slope in the final reaction mixture will be roughly in the 40-70 range. If you had a 0.025 mg/ml DAGK solution which is 100 U/mg using the DHG assay, you would add about 4 microliters to 500 microliters to get a slope of about 50 in the 200 microliter well (see below).
11. Transfer 2 X 200uL of each reaction mixture to two microplate wells. By doing two wells, you get two measurements. If they do not give the same DAGK activities it is a sign of trouble. The transfering should be done using either 250 or 500 microliter fixed needle syringe or using a piston displacement pipettor. Do not use a pipetteman. Avoid bubbles in the well.
12. Open drawer and carefully put plate in. Then, click on "Read" button to start run.
13. You should assume that the first part of the run (0-300 seconds) is a time during which the temperature is equilibrating. The real data starts after this.You can monitor progress in individual wells by clicking on the well. You can also use the "reduction" menu to change the time range of the points actually displayed.
14. Wait for assay to finish to your satisfaction (you want at least 300 seconds of linear slope, with the beginning being sometime after 300 seconds.).
15. Save your data to a file.

Data Analysis

10. Double click on the reaction well of interest. Determine which range of times represent a linear slope (constant reaction rate). In "reduction" menu type in that range of time (lag time and end time). Make sure "absolute value" has been selected and that "slope" has been chosen under kinetic reduction. Click OK.

11. Record slope given. Slopes have uncertainties of about plus/minus 2. Thus, slopes less than 20 have a large relative uncertainty. Also, slopes greater than 300 can not be trusted or slopes measured too near to the point where all of the NADH was consumed. The OD should be less than 2.0 for the initial and final points.

12. To calculate U/mg , multiply your slope by the "Plate Conversion Factor" (see below). Then divide by the number of microliters added to the original 0.5 ml of reaction mixture. Then multiply by 2.5 (since the 200 microliters actually in any plate is 2/5 of the original 500 reaction). Then, divide by the mg/ml in the DAGK stock solution used.

13. "Plate conversion factor": This number is to convert from slope on the microplate reader to slope on the spectrophotometer. This still needs to be very accurately determined. A value of 0.057 has been determined and independently confirmed.

RECONSTITUTIVE REFOLDING

Reference: B. M. Gorzelle, J. K. Nagy, K. Oxenoid, W. L. Lonzer, D. S. Cafiso, and C. R. Sanders. (1999) Reconstitutive refolding of diacylglycerol kinase, an integral membrane protein. Biochemistry 38, 16373-16382.

MATERIALS

- Dodecylphosphocholine (DPC) from Anatrace ("Fos-Choline-12") or Avanti Lipids.
- Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) from from Genzyme, Anatrace, or Avanti Lipids.
- Spectra-por **1.1** MWCO 8000 dialysis tubing.
  As of 2010, it appears that Spectra-Por 1.1 tubing is no longer called "1.1", but is still sold by Spectra-Por as "Biotech Dialysis Tubing. Regenerated Cellulose. 8000 MWCO". Cat. no. 129015 (10 mm flat width) or 129020 (16 mm flat width).

3/2014 Note: According to Kate Mittendorf, the following tubing is the closest thing current sold to the now defunct tubings described above. Yes, this really does matter.

Spectra/Por Biotech Dialysis Membrane
Biotech RC Tubing
MWCO: 20 kD
Nom. Flat Width: 16.00 mm
Diameter: 10.00 mm
Vol/Lg: 0.79 ml/cm
Part Number: 133342

PREPARATION OF DAGK

The last two steps of the standard DAGK purification protocol (equilibration with DM and elution in DM) are now replaced with the following two steps:

(1) equilibrate column with 2 X 1 column volumes of 0.5% DPC in water.
(2) elute DAGK with 0.5% DPC solution plus 0.25 M imidazole, pH 7.8.

0.2 mM DTT can also be included as a reducing agent, if necessary in the above steps, IF you are not using regenerated resin

Some DAGK mutants can be frozen in liquid N_2 and thawed in DPC solutions with no loss of activity being observed. However, cases have documented where freeze thaw of DAGK in DPC *does* seem to result in a loss of activity. Thus reconstitution of DAGK should be undertaken *immediately* following DPC-based purification with no freeze/thaw of the purified DAGK solution. For long term storage of DAGK, it is better to just leave the enzyme on Ni-agarose resin until it is actually needed.

PREPARATION OF DPC-POPC STOCK SOLUTION

1. Into a Falcon tube, add: buffer (50mM HEPES, 300mM NaCl, 1mM EDTA, pH 8.0)
   POPC to 50mM
   DPC to 200mM
2. Repeatedly freeze tube in liquid nitrogen and then thaw while bath sonicating. Do this until solution is clear, between 10-20 cycles.

3. Store at -80°C.

RECONSTITUTION

1. Prepare dialysis buffer at 1X concentration in a large beaker. Use a ~1000 volume excess of buffer over your total sample volume. Add DTT fresh to this solution to a concentration of 0.2 mM (30 mgs/liter).
   Dialysis Buffer **100X** stock: 1 M imidazole, 50 mM EDTA, pH 6.5
2. Add enough DPC/POPC stock to bring the DAGK:POPC mol:mol ratio to 1:120. (ca. 170 microliters of stock per mg of DAGK present).
3. Transfer the solution to dialysis tubing and seal the tubing. Double clip both ends or use one clip and one knot per end. Use a weighted clip on the bottom end. Refolding goes best when there is a large tubing surface area to volume ratio so that dialysis goes very fast. Thus, leave a lot of empty space in your tubes (but no bubbles). For example, very good results have been obtained when about 3 mls of solution were placed in about 6 inches of 16 mm tubing.
4. Do a total of at least four batches (3 changes) of buffer at room temperature, with each cycle lasting 18-24 hours. *It has been observed that refolding takes place only very late in the reconstitution procedure (after the appearance of cloudiness) and so it is critical not to stop dialyzing prematurely.* Stir the dialysis solution so there is a gentle flow of solution around the dialysis bags. Periodically (once or twice a day) mix the contents of the bag to disrupt concentration gradients that may have built up within the tubes and to disrupt any film which may have formed on the inner surface of the tubing, which will hinder dialytic flow.
5. DAGK in POPC vesicles can be frozen in liquid N2 and thawed without loss of activity. Store at -80°C.

ASSAYING SOLUTIONS FOLLOWING DIALYSIS

Protocols for the mixed micellar and vesicular assays are found in the Sanders lab protocols. Here we will comment only on how to prepare the DAGK solution from DAGK/POPC vesicles.

For vesicular assay: make sure the vesicles are well-mixed and simply use a syringe to remove a little which is added to a cuvette with a pre-incubated detergent-free DBG assay mixture to initiate the reaction.

For mixed-micellar assay: make sure vesicles are well-mixed and remove 100 microliters and mix with 0.9 ml of 1% DM. Make sure it is well-mixed and remove a little and add it to the standard mixed-micellar assay mixture to initiate the reaction.

To quantitate the DAGK concentration: the 1:10 dilution you just used for the mixed micellar assay can be subjected to a direct A280 measurement, *but only after adding 5 microliters of formic acid to clarify the solution, which will often be slightly cloudy.* Do not use DAGK samples which contain formic acid to do activity assays.

OTHER PROCEDURES

SDS GEL ELECTROPHORESIS

Invitrogen NuPAGE 4-12% Bis-Tris gels with MES running buffer.

You will need:

NuPAGE pre-cast gels from Inivtrogen (4-12% or 10% Bis Tris)
4X LDS sample buffer
1X MES Runnung buffer
Stain (Coomassie Blue)
Destain

Coomassie Blue Stain, 500 mL
1 g Coomassie Blue (Brilliant Blue R-250)
215 mL MQH_2O
35 mL glacial acetic acid
250 mL ethanol (100%)

Destaining solution, 1 L
590 mL MQH_2O
55 mL glacial acetic acid
355 mL ethanol (100%)

Gel-drying solution, 1 L
50 mL glycerol
250 mL ethanol (100%)
700 mL MQH_2O

1. Prepare samples. Add 4X LDS sample buffer to your samples to obtain 20 µL total volume. Try to load 3-4 µg protein per lane if the gel will be stained with Coomassie Blue stain. Silver stain can detect about 20 ng protein.
2. Dilute 20X running buffer stock to 1X. Running buffer can be saved and reused 6-7 times. However, if gel is to be used for silver staining or Western blotting, be sure to use fresh buffer.
3. Assemble gel apparatus. If running only one gel, insert plastic gel blank in

second gel space.

4. Cut open pre-cast gel bag. Remove white tape and carefully slide comb out.
5. Rinse wells with water or running buffer. Fill them again with buffer and put them into gel apparatus.
6. Fill inner buffer chamber with buffer so that tops of wells are submerged.
7. Load samples using gel-loading pipet tips.
8. Fill outer buffer chamber with buffer. It is important when running NuPAGE gels to fill the outer chamber almost to the top because they generate lots of heat.
9. Run gel(s) at 200V for 30 min (lower voltages lead to better resolution!).
10. Turn off power and remove gels from apparatus.
11. Break plastic plates apart by inserting gel knife between them.
12. Use gel knife to cut “foot” off of gel.
13. Unless gel will be used for Western blot, place it in Coomassie Blue stain for 1 hour to overnight.

1. Pour stain back into its container. This solution can be reused.
2. Add destaining solution to gel. Stain will be removed more quickly if a KimWipe or piece of paper towel is placed in the dish along with the destaining solution. Change destaining solution as often as you like. When gel is sufficiently destained, discard destain solution. Keep gel in MilliQ water until you are ready to dry it. If gel gets overly destained, it can be re-stained.
3. To dry gel, use Novex drying stand.
4. Soak your gel and two cellophane sheets in Gel-drying solution for a few minutes.
5. Place the gel between two cellophane sheets. Avoid getting any bubbles in the cellulose sandwich, as they may cause the gel to crack during drying.
6. Clamp drying stand closed. Allow to stand on bench top 24-48 hours.

SILVER STAINING

Use Invitrogen’s NuPage SilverXpress Silver Staining Kit.
Follow directions in materials enclosed with the kit—but here are a few tips:

This kit can detect 0.8-1 ng protein (50 is ideal).
Use clean containers that are designated specifically for silver staining.
Set shaker to about 60 RPM.
Use MilliQ water, and make all solutions fresh on the day of staining. If you want a really nice-looking gel, use water fresh from the MilliQ purifier, not water that has been sitting in the carboy.
Silver staining can be done to a gel which has previously been Coomassie stained; in fact, it may give better results. If gel has been Coomassie stained, omit the fixing step from the solver stain protocol.
Don’t touch the gel with your hands or with metal objects.
If you can’t finish the whole staining process at once, the gel can be left in sensitizing solution overnight. Don’t skip or shorten any steps. Really.

Summary of procedure for NuPAGE Bis-Tris gels:

1. Fixing. 10 minutes. 90 mL water + 100 mL methanol + 20 mL acetic acid
2. Sensitizing. 2 X 30 minutes. 105 mL water + 100 mL methanol + 5 mL sensitizer (enough for both washes)
3. Wash. 2 X 10 minutes. 400 mL water (enough for both washes)
4. Staining. 15 minutes. 90 mL water + 5 mL Stainer A + 5 mL Stainer B

5. Wash. 2 X 5 minutes. 400 mL water (enough for both washes)
6. Developing. 1-15 minutes, until you like how it looks. 95 mL water + 5 mL developer
7. Stopping. 10 minutes. 5 mL stopper, add directly to developing solution
8. Wash. 3 X 10 minutes. 600 mL water (enough for all three washes)

DETECTING HIS-TAGGED PROTEIN EXPRESSION IN E. COLI CELLS

Method A.

Preparation of Samples for Western Blot Analysis of His-Tagged Protein Expression

(This approach will completely solubilize your cells pellets so that you can analyze expression of the protein in whole cell lysates. It will solubilize the cells better than just SDS sample buffer and samples will run on the gel without any viscosity issues. This method does not heat or boil the samples.)

1. Take 1 mL cell culture samples (pre-induction,harvest) and record OD_{600}.
2. Spin down cells in a microcentrifuge for 2-3 minutes, remove supernatant and store pellets at -80 °C, if not continuing immediately.

3. Prepare Lysis Mix (scale up/down depending on how many samples you have):
   1 mL Lysis Buffer (50 mM Na phosphate pH 7-8, 300 mM NaCl, 0.5 % DM)
   20 µL Lysozyme/DNase/RNase mix (100/10/10 mg/mL)
   5 µL Mg acetate, 500 mM

4. Resuspend cell pellets in Lysis Mix. Use 100 µL Lysis Mix for each OD_{600} unit. For example, if your measured OD_{600} is 1.2, use 120 µL Lysis Mix; if OD_{600} is 0.9, use 90 µL Lysis Mix.
5. Tumble or nutate resuspended samples for ~ 15 minutes at room temperature.
6. Add lauroylsarcosine to 1% (from 10% stock), then tumble another 5-10 minutes.
7. Add 4X LDS sample buffer to 1X. Mix by vortexing. Add DTT if needed.
8. Load 10-15 µL onto 4-12 % Bis Tris NuPAGE gel.
9. Run at ~120V for 1 hour.
10. Stain with Commassie Blue or SimplyBlue Stain, or transfer to nitrocellulose membrane for western blot detection.

Method B.

This is a general Sanders Lab method for preparing samples for LDS-PAGE. SDS may not be enough to completely solubilize your pellets and some samples may be too viscous to load and run on a gel. Heating the samples can help.

Use Invitrogen NuPAGE 4-12% Bis-Tris LDS gels with MES Running Buffer.

1. Grow cells, taking 1mL aliquots of culture just before induction and just before harvesting. Be sure to measure A600 at the time of induction. Immediately freeze aliquots in liquid N_2 and store at -80 °C.
2. Prepare 1 or 2 identical sets of samples to be run on 1 or 2 gels (one for Coomassie stain, one additional one for Western blot if desired). Keep in mind that many of our membrane proteins are not oversexpressed at a high level and are very difficult ot distinguish from all other proteins in a whole cell lysate sample. To confirm expression, Western Blot is usually required.
3. To prepare gel samples, put 300 µL cells in Eppendorf tube (adjust amount accordingly if the A600 of the culture at induction was other than 0.8-1.2).
4. Spin 2-3 min in a microcentrifuge.
5. Discard supernatant.
6. Resuspend pellet in 40 µL 1% SDS in lysis buffer.
7. Add 40 µL 4X LDS loading buffer.
8. Add 20 µL of freshly prepared 200 mM DTT (31 mg/ml)
9. Bath sonicate 1 min.
10. Divide sample into two aliquots.
11. Incubate 1 aliquot for 30 min at RT. Incubate the other aliquots for 2 minutes in boiling
 water. Both will be electrophoresed. Some proteins show up on gels only after boiling.
 Others show up only if they are not boiled.
12. Vortex briefly.
13. Spin down briefly.
14. Load 5-20 microliters of sups onto gel. Don't worry of sample is a little "stringy".
15. Use SeeBlue Plus2 molecular weight marker from Invitrogen. Load 5 µL of marker.
16. Run gel at 200V for 30 min.
17. Coomassie stain one of the gels. The other (optional) gel is used for Western blotting.

Native Gel Electrophoresis of Membrane Proteins

unpublished method developed by Chuck Sanders and Dehui Mi

Use at your own risk. We don't claim this is a robust method.

1. Assemble Invitrogen XCell *SureLock* MiniCell gel apparatus. Insert empty gel cassette into the apparatus.

2. Pour the Separating Gel Mixture (For both separating and stacking gel, the mass ratio of acrylamide to N'-N'-methylenebisacrylamide is 36.5 : 1, and their molar ratio is 79:1. The stock Acryl / Bis solution is made by dissolving 29.2 g of acrylamide and 0.8 g N'-N'-methylenebisacrylamide in 100 ml of water. The detailed recipes for both gel mixtures are shown in the attached table.) into the empty cassette up to the third raised bar/ridge from the bottom of the cassette. (This is 1 cm below the bottom of the wells if you place a comb completely into the cassette and mark the bottom of the wells.) Immediately and very carefully add a layer (600 μl) of water on top of the separating gel. Allow the gel to polymerize for 45 minutes.

3. Pour out the overlay water with the help of a piece of Kimwipe. Place a comb on top of the gel cassette and tilt it so that the teeth are at a slight (~ 10°) angel. This will prevent air from being trapped under the comb teeth while the Stacking Gel Mixture are poured. Pour the Stacking Gel Mixture (4 % stacking gel made with the stock solution of Acyl : Bis = 30 % : 0.8 %) down the cassette nearest the upturned side of the comb. Pour until all the teeth have been covered by solution. Capillary action of the gel solution will help fill up the space between the teeth. The comb is properly seated when the T portion of the comb rests on top of the cassette. The above method works well for combs with finer teeth (e.g. the 17-well-comb). For combs with wider teeth (e.g. the 12-well-comb), however, it is better not to tilt the comb at an angle. Just let the comb sit straight on top of the cassette with the T portion of comb is still 3 mm above the top of the cassette. Pour the Stacking Gel Mixture down the cassette from either side of the comb until the solution has covered the teeth at half way of their height. Then gently push the comb straight down to seat it properly on top of the cassette.

4. Prepare samples with the desired concentration so that there will be at least 2 μg of protein per well. Mix samples and the Native Sample Buffer in 1:1 ratio. Protein solutions should be kept on ice.

5. When the gel is ready (finished polymerization, which takes about 30 minutes), take it out from the apparatus and remove the comb. Rinse the wells several times with either distilled water or the Native Running Buffer. Peel off the tape on the bottom of the gel cassette to expose the foot of the gel. Put the gel cassette back into the apparatus and take it to the cold room.

6. Fill the chambers of the apparatus with pre-chilled Native Running Buffer.

7. Load samples into wells. For 12 well gels, the maximum volume per well is 20 μl. For 17 well gels, the maximum volume per well is 15 μl.

8. Place the apparatus in a plastic pan containing water and ice. Pre-chill the water in the cold room the night before. The level of the water in the pan should be just above the bottom of the sample wells.

9. Put on the lid of the apparatus and run the electrophoresis at a constant 70 volts. The current will be 20-25 mA per gel and the run should take about 5-6 hours, which is the time it takes for the tracking dye to migrate to the bottom of the gel cassette. The running buffer can be reused for no more than 5 times. As it is reused, the run time becomes longer. For example, the first run with freshly made running buffer takes 5 hours 10 min, the second run will take 5.5 hours, and so on.

10. Turn off the power supply and take out the gel. The Native Running Buffer can be saved and reused up to 5 times.

11. The gel is fixed and then stained with Coomassie staining solution from 2 hours to overnight. Preferably overnight.

Destain the gel using standard procedures

Solutions for Native PAGE of Membrane Proteins

Stock Solutions

1.5 M Tris-HCl pH 8.8, make 100 ml

0.5 M Tris-HCl pH 8.8, make 100 ml

1 M Tris-HCl pH 8.8, make 100 ml

(10 x) DM (β-n-decyl-maltoside from Anatrace) solution, make in 1 ml aliquots as needed
DM 0.01 g, dissolve in 1 ml dd H_2O. Final concentration is 1 %.

Separating Gel Mixture, in 0.375 M Tris-HCl pH 8.8

Final Gel Percentage	6 %
dd H_2O	4.445 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
Stock Acyl / Bis (30 % / 0.8 %)	2 ml
(10 x) DM solution	1 ml
10 % (wt/vol)APS	50 μl
TEMED	5 μl
Total	10 ml

Stacking Gel Mixture, in 0.125 M Tris-HCl pH8.8

Final Gel Percentage	4 %
dd H_2O	1.557 ml
0.5 M Tris-HCl pH 6.8	0.375 ml
Stock Acyl / Bis (30 % / 0.8 %)	0.75 ml
(10 x) DM solution	0.3 ml
10 % (wt/vol) APS	15 μl
TEMED	3 μl
Total	3 ml

Solutions for Native PAGE of Membrane Proteins (continued from previous page)

Native Running Buffer, in 1 liter, pH 8.3

Tris Base	3 g
Glycine	14.4 g
DM	0.1 g

* Mix Tris and Glycine, adjust pH to 8.3. Then add DM.

Native Sample Buffer

1 M Tris-HCl pH 8.8	0.062 ml
1 % (wt/vol) Bromophenol-Blue	0.01 ml
Glycerol	0.2 ml
dd H_2O	0.628 ml
(10 x) DM solution	0.1 ml
Total	1 ml

<u>Western Blot Detection of His-tagged Proteins</u>

(updated by AH 4/2012)

This protocol is based on Protocol 8 in the Qiagen Detection and Assay Handbook. It has been optimized for anti-His (Abgent) as the primary antibody, and goat anti-mouse/AP conjugate (Cell Signaling Technologies, Goat anti Mouse IgG, γ-chain specific) as the secondary antibody. Use flat-bladed tweezers to handle the membrane. A small yellow tip box works well for this procedure. Use ~30 mL of solution and wash for 10 minutes. Gently agitate on platform shaker during each wash/incubation step.

1. Run SDS-PAGE and transfer proteins from gel to nitrocellulose membrane according to the instructions that came with the gel apparatus.
2. **Wash membrane twice in TBS.**
3. **Incubate for 1 hr in blocking buffer.**
   If it is desired to stop for the day, leave blot in blocking buffer overnight at 4°C.
4. **Wash membrane twice in TBSTT.**
5. **Wash membrane in TBS.**
6. **Incubate membrane in blocking buffer with anti-His antibody [add 10 μL stock solution (Abgent anti-His) to 30 mL blocking buffer] and incubate for 1-2 hours.**
7. **Wash membrane twice in TBSTT.**
8. **Wash membrane in TBS.**
9. **Incubate membrane in blocking buffer with anti-mouse/AP conjugate [add 10 μL antibody stock solution (Cell Signaling) to 30 mL blocking buffer] and incubate for 1-2 hours.**
10. **Wash 4 times with TBSTT.**
11. **Stain with AP staining solution until the signal is visible. <u>Do not shake blots during color development</u>.**
12. **Stop the chromogenic reaction by rinsing twice with water.**
13. **Photograph/scan the membrane as soon as possible. It will be OK if covered in water and kept away from light. If dried and exposed to light the bands will fade.**

NuPAGE Transfer Buffer (1L):

Transfer Buffer (20X)	50 mL
Ethanol	100 mL
Deionized H_2O	850 mL

To prepare 20X Transfer Buffer, dissolve the following in ~100 mL deionized H_2O:
Bicine 10.2 g
Bis-Tris 13.1 g
EDTA 0.75 g
!! Mix well and adjust the volume to 125 mL. Adjust pH to 7.2, if needed.

Western Blot Solutions (all amounts given per L):

Tris Buffered Saline (TBS)
(20 mM Tris, 140 mM NaCl, pH 7.5)
2.42 g Tris base
8.18 g NaCl

TBS + Tween/Triton (TBSTT)
(0.1 % Tween 20, 0.2 % Triton X-100 in TBS)
for 1 L
1 mL Tween 20
2 mL Triton X-100

Blocking Buffer (make fresh – you will need 100 mL for one blot)
3% Bovine Serum Albumin (fraction V, Sigma A-7906) in TBS
3 g BSA in 100 mL TBS.

Staining solutions (for step 11.)

The complete **AP Staining solution** is Buffer A with 0.33 mg/mL NBT and 0.166 mg/mL BCIP.

To prepare the final staining solution, add 198 µL 5% NBT stock and
100 µL 5% BCIP stock to 30 mL Buffer A.

See next page for how to make NBT and BCIP….

Individual components:

1. **Buffer A (AP Staining Solution)**:
   100 mM Tris-Cl, (start with Tris base and pH to 9.5 with HCl)
   100 mM NaCl
   5 mM $MgCl_2$

2. **NBT stock** is 5% NBT in 70% DMF
   10 mg NBT (1 tablet, Sigma)
   140 µL DMF
   60 µL MQH_20
   can be stored in aliquots at -20 °C

3. **BCIP stock** is 5% BCIP in 100% DMF
   25 mg BCIP (1 tablet, Sigma)
   500 µL DMF
   can be stored in aliquots at -20 °C

ISOLATING MEMBRANES FROM E. COLI

1. Lyse cells using Sanders method.
2. Tip sonicate 5-10 minutes on ice, 25% duty cycle, 30% power.
3. Spin 20 minutes at 5000 g.
4. Supernatant contains the membranes. Pellet is cell debris.
5. Spin the supernatant in an ultracentrifuge for 2 hours at 60000 g. The pellet will contain the plasma membrane.

NOTES ON FREEZE-THAWING DAGK SOLUTIONS

I (CS) have frozen and thawed a variety of micellar **LOW SALT ONLY!** DAGK solutions and have never observed a loss of activity for DAGK in DM. **This not true for DPC solutions, where freeze-thaw should generally be completely avoided.** Even for DM solutions, repetitive freeze-thawing of a single DAGK solution should be generally avoided. Thus, when DAGK is to be stored, it should be generally be stored as a series of aliquots (which can be removed, used and then discarded one at a time) rather than as a pool which is thawed, a little removed, and the rest re-frozen until the next experiment.

CONCENTRATING MICELLAR DAGK SOLUTIONS

DAGK in detergent micelles can be concentrated using Amicon Centricon centrifuge cartridge concentrators. Concentrations of up to 3 mM are possible without activity loss or aggregation being induced. This method does not work for DAGK in lipid vesicles. For large volume solutions, Centricon Plus-20 (MWCO 10 kD) concentrators are used. For volumes of 2 ml or less, Centricon YM-50 (MWCO 50 kD) cartridges are used. Samples are spun at maximal speed in a Clay Adams DYNAC table top centrifuge at room temperature. It often takes only about 15 minutes to concentrate with the larger cartridges, longer (ca. 1/2 hour) with the small cartridges. Usually I wrap tubes with a Kimwipe before putting cartridge into rotor.

CONCENTRATING DAGK IN LIPID VESICLES

DAGK in lipid vesicles can be concentrated by placing an open glass vial containing the vesicles in a bath of 20-40 deg C water and blowing a stream of clean air across the top of the sample. The bath is needed to keep the solution from cooling down due to the enhanced rate of evaporation. Typically, Argon which has passed through an drying agent and then filtered through glass wool in a micropipette is used as the air source.

GLUTARALDEHYDE CROSS-LINKING OF DAGK MUTANTS

Reference: O. Vinogradova, P. Badola, L. Czerski, F. Sonnichsen, and C. R. Sanders. Biophysical Journal 72, 2688-2701 (1997)

Purpose: Normal wild type DAGK will get covalently linked by glutaraldehyde to form primarily trimeric DAGK which can be observed in SDS-PAGE. This reflects the fact that DAGK functions as a homotrimer. Some DAGK mutants may not fold correctly and assemble into homotrimers. In this case, covalent homotrimers would not be expected to form in the presence glutaraldehyde. Thus, we use glutaraldehyde cross-linking followed by SDS-PAGE as one way of testing for correct folding of DAGK. (for examples of data, see Biophys J. 72, 2688-2701 or Biochemistry 38, 16373-16382).

Glutaraldehyde cross-linking buffer: 2% decyl maltoside
20 mM phosphate
50 mM NaCl
1 mM EDTA
1 mM DTT (add fresh on day of use, optional)
pH 7.5

1. When cross-linking a mutant, always run a parallel reaction (identical conditions) on WT, C46, or Cysless as a standard.

2. Add DAGK to cross-linking buffer to 25 µM (roughly 0.35 mg/mL), for a final volume of 200 µL.
3. Add glutaraldehyde to 16 mM from 25% aqueous stock solution (Sigma, store at -20˚C). {25% solution is 2.5 M, add 3.2 microliters per 500 microliters of solution).
4. Incubate for fixed period of time (2-24 hours, but always do the same amount of time—little additional reaction occurs after 2 hours) at room temp with shaking, ~300 RPM.
5. Run cross-linked samples on SDS-PAGE. Remember to use C46, Cysless or WT as control.

Note: Provided that there is no amine (besides protein) present in the buffer, GA cross-linking works very well in variety of different buffers and pH. So, conformity to the above protocol is not very critical.

DESALTING OF DAGK / PREP OF SAMPLES FOR FAR-UV CD OF DAGK

The key to far-UV CD is to have a clear solution which also does not have anything in it (besides protein) which absorbs in the 180-250nm range. Even if any such absorptive substance produces no CD signal, it can strongly absorb both components of the polarized light making acquisition of a CD spectrum impossible (the equivalent of trying to take standard UV absorbance measurements on a solution with A > 3.0). For DAGK, one would have to worry especially about imidazole. For far UV CD, Olga made samples in the range of 0.2 mg/ml. Special quartz cuvettes are used having 1 mm (instead of the usual 1 cm) path lengths. Olga's Biophysical Journal paper has additional info on how she made measurements and also has representative spectra. One way to produce DAGK samples for near-UV CD would be to purify the enzyme using something other than imidazole as the eluting agent. This can be done. However, for our studies we may more often be interested in taking a CD spectrum of a sample eluted the normal way and subjected to other experiments (activity measurement, cross-linking, disulfide mapping, etc.)… so that we have a CD spectrum of EXACTLY the same batch of DAGK as used in these other experiments. For this, a better strategy would be to de-salt the DAGK.

Desalting Buffer: 1% decyl maltoside
100 mM NaCl
20 mM phosphate
pH 7.0

1.Prepare 3 ml of Biogel P-4 resin by incubating with DM-containing buffer for 4 hours at room temperature.
2.Pour off the excess solution (with any non-sedimented suspension).
3.Load it on to a 0.7 cm diameter column. Make sure the column has been well-equilibrated in buffer before applying the protein (flow at least 3 column volumes of DM buffer through it if it has not already been equilibrated).
4.Apply 0.5 mg of DAGK to the column in as small a volume as possible (1 ml or less). The starting DAGK should be concentrated enough so that even when it is diluted by the desalting column it will be concentrated enough upon elution to use directly in CD.
5.Once the sample flowed into the column, "chase" the sample in with about 0.25 ml of DM/buffer.
6.Apply a small (0.1 ml) volume of 3 mM tyrosine in buffer. If you can cleanly separate the tyrosine from the DAGK (elution judged by the UV-detector/chart) then you can be sure DAGK is completely separated from imidazole or other small molecules.
7.Collect only the first ¾ of the DAGK peak (ideally the top ½ of the total peak, not saving the "wings").
8.Once all of the tyrosine is eluted from the desalting column, let another 3 bed volumes of buffer continue to flow through (to make sure all of the imidazole is completely eluted).
9.Now the column is ready to use again: you don't have to pour a new column. Only use a column 2 times before making a new one! We have observed that resolution really degrades with repetitive use of the same column (this was true whether BioGel or Sephadex resins were used).

GENERAL PROCEDURE FOR DESALTING MEMBRANE PROTEIN SAMPLES

Take protein that has been purified into detergent + 250 mM imidazole. Adjust pH to 6.5 and add EDTA, as usual. Generally, you will want to now concentrate your sample to roughly the concentration that is desired for the experimental that the protein will eventually be used for (NMR, for example).

Now, prepare a desalting column. For a 0.2-2 ml protein sample, use a 0.7 or 1 cm diameter column and a resin bed that is at least 10 times taller than its diameter. (E.g., 1 cm X 10 cm is good). The volume of resin in the column should be >5 X the protein concentrated protein solution that will be applied to the column. (If you have 1-2 ml to de-salt, 10-15 mls of swollen resin should be about right).

Use Bio-Gel P-4 resin (fine), which has a MW cut-off of 4 kDa. Protein will elute first in the "void" volumn, while buffer/salt will elute last in the "wash" volumn. 1 Gram of dry resin will swell to about 3.5 ml. Weigh out the amount of resin you need (plus 20%) and mix with elution buffer. Gently disperse the resin so it is in contact with the solution. Allow the resin to swell for at least 4 hours at room temperature, with occasional stirring (but do not grind the beads). Load resin into column and allow bed to settle under flow.

Elution buffer: The elution buffer should contain the same detergent at the same concentration that the protein was eluted in. The choice of which buffer to use depends on the experiment the sample will be used for.

Now, you are ready to run the column. The key to any size exclusion column (including desalting) is to have a small loaded sample volume compared to the volume of the resin in the column. Tall skinny columns give much better resolution than short fat column.

Load the protein sample to the column and elute, monitoring A280. Once the sample flows into the top of the resin bed, "chase" the sample in with about 0.5 ml of elution buffer and then start the elution, proper.

The protein will come off first, followed by the salt (which will not have much A280). *An easy trick you can do to make the salt peak visible is to add tyrosine to a concentration of 0.5 mM to your APP sample before you load it on the column. Tyrosine is A280-visible and will run with the salt peak, making it detectable..*

DAGK STABILITY DETERMINATION USING SDS TITRATION, CROSS-LINKING, AND SDS-PAGE

Reference: Nagy, J. K. and Sanders, C. R. (2004) Destabilizing Mutations Promote Membrane Protein Misfolding. (Accelerated Publication) Biochemistry 43, 19-25.

Overview of the method

The goal of this method is to determine the thermodynamic stability of DAGK by examining the concentration of the denaturing detergent SDS which is required to convert the native trimer into monomeric form. Stable DAGK is monomerized only at high SDS concentrations, unstable DAGK comes apart at lower concentrations.

DAGK in DM/POPC mixed micelles is titrated with SDS, followed by GA cross-linking at each SDS concentration. Samples are then analyzed by SDS-PAGE.

Procedure

DAGK Stock: DAGK that has been reconstitutively refolded into POPC vesicles (1:120). Note: this means a 1 mg/ml DAGK/POPC sample contains 8.6 mM POPC.

DM Stock: 5% decylmaltoside in DAGK assay buffer (108 mM)

SDS Stock: 5% SDS in DAGK assay buffer (174 mM, store at room temperature)

GA Stock: Use fresh 25% solution (stored as frozen solution).

Buffer: DAGK assay buffer (75 mM PIPES, 50 mM LiCl, 0.1 mM EGTA, 0.1 mM EDTA, adjust pH to 6.9 using LiOH)

- The final cross-linking reaction mixture will in each case be 75 microliters and will contain DAGK stock, DM stock, SDS stock (or not), and GA. Use small eppendorf tubes. The final DAGK concentration is always 0.2 mg/ml (meaning that the final POPC concentration is 1.7 mM), the DM concentration is always 12 mM, and the GA concentration is 35 mM.

- First, mix DAGK stock, DM stock, and the appropriate volume of buffer. Mix so that the vesicles dissolve. Then add the SDS solution and mix samples for 1 hour. Then add GA and mix overnight. Then, run SDS-PAGE.

- The volumes of DM and GA to use for each reaction are always the same: 8.3 microliters of DM stock and 1 microliter of GA stock. The amount of SDS is varied. The amount of buffer used depends on what volume of DAGK stock is used and the amount of SDS.

- For each set of reactions, it is probably a good idea to run a control GA reaction which contains Cysless or C46 and DM/POPC, but no SDS… just to make sure the cross-linking reaction is working well.

Here are amounts of SDS to use for each reaction set:
(Mol% is calculated based on 2 mM free DM such that the total micellar DM concentration is 10 mM, while the POPC concentration is 1.7 mM)

Microliters of SDS	Conc. in mM	Mol%
0	0	0
0.6	1.4	10
1.2	2.8	20
2.1	5.0	30
3.3	7.8	40
4.9	11.7	50
7.4	17.6	60
19.7	46.8	80

SDS-PAGE: Mix 25 microliters from each reaction with 15 microliters of Invitrogen NP0007 Bis-Tris Loading Buffer. Mix and load 20 microliter aliquots into the wells of 4-12% Bis-Tris gels. *There is no need for molecular weight standards, but remember to run the control sample.*

CHEMICAL MODIFICATION OF FREE THIOLS IN DAGK WITH DTNB

Reference: Czerski, L. and C. R. Sanders. Thiol modification of membrane proteins: dependence upon protein site membrane disposition and reagent hydrophobicity. FEBS Letters 472, 225-229 (2000)

We wish to quantitate exactly how many moles of free -SH are present in DAGK solutions relative to the moles of DAGK present. This can be determined by measuring the number of moles of a thiol-specific reagent (DTNB) which react with a given amount of DAGK. We would also like to learn something about the accessibility of the free -SH groups at different positions in DAGK. This can be determined by measuring the rate at which the thiol-specific reaction takes place for each mutant.

1. Purify DAGK in absence of any reducing agent (mercaptoethanol, TCEP, or dithiothreitol. Any thiol or reducing agent present will give a false positive with the DTNB reaction. We also want to look at DAGK under conditions where it is fully catalytically active. This can be accomplished by using detergent solutions with 8 mol% tetradecanesulfonic acid (TDS) present. TDS is a negatively-charged lipid-like detergent which is a good activator of DAGK. Ultimately, we want to run a 1 ml modification reaction which contains 0.015-0.1 mM DAGK (0.21-1.4 mgs/ml, the more concentrated end being preferred). Bonnie has a chart for all of her mutants on resin which tells what yield she got from her batches and you can use this as a guide for deciding how many mls of resin to use for each mutant.

2.Wash the DAGK on-resin with 1% DM (21 mM) plus 8 mol% TDS (0.08 X 21 mM). Wash until you are sure any residual reducing agent has been removed (about 5 X 1 column volume).

3.Elute the DAGK with 1% DM plus 0.25 M imidazole, pH 8.

4.Quantitate DAGK in the pool by measuring A280 (1 mg/ml solution = 1.8 O.D. units). Use the elution buffer to zero the cuvette first.

<u>Running the modification reaction</u>

The reagent dithio-bis-p-nitrobenzoic acid (DTNB) reacts with free RS^- to release one mole of p-nitrophenylthio anion per mole of thiol, which is colored. It has a Molar absorptivity =13,600 at λ=412 nm Thus, a 0.1 mM concentration of free -SH should lead to a absorbance reading of 1.36 at 412 nm.

DTNB Buffer: 200 mM imidazole
1% DM
8 mol% TDS
pH 7.0

DTNB Stock: 10 mM DTNB in buffer (always make fresh on day of reactions, keep on ice)

1. The DTNB solution is not completely stable. It becomes more yellow as it sits around. It is important to get an accurate starting absorbance. In cuvette, mix 0.1 mL of DTNB stock with (0.9-x)mL of buffer (where x=volume of DAGK to be added).
2. Place cuvette in spectrophotometer at 20˚C and allow temp to equilibrate.

3. Measure and record A412. This starting A412 should be corrected for dilution by dividing it by the number of ml actually present in the cuvette at this point (since it is going to be diluted when the DAGK solution is added).
4. Add x mL of DAGK solution, mix, and monitor A412 vs. time until absorbance reaches a plateau. This could take an hour or more.
5. Save time trace.

Correcting for Baseline Reaction

During the time it takes for the free -SH to react with DTNB, there will be some spontaneous reduction of DTNB leading to color development (which will add to the total A412 you measure). The best way to correct for this would be to run a two cuvette reaction where one cuvette was the blank and one was DAGK and you subtract the blank from DAGK. However, this may not be possible and so the best you can do is to run a blank reaction with 0.1 ml of DTNB stock and 0.9 ml of solution 2. From this reaction one can get the background rate (measured as change in A412 per minute). Using this measurement, the final absorbance in a real DAGK run can be corrected:

true A412 due to DAGK reaction = final observed A412 - (baseline change in A412 per minute X number of minutes of reaction).

Based on experience with different buffers, the baseline rate at pH 7 and 20 degrees is known to be about 0.00023 OD/minute (0.0017 OD/minute at pH 8.0 and 25 degrees). This is not real fast, but high enough to introduce a significant error for reaction which may take many minutes.

Calculations

Total free SH in the DAGK stock (molar) =
[final (corrected) absorbance - initial (corrected) absorbance]/ [13,600 X ml stock used]

Rate of reaction: This is inversely proportional to the $t_{1/2}$: the time at which A412 development is exactly ½ the maximal development.

This is equal to $[(\text{starting A412} + \text{final A412})/2]^{-1}$.

<u>LABELING, RECONSTITUTIVE REFOLDING (RR), AND TESTING OF SINGLE-CYS DAGK MODIFIED WITH MTSL</u>

(SPIN LABEL FROM TORONTO RESEARCH CHEMICALS)

NOTE: for any of the mutants which are known to be especially difficult to refold: instead of using DPC as specified below, use CFOS-9 (CFOS-3) at purification and reconstitution steps (use separate dialysis solutions for the CFOS-3 samples). You can cut down the total dialysis cycles/time if CFOS-3 is used (as you showed previously).

1.Purify ca. 2-3 mgs of each mutant in DPC with 0.1 mM DTT (or DTE) being present at both equilibration and elution steps. (You want to make sure that the Cys on the DAGK are fully reduced.) When you purify, try to save only the concentrated part of each DAGK peak... you want the DAGK to be relatively concentrated (>2 mg/ml) so that you don't have to use too much MTSL solution. Purify into 1.5 ml eppendorf tubes.
2.Quantitate the mg/ml of DAGK in each pure pool.
3.Make up a fresh 75 mM MTSL solution by dissolving 10 mgs of MTSL (it its vial) by methanol.
4.Add MTSL to each DAGK pool in the eppendorfs, to a concentration of 2 mM MTSL. The free Cys thiols on DAGK will react with MTSL to form disulfide bonds. MTSL will also react with DTT present (but the product of this reaction will not interfere with Cys modification and will be removed during dialysis).
5.Put reaction mixtures on the rotating shaker and rotate them fast for 30 minutes at room temperature (as for GA cross-linking reactions). Then put them in the 37 degree incubator and let them sit there for 3 hours.
6.Now, add DPC/POPC mixture with NO REDUCING AGENT according to the standard RR protocol. Mix and transfer solution to the standard dialysis tubing for reconstitutive refolding. Now, dialyze each vs. 5 total batches of 10 mM imi, 50 micromolar EDTA (NO reducing agent, pH 7.8). Overnight, all day, overnight, all day, overnight. Record when samples become cloudy.

FLUORESCEIN LABELING OF MEMBRANE PROTEIN ASSOCIATED WITH NI(II)-RESIN

PROTOCOL DEVELOPED BY CHUCK ELLIS

The general procedure for labeling proteins (or other macromolecules that contain primary amine groups) with NHS-fluorescein is found on pages 306-307 of Bioconjugate Techniques by Greg T. Hermanson (Academic Press, 1996).

The general procedure for binding 6xHis-tagged proteins to Ni-NTA-coated microplate wells is found in Qiagen's Protocol 12 on pages 70 through 73 of the QIA*express* Detection and Assay Handbook (October 2002 edition).

A combination of the two procedures was used in developing the following protocol which was developed specifically for labeling proteins which are associated with immobilized Ni(II)-resin in micro-well plates. This protocol can presumably be adapted to tagging proteins associated with loose resin. Indeed, please be aware Ni(II) microwell plates sold by Qiagen are coated with albumin. Thus, the following procecure labels both the protein bound to the nickel resin and also non-specifically-bound albumin also present in the wells- that is why this turned out NOT to be a good route for quantitating protein expression based on microwell fluorescence following labeling. Keep in mind, too, that this is a non-specific labeling method which will normally multiple sites in the targeted protein—this will often result in protein inactivation.

Here’s the protocol:

1. Add 0.200 ml of clear cell lysate containing poly-His-tagged protein to each well and shake the microplate at room temperature for 1 hour to bind the protein to the Ni-NTA groups.

2. Wash wells 4 times with Na-PBS-DM (50 mM sodium phosphate, 150 mM NaCl, 0.5% DM, pH 7.5). Soak wells for 1 minute per wash, and dry the wells by tapping the microplate on paper towels.

3. Add 0.190 ml of Na-PBS-DM followed by 0.010 ml of 2.1 mM NHS-fluorescein (0.5 mg NHS-fluorescein in 0.5 ml DMSO; store protected from light) to the wells. Wrap the microplate in aluminum foil and shake at room temperature for 1 hour to bind the fluorescein to the protein.

4. Wash wells 4 times with Na-PBS-DM. Soak wells for 1 minute per wash, and dry the wells by tapping the microplate on paper towels.

The rest of the protocol involved washing the wells 9 times with 40 mM imidazole (in Na-PBS-DM), eluting the fluorescein-tagged protein either with 250 mM imidazole (in 0.5% DM, pH 7.8) or with 100 mM EDTA (in Na-PBS-DM), transferring the eluant to a black Costar microplate, and finally detecting the fluorophore in a FLUOstar microplate reader (485 nm excitation filter, 520 nm emission filter).

An alternate method may be to do the 40 mM imidazole elution step before doing the NHS-fluorescein step in order to elute all of the non-polyHis-tagged proteins from the column before doing fluorescein labeling.

ALKYLATING A SINGLE-CYSTEINE MUTANT OF A GENERIC PROTEIN WITH A GENERIC BROMOACETYLATED REAGENT

Reference: 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.*

<u>Modification Reaction</u>

The following procedure is based on our experience with 1-bromo-3-trifluoropropanone (BrCH2-CO-CF3) and the membrane protein diacylglycerol kinase. "It should work" with BABE and a water soluble protein. In our experience, this is a pretty specific reaction for thiols.

Some folks who do BABE modification, do the reaction using a pre-formed BABE-metal ion complex. I really don't see the point of that unless your protein has a high affinity metal ion binding site in it. It would seem (to me) to be preferable to add a stoichiometric amount of the desired metal ion just before NMR.

Reaction buffer: we use 0.2 M imidazole, pH 7.8 because that is what we have following purification of our polyHis-tagged protein. A more ideal buffer would be pH 7.8, 100 mM Hepes. What is most important is that there be no thiols in the buffer.

Protein solution: The protein should be solubilized in reaction buffer. The reaction efficiency will not be dependent upon protein concentration. The prime advantages to having a more concentrated protein solution (say, 1 mM) to start with are (1) that you will have to consume less of your bromacetyl reagent to modify a given number of mgs of protein, (2) only a small volume will need to be removed after reaction to determine the % remaining free thiols (see last section).

Stock solution of the bromoacetyl reagent: Depending on the reagent, there may be no need to make a stock solution. It may be possible to simply weigh out the solid and add it directly to the protein solution. This is preferred. If not possible (for example, if it's a viscous oil), then make a fresh stock solution in the same buffer your protein is in. (100 mM - 1 M, depending on solubility). Ideally, stock solutions should be made fresh just before the labeling procedure. However, if the reagent is scarce or expensive, there probably is no problem with storing over a period of weeks at -80 deg. C.

Add the bromoacetyl reagent to the protein solution at room temperature such that the concentration of the labeling reagent is 10 mM. You can run the reaction in a glass or plastic vial with a cap. Mix the solution for 1 hour at room temperature by attaching the tube to a rotator or shaker.

Following reaction, you must now remove the XS reagent. This can be done by dialysis (the most straightforward) or by running a desalting column. One could also use a centrifugal concentrator and do multiple concentration/dilution cycles until the concentration of XS reagent is minimal. The only disadvantages of dialysis is that some sample dilution will take place. The disadvantages of a desalting column are (1) dilution and (2) that if one is not careful, resolution of the protein from the XS reagent may not be complete (however, since BABE has a benzene group in it, this would be easy to follow with a UV detector). Let me (CS) know if there are any questions about any of this.

<u>Quantitating the % Modification of Protein Cys Residues</u>

It may be possible to do this by NMR if all of the protein peaks are assigned and one can identify peaks from the Cys-attached BABE and compare them with the integrals of individual protein peaks. However, here is a chemical analysis procedure which can be used instead.

The number of moles of free SH in your protein before and after chemical modification can be determined by measuring the number of moles of a thiol-specific reagent (dithio-bis-nitrobenzoic acid, DTNB) which reacts with a given amount of protein.

Running the DTNB reaction

The reagent dithio-bis-p-nitrobenzoic acid (DTNB) reacts with free RS^- to release one mole of p-nitrophenylthio anion per mole of thiol, which is yellow. It has a molar absorptivity =13,600 at λ=412 nm Thus, a 0.1 mM concentration of free -SH should lead to a absorbance reading of 1.36 at 412 nm if it reacts to 100%. Neither DTNB nor the thiobenzoate product should undergo any reaction with BABE, so you should be able to run this reaction using aliquots from the protein/BABE reaction mixture without remove the XS reagent first.
Solutions Needed:

1. Protein solution in pH 7.8 reaction buffer.
2. Buffer for DTNB: use same pH 7.8 buffer as used for the alkylation reaction.
3. 10 mM DTNB in buffer (always make fresh on day of reactions, keep on ice).

If you have reason to believe that the thiol you are trying to alkylate is buried in the protein where it is somewhat inaccessible to reagents like DTNB, it is possible to run this reaction in the presence of denaturing agents like urea. Just add this to the buffer.

The final reaction volume will be 1 ml. Of this 1 ml, 0.1 ml will always be your DTNB stock (solution 3), (0.9 - x) ml will be buffer (solution 2), and x ml will be protein solution, where x is never more than 0.5 ml. The amount of protein solution you'll use depends on the concentration of your original protein solution. The ideal final protein solution for a single-Cys mutant in a 1 cm cuvette would be 0.1 mM. However, one could probably get by with even less (perhaps 0.025 mM), if protein is scarce.

Measuring the starting absorbance: The DTNB solution is not completely stable… as it sits around it will gradually become more and more yellow. It is very important to get an accurate starting absorbance . To do this:

Zero the spectrophotometer using your cuvette with only buffer in it. Then, in the same (clean and dry) cuvette mix 0.1 ml of solution 3 and (0.9-x) ml of solution 2. (The final volume at this point will be 0.5 ml or higher). Place in the spectrophotometer at 30 deg. C. and allow temp to equilibrate. Then, measure and record A412. This starting A412 should be corrected for dilution by dividing it by the number of ml actually present in the cuvette at this point (since it is going to be diluted when the protein solution is added).

Now, add in x ml of protein solution, mix thoroughly and monitor A412 as a function of time until the absorbance plateaus (This could take as long as an hour or more). The time trace should now be saved as a file.

Correcting for Baseline Reaction

This is only necessary if sites react slowly so that you have to wait many minutes for the reaction to come to completion. If the reaction occurs quickly (within a few minutes, the amount of baseline reaction should be negligible).

During the time it takes for the free -SH to react with DTNB, there will be some spontaneous reaction of DTNB leading to color development (which will add to the total A412 you measure). The best way to correct for this would be to run a two cuvette reaction where one cuvette was the blank and one was protein and you subtract the blank from protein. However, this may not be possible (if you have a single beam spectrophotometer) and so the best you can do is to run a blank reaction with 0.1 ml of DTNB stock and 0.9 ml of solution 2. From this reaction one can get the background rate (measured as change in

A412 per minute). Using this measurement, the final absorbance in a real protein run can be corrected:

true A412 due to protein reaction = final observed A412 - (baseline change in A412 per minute X number of minutes of reaction).

Based on experience with different buffers, the baseline rate at pH 8 and 25 degrees is known to be about 0.0017 OD/minute at pH 8.0.

Calculations

Total free SH in the protein stock (molar) =

[final (corrected) absorbance - initial (corrected) absorbance]/ [13,600 X ml stock used]

SYNTHESIS OF SHORT CHAIN DIACYLGLYCEROL

Reference: Czerski, L. and C. R. Sanders. Functionality of a membrane protein in bicelles. Analytical Biochemistry 284, 327-333 (2000).

This procedure is written specifically for diacetylglycerol, but can be easily adapted to longer-chained DAGs. For short chained DAGs especially, I prefer the anhydride method.

In 1 ml of toluene in a round bottomed flask mix 0.5 g (0.4 ml, 2.7 mmoles) of sn-3-benzyl glycerol (Sigma) {same as Aldrich R-(+)-3-benzyloxy-1,2-propanediol. cat. no 438960-1G}, 0.7 g (0.65 ml, 6.9 mmoles) of acetic anhydride, 1 gram (1.35 ml, 7.7 mmoles) of diisopropylethylamine, and a couple of crystals of dimethylaminopyridine (DMAP). Stir under Ar or N2 at about 80 degrees for 1.5 hours (use a heat mantle with some sand and stick a thermometer in the sand). The reaction mixture will take on an amber color. (Note, when I make dibutyrl or hexanoyal, I don't use a solvent... no toluene)

The reaction products can be viewed using glass-backed silica gel TLC. Among various visualization methods, a simple one is to dip the dried plate into 50% sulfuric acid, let the plate drip dry and then gently dry the plate with a heat gun until the wetness disappears (you may see spots at this point). If you now let the plate sit for a while at room temperature, spots will appear as "grease spots" on the plate. {This kind of depends on exactly what type of silica gel plate you are using. An alternative protocol is to dip in the acid and then put on a hot plate to char. A Corning hot plat at a setting of 4 seems to work well} If 4:1 hexane:ethyl acetate is used to develop the plate benzyldiacetylglycerol will exhibit an Rf of about 0.3.

To the RT reaction mixture add 25 ml CHCL3. Extract the organic phase 2X with 0.15 M formic acid in water, 2 X with water satd. with NaHCO3, and then 2X with water. Dry the organic phase by adding some MgSO4 (until it doesn't clump) and then filtering out the solid. Rotovap the organic phase to dryness.

At this point we ran flash chromatography (this may not be necessary) using a ca. 1.5 X 20 cm bed of flash-chrom. grade silica gel and eluting with 5:1 hexane:ethyl acetate. Monitor eluate with TLC (take ca. 7 ml fractions). Pool fractions containing the major product and Rotovap. Remove residual solvent by placing rotovapped product under high vacuum for 1/2 hour. H1 NMR of the product in CDCl3 can be used to confirm you have right product (note that you will see residual ethyl acetate in your spectrum- don't worry about this).

To remove the benzyl protecting group, use catalytic hydrogenation. Rather than get a big cylinder of H2 gas, you can get a lecture bottle (you have to order a valve separately) from Aldrich which will last for ca. 4-5 reactions. Big balloons can also be purchased from Aldrich.

Dissolve 100 mgs of sn-3-benzyl-1,2-diacetylglycerol in 3 ml THF in a 25 ml RB flask. Add 100 mgs of 5% Pd on carbon (Aldrich). Seal the flask with a double septum (use new septa). Make a H2 glass balloon (see drawing) with a needle attached and stick the needle into the septa. Now, purge the system of O2 by pumping Ar (or N2) into the flask (through another needle in the septa) to partially inflate the balloon. Then allow gas to escape through a needle in the septum (do this 3 times). Next, stick in a needle attached to a tube connected to the H2 bottle (prepurge the tube with H2) and pump in H2 gas to partially inflate the balloon. Let the H2 gas out once and then fully inflate the balloon and stir reaction vigorously at room temperature under the H2 balloon. Let reaction go for three hours at RT (if the H2 balloon deflates very much you can refill at any point).

After three hours run a tlc to make sure the reaction has gone to completion. We used 8:1 CHCl3:MeOH. The product shows up with an Rf of about 0.7, unreacted precursor elutes with the solvent front.

The product mixture should be filtered to remove the catalyst and dried with a rotovap. Then redissolve the oil in 8:1 CHCl3:MeOH (a higher ratio should probably be

used so that the product Rf is in the 0.3-0.5 range) and flash chromatograph using the same solvent system to get the pure sn-1,2-diacetyl glycerol. For a 100 mg reaction, a 1 cm X 20 cm bed should suffice. Rotovap to remove the solvent to produce a colorless liquid. Residual solvent can be mostly removed by additional drying under high vacuum, but beware that the molecular weight of diacetylglycerol is low enough that you may lose some of it as well under vacuum. Store product in low temp freezer... with time some isomerization to the 1,3 isomer will take place.
For dipropanoylglycerol: use 7:1 hexane:ethyl acetate for purifying the benzylated product; we used 8:1 $CHCl_3$:MeOH for final product, but I would recommend switching to much higher ratio to get the Rf down.
For dibutyrlglycerol: Benzylated product can be flash-purified using 6:1 hexane:EA (Rf is 0.4 in 13:1 hexane:EA). To purify the final product, run flash chrom using 5:1 hexane:ethylacetate {This may be a little too stringent… 4:1 may work better}. You may get two major products off of the column (the higher Rf one has an Rf of ca. 0.3). It is actually the lower Rf product which is the correct one. The high Rf one may be the 1,3 isomer.
For dihexanoylglycerol: Same general procedure as above, but use hexanoic anhydride. The 3-benzyl-1,2-dihexanoylglycerol compound will have an Rf of about 0.5 in 10:1 hexane:ethyl acetate. Use 3.5:1 hexane: ethyl acetate for following debenzylation and for purification. The Rf of dihexanoylglycerol in 2.5:1 hexane:EA is is ca. 0.33.

Monitoring DAGK Insertion in Vesicles using Microplate Reader

Reference: Nagy, J. K., Lonzer, W. L., and Sanders, C. R. (2001) Kinetic study of Folding and misfolding of diacylglycerol kinase in model membranes. *Biochemistry 40,* 8971-8980.

Insertion Buffer

To PIPES Assay buffer, add:
1 mM DTT
1 mM PEP
0.5 mM NADH
3 mM ATP
15 mM MgAcetate

Add 7.7 uL of DBG to 10 mL of insertion buffer and tumble/sonicate for ca. 30 min.

Make a solution of 20 mM POPC in the insertion buffer + DBG and extrude through 50 nm membrane.

In a 1.5 mL eppindorf, mix 450 uL insertion buffer + DBG, 50 uL preformed vesicles and 20uL PK/LDH

To cap of eppindorf, add appropriate amount of DAGK stock (0.3ug).
Close caps and shake vigorously for ca. 5 sec
Add 200uL to microplate and read at 30 C for ca. 3 hours.

Set up microplate reader as you would for any kinetic run but adjust time to 3 hours

Processing data from DAGK Insertion Reactions

Always have a control lane containing all reaction components except DAGK

Save file as .pda (softmax data file)

To save file as .txt to work with in Axum:

Click on microplate reader icon on toolbar.
Make sure 'export format' is in TIME and click on 'include labels'
Next, under the file menu, choose EXPORT
Export as .txt and be sure that 'plates and cuvettes' and 'all' are selected

The text file can be opened in Excel and the columns containing text, time and temperature readings can be deleted. Also delete any columns not containing relevant data. Resave as .txt

Open Axum and import file.
You should have two columns, one with the 'blank' data and one with reaction data. Label these columns 'blank' and 'A340'
Create a column called 'time'

under 'data' on the toolbar, go to fill and fill the time column

Now, plot the blank and A340 vs time and see where the reaction ends

delete any unnecessary data in the data sheet

1. make a column called ‘correction’

2. in transform box, type: correction= (# in blank row 1)-blank;

3. make a column called ‘corrected’

4. in transform box type: corrected = A340+correction;

5. plot time vs. corrected and do a nonlinear LS fit under 'tools' on toolbar:

    dependent variable: corrected
    model: p0+p1*time+p2*time^^2
    set all parameters equal to ‘1’: ie p0=1, p1=1, p2=1
    in ‘results’ select data sheet, predicted values and residuals to be displayed

in the data sheet, you will now have a column called 'parameters' and you will be given predicted values for p0-p2
Now, reselect nonlinear LS fit

6. redo model: p0+p1*time+p2*time^^2+p3*time^^3+p4*time^^4

    use p0-p2 parameters from above and set p3 and p4 equal to ca. 1e-10 and 1e-13.

    Generate new parameters

you will see the parameters in your 'param' column change

7. Now, take first derivative of the model p0+p1*time+p2*time^^2+p3*time^^3+p4*time^^4 ,

Chose 'transform' from the toolbar and type:

    activity= -((First derivative)*8000/micrograms used in reaction);

    use the parameters generated above in equation for derivative

NOTES:
don't forget to use semicolons at the ends of all sentences when working in the transform dialogue box.

Low Temperature Expression of Membrane Proteins (V2R) in *E. coli*

Anne Karpay, Changlin Tian, and Charles R. Sanders

*Reference: This is an improved version of a protocol originally presented in the supporting materials to a 2006 JACS communication from the Sanders lab: Tian, C., Breyer, R. M., Kim, H. K., Karra, M.D., Friedman, D. B., and Sanders, C. R. (2005) Solution NMR spectroscopy of the human vasopressin V2 receptor, a G protein-coupled receptor. J. American Chemical Society **127,** 8010-8011.*

Expression and Purification of the Human Vasopressin V2 Receptor (V2R)

Standard subcloning procedures were used to amplify the full length human V2R gene by polymerase chain reaction (PCR). Codons for the first two N-terminal amino acids (Met, Leu) were removed. The resulting PCR product was digested with *Nde* I and *Xho* I restriction endonucleases (New England Biolabs, Beverly, MA), and the product was ligated into a pET21b (EMD Biosciences, San Diego, CA) vector with complementary restriction endonuclease sites. The ligation products were used to transform NovaBlue *E. coli* (EMD Biosciences) cells, and successful transformants were selected using ampicillin resistance. The correct DNA sequence of the new construct was verified by the DNA Sequencing Facility, Vanderbilt University. The final construct encodes amino acid residues 3-371 of human V2R plus a carboxyl-terminal: -LEHHHHHHHHHH tag.

Expression of the tagged receptor is under control of an isopropylthiogalactoside (IPTG)-inducible promoter. To express V2R in *E. coli*, BL21-(DE3)-Codon Plus-RP cells (Stratagene, La Jolla, CA) were transformed using the V2R/pET21b expression vector described in the above paragraph. Successful transformants were grown in ^{15}N-labled M9 minimal media in the presence of 100 μg/mL ampicillin and 50 μg/mL chloramphenicol, and supplemented with a multi-vitamin (CVS Spectravite, 0.1 tablet per liter of medium). This culture was incubated at 37°C, 250 rpm, to an OD_{600} = 0.7, and then incubated at 12°C with 240 rpm for approximately 2 hours. When OD_{600} reached 1.0, protein expression was induced using 1 mM IPTG and rotary shaking was continued for 36 hours at 12°.

Cells harboring recombinant V2R were harvested by centrifugation and then resuspended in approximately 10 mL lysis buffer (70 mM Tris-HCl, 300 mM NaCl, pH 8.0, with 2 mM 2-mercaptoethanol and protease inhibitor cocktail--50 μL; Sigma # P8849, St Louis, MO) per gram of cells. Cells were then frozen in liquid nitrogen. The cell suspension was thawed for 30 minutes at 4°C, and additional lysis buffer, reducing agent, and protease inhibitor were then added to reach a ratio of 20 mL lysis buffer for each gram of wet cells in the starting pellet. The lysate was passed through an EmulsiFlex C3 High Pressure Homogenizer (Avestin, Canada) three times. After this magnesium acetate (to 5 mM), DNase (to 0.02 mg/mL), and RNase (to 0.02 mg/mL) were added, and the lysate was rotated at 4 °C for 1-2 hours. The detergent, 1–myristoyl–2–hydroxyl–*sn*–glycerol–3–phosphocholine (LMPC; Avanti Polar Lipids, Alabaster, AL) was then added to the lysate to a concentration 1.0 %, and the solution was rotated at 4°C for 4 hours. The lysate was then centrifuged at 4°C at 40,000 ×*g* for 20 minutes. The supernatant (which includes the plasma membrane) was incubated with 2 mL of TALON metal affinity chromatographic resin (Clontech Laboratories, Mountain View, CA) per liter of original culture and rotated at 4°C, for 1 hour. The resin was then packed into a gravity-flow column attached to a UV-detector (280 nm), and washed with 8-10 column volumes of binding buffer (20 mM Tris-HCl, 200 mM NaCl, 0.1

% LMPC, pH 8.0). Impurities were then eluted using a “wash” buffer (20 mM Tris-HCl, 200 mM NaCl, 0.1 % LMPC, 30 mM imidazole, pH 8.0) until the monitored OD_{280} returned to baseline after approximately 8-10 column volumes. V2R was then eluted with a pH 7.0 buffer containing 250 mM imidazole and 0.2 % LMPC. All chromatographic buffers also contained 2 mM 2-mercaptoethanol.

(Note, in the above protocol, we don’t isolate the plasma membrane before doing detergent extraction. If you want to first isolate the membranes do the following. After lysis do a low speed spin using a regular centrifuge to remove unlysed cells and other junk. Then, the supernatant in an ultracentrifuge for 2 hours at 60000 g. The resulting pellet will contain the plasma membrane.)

The V2R-containing eluate was iteratively concentrated using an Amicon Ultra-15 centrifugal filter device (10 kDa molecular weight cut-off; Millipore, Bedford, MA) and diluted with buffer (20 mM Tris-HCl, pH 8.0, 0.1% LMPC) to remove imidazole and reduce volume in preparation for cation ion exchange chromatography. The solution was loaded onto a HiTrap SP/HP Sepharose column (GE Healthcare) and purified with a 0-1 M NaCl gradient (20 mM Tris-HCl, pH 8.0, 0.1 % LMPC). An elution profile for this column is shown in Figure S1. Typical yields of V2R were 1-2 mg of pure protein per liter of media. Shown in Figure S2 are SDS-PAGE results for V2R purified by this protocol (lane 3) compared to results for V2R purified using the same protocol, except that Ni(II)-NTA agarose rather than Co(II)-TALON resin was used at the metal ion affinity chromatography step.

To prepare purified V2R in LMPC micelles for NMR, EDTA and dithiothreitol were added to 2 mM and D_2O was added to a concentration of 10 %. The pH was adjusted to 5.0 using acetic acid and the solution was then concentrated using an Amicon Ultra-15 centrifugal filter device (10 kDa molecular weight cut-off; Millipore, Bedford, MA). By this operation, both V2R and the detergent LMPC were concentrated. Samples were then transferred to 5 mm NMR tubes and subjected to NMR at 600 MHz and 15ºC. The TROSY spectrum of V2R prepared according to this protocol is shown in the right panel of Figure S3. Shown in the left panel of Figure S3 is the spectrum of V2R that was prepared (from the same pool of LMPC-extracted cells) using an identical protocol as for the right panel, except that Ni(II)-NTA agarose was used instead of Co(II)-TALON resin for the metal ion affinity chromatography step.

Sanders Lab Protocols for Preparing 15N-Labeled PolyHis-Tagged Water Soluble Proteins.

Chuck Sanders, Vanderbilt, Autumn 2005

This protocol gives the standard methods for preparing 15N-labled minimal medium for growing *E. coli* from which protein will be purified for NMR. Using the protocol below, you will get identical results using either 15N-labeled or unlabeled medium—the presence of 15N does not change anything.

15N-ammonium chloride can be purchased from Sigma or from Cambridge Isotopes Lab. It is not radioactive and so no special paperwork or ordering procedure is required.

The following protocol is based on the assumption that ampicillin is the appropriate antibiotic. The protocol is also based on the assumption that the expression plasmid is IPTG-inducible. Depending on what cell type/vector you are working with some adjustments or additions may be required.

UNIFORMLY ^{15}N-LABELED CULTURES USING H2O

First grow a small liquid culture (3-6 ml of LB medium in 15 ml sterile Falcon tube) from one plate colony. (LB = Luria broth). This small liquid culture is then 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*
   A. Add the following amounts per liter of medium:

Na_2HPO_4	6 g (12.8 g if the 7-H_2O hydrate form is used)
KH_2PO_4	3 g
NaCl	0.5 g
$^{15}NH_4Cl$	1.0 g

   B. Adjust pH=7.0
   C. Autoclave
   D. Let medium cool to room temp, then add sterile:

$CaCl_2$	1 ml/L
$MgSO_4/7H_2O$	1 ml/L
40% Glucose (dextrose)	10 ml/L
Ampicillin	100 mg/L
Vitamins (see below).	2 ml/L

Stock solution of $CaCl_2$ is prepared by dissolving 1.47g of $CaCl_2$ in 100 ml of dd H_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 dd H_2O. Filter sterilize.

40% sterilized Glucose can be ordered or can be prepared by sterile filtration

Ampicillin stock is filter-sterilized.

Vitamin prep: smash 1 vitamin (Spectravite, multivitamin/multimineral from CVS Pharmacy- yes, it does matter which type of vitamin) *and mix with 20 mL of H2O. Mix, bath sonicate, and remove insoluble junk by low speed centrifugation. Filter with steriflip.*

2. Grow 2-5 mL LB/amp cultures overnight with shaking at 37 °C.
3. Use 1 tube of LB to inoculate each large flask of minimal medium.
4. Grow with shaking at 37 °C until OD_{600} = 0.8-1.0 (usually about 6 hours).
5. Induce with 0.2 g/L IPTG (or whatever inducing agent is appropriate).
6. Continue shaking at 37 °C from 3 hours to overnight.
7. Harvest cells by centrifuging. Immediately freeze for storage or proceed to cell lysis/protein purification.

Variations of the above: Some proteins only are well expressed when the cells are grown and induced at lower temperatures (30, 20, or even 12 °C). In this case, it is usually OK to first grow cells at 37 °C until OD_{600} is about 0.6. At this point, switch to a lower temperature, allow cells to acclimatize for two hours at the lower temperature, and then induce. At very low temperatures, you may want to allow induction to proceed for as long as two days before harvesting.

With any new protein, some systematic testing of expression conditions can be very helpful to optimize expression.

Some expressed proteins get proteolyzed with time. In this case, the amount of time between induction and harvesting must be reduced. At 37 °C, 3 hours of induction (no more) is sometimes optimal to avoid proteolysis.

HARVESTING CELLS

Harvest cells by centrifuging at 4 °C at for 15 minutes on any preparative Beckman or Sorvall centrifuge. Generally spin at 80% 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. The resulting 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 for later reference.**

PURIFYING WATER SOLUBLE POLY-HIS-TAGGED PROTEINS FOLLOWING *E. COLI* EXPRESSION AND PREPARING NMR SAMPLES FOR INITIAL NMR FEASIBILITY TESTING

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These protocols are based on starting with > 1 gram of pelleted *E. coli* cells, in which a polyHis-tagged protein has been overexpressed into the cytosol. A different protocol is required if the protein is a membrane protein and/or if the protein is expressed into inclusion bodies. There is also a separate protocol for doing expression screening of small (10 mL) cultures. The following protocol can be applied both to labeled (for NMR) and unlabeled protein. The lysis/purification method is independent of what medium the *E. coli* cells were grown in.

For NMR feasibility testing uniformly 15N-enriched protein is required. An appropriate same will be >0.5 mM in concentration and have a volume of ca. 0.6 ml. For a 20 kDa protein, this means at least 6 mgs of protein are required.

LYSIS PROCEDURE

1. Take *E. Coli* cell pellet 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 well.

2. Add PMSF (phenylmethylsulfonyl fluoride- a poison!) from a 20 mg/ml stock solution in isopropanol (this can be stored indefinitely in the freezer) to a concentration of 20 mgs per 100 ml of sup (1.1 mM). PMSF is a protease inhibitor which will help keep protein from getting chewed up. *Because PMSF will inhibit mostly the Ser/Thr proteases, it may be preferable to instead use a protease inhibitor cocktail that is broadly specific towards a variety of proteases. Such cocktails are available from a number of vendors and can be used according to manufacturer specifications.*

3. To the suspended cells add the following:

   lysozyme powder 0.2 mg/ml
   powdered DNase and RNase 0.02 mg/ml each
   MgAcetate to 5 mM from a 500 mM stock (stock: 11 g/100 ml)

   Seal the container and incubate for ½ hour at room temperature with tumbling (no stir bars!).

   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. Tip sonicate at 50-75% power, 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. An alternative to a sonication is a shear-based disruption device such as a French press.*

5. Add dithiothreitol (DTT) to a concentration of 0.25 mM (5 mgs per 100 ml). DTT is a reducing agent which will help keep the Cys thiol groups from getting oxidized.
6. Centrifuge extracted lysate at 30,000 x *g* for 20 minutes. If the expressed protein goes into inclusion bodies, it will spin down with the pellet. If the expressed protein is cytosolic, it will remain in the sup. Save the sup. THE REST OF THIS PROTOCOL IS BASED ON THE ASSUMPTION THAT THE EXPRESSED PROTEIN IS IN THE SUP! SDS-PAGE/coomassie and Westerns can be run on the pellet to check to see if any of the protein of interest is in inclusion bodies.

Note About DTT

Dithiothreitol is a reducing agent which is typically used in concentrations of 0.1 to 1 mM to keep 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).

PURIFICATION SOLUTIONS

0.25 mM dithiothreitol (DTT) should be added to all solutions listed below on the day the buffer will be used. *Regenerated resin sometimes cannot be used in the presence of DTT, new resin must often be used. (If there is a problem you will know it because the elution solution will turn brown when DTT is passed over the column).*

-Lysis Buffer:
75 mM Tris-HCL (Tris is the buffer component, make this starting with Trizma base)
0.3 M NaCl
0.3 mM EDTA (chelating agent to scavenge multivalent metal ions.
10 micromolar BHT (B-hydroxyltoluene, a free-radical scavenger, optional)
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
10 micromolar BHT (optional)
pH = 7.5

-Wash Buffer:
Buffer A + 0.04 M imidazole, pH 7.8

-Rinse buffer:
Buffer A

-Elution buffer
0.25 M imidazole (make sure a high grade of imidazole is used, see below)
pH 7.8

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.

Ni^{2+} RESIN INCUBATION AND ELUTION

7. Ni-NTA Superflow resin should be equilibrated by rinsing with buffer A. Use about 1.2 ml of packed resin for every gram of wet cells in the lysate. Pack resin into a column and rinse twice with 2 bed volumes of buffer A.
8. Transfer the resin into a tube containing post-centrifugation supernatant from the lysed cells (on ice).
9. Tightly close the lid and rotate the tube for ½ hour (no longer) in the cold room. During this time the polyHis-tagged protein will bind to the nickel resin.
10. Following incubation, isolate the resin by centrifugation of the solution. 3,000-5,000 x *g* in a tabletop centrifuge for 15 minutes should be fine.
11. Pour off the supernatant (try not to lose any of the resin) and either freeze the resin in liquid N_2 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 Buffer A. Freezing protein-on-resin and storing at -80 °C usually does no harm to the protein of interest, at least when storage is only for a matter of days.
12. Turn on chart recorder/UV detector and start monitoring A280.
13. Wash column with cold 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 that 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.
14. Elute the protein with elution buffer. The target protein will elute as a sharp band that can be followed at A280 using the detector/chart recorder. Only this band needs be collected. Remember to tare your collection vial while it is empty so you can measure the volume of protein solution eluted.
15. Weigh the eluted protein solution.
16. Zero a quartz cuvette on spectrophotometer using elution buffer at 280 nm. Clean and dry the QUARTZ cuvette and then measure A280 of your eluted protein solution. If A280 is greater than 2.0, then take dilute 200 microliters of your eluted protein solution and dilute 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 a web site:
    http://us.expasy.org/tools/protparam.html

    This program will provide you with "Abs 0.1% (=1 g/l)", which is the absorbance at 280 nm of a 1 mg/ml solution of the protein. Divide your observed A280 by this number to get the actual mg/ml of protein solution.

You can now calculate both the mg/ml of your protein solution and the total mgs of protein.

Verify protein molecular weight and purity by running SDS-PAGE, followed by Coomassie staining. *I recommend that you do NOT boil or heat your sample before loading on gel, but do keep a reducing agent in your sample buffer.*

Making NMR Samples Starting From Pool Prepared As Described Above

- Protein needs to be 15N-labeled.
- Purify protein off of Ni(II)-agarose as described above.
- Measure weight of solution and A280.
- Add D2O to sample to a concentration of 10%.
- 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. 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).
- Concentrate the solution using either a 2-15 ml capacity centrifugal cartridges that are available from Millipore. (We like the Amicon-Ultra Centrigual Filter Device series). Make sure the molecular weight cut-off of the filter is safely below that of your purified protein (by a factor of at least 2).

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.

Transfer the desired volume (normally 550 microliters) to a high quality 5 mm 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).

Run NMR.

Procedure to make a micelle-like sphere in pymol

Congbao Kang

1. Open pymol and load the structure pdb file in and show cartoon or other formats.
2. Create an atom in pymol (any kind of atom), show this atom in sphere: One way is type in the command editor.attach_amino_acid('pk','ace'),to generate ace molecular, there are three new items are generated on the right side of the screen. Click the "ace" to hide everything, then click on "pk1" and show it in sphere, this item will be shown in sphere mode. This sphere will be the center of your structure.
3. Set the diameter of the sphere scale to be 10 which should be about 36 A in diameter (this should be confirmed by measuring the length of helix inside the sphere, *because other setting of sphere will also affect the diameter*, or you can make two atoms at the same time and let them sitting side by side and measure the distance between the two atoms). Just type in set sphere_scale, 10 (you can always change this as what you like, but the diameter of the sphere should be measured again).
4. Set the sphere transparency to 40% or different which can make the helix structure inside the micelle visible. Type in set sphere_transparency, 0.4.
5. Hide the small label inside the ball. Because we build a new molecular, after set the transparency, you will see one small black "ball like item" which is used for generation of another molecular. Click on pk1 and any other atoms on the protein, this small label will disappear.
6. Move the helix to anywhere you want if want to and save the png file or the state file for later use.
7. To make a better looking sphere, you can also use the ray function, just type ray in command line, then save the rayed figure.

Plotting NMR Spectra for Publication (K. Oxenoid, H.-J. Kim, C. Mobley) (Last update: March 2004)

A. Making TROSY (or HSQC) spectra for a publication.

1. Displaying a single spectrum on one figure
   - 1.1. Process the data appropriately using NMRpipe and open it in the NMRView.
   - 1.2. Adjust the PPM range and reference, if you have to, according to your interest.
   - 1.3. Make postscript file using plot option in NMRView with these parameters: width 8.5-10 (10 is usually the best unless you want to emphasize certain peaks), height 7, linewidth 0. Two things are important in the parameters. First, set the height to 7 (default is 10). Default value is OK only in some cases. So use 7. Second, keep the linewidth 0 if you are dealing with very crowded and zoomed-out spectra, while you can increase it up to 1 (0.5 is OK too) if you are dealing with simple and zoomed-in spectra. The higher the linewidth is set to, the thicker the lines become and you cannot see the individual contour levels.
   - 1.4. Labels (if I have labels), and ppm numbers appear to be bigger. To fix them, either run a small script that can be found in ~/spectra/ called modps.com (i.e. "modps.com spectrum.ps") and that simply changes two entries in the postrscript header or change them in Adobe illustrator or related programs.
   - 1.5. Get the your_spectra.ps in PC computer. Using NMRView on the lab computers automatically saves your spectra files to your structbio account. To access them first login on the PC using your account, (using another person's account makes things more difficult for some applications) and go to network connections and click on 'computers near me' and then click on 'sbserv'. You should see a file with your login name, click on it. Then right click on the .ps file that you want to open and choose open with correl draw. When the correl screen comes up, select 8MB and load text as text (when the loading screen comes up this will make more sense). Then hit OK and wait for the file to load. When you see your spectra in the correl window, hit the OK button on the loading window again to make it disappear. From here you can use correl to adjust parameter such as the titles and figure legends, if you did not do this in step 1.4.
   - 1.6. When you have the spectra the way you want it, make the .ps file into a .pdf file. The easy way to do this is to select the print option in Correl draw and under destination select Adobe pdf. You will be asked to give the file a name and then just hit save and the file will converted into pdf format. The adobe pdf program usually come up after this, but, unless you want to use that program to make additional modifications, all you need to do is close the window.
2. Overlaying spectra in one figure
   - 2.1 Repeat step 1.1 and 1.2.
   - 2.2 Ovelaying can be done in two ways. One (2.3) is overlay them in the NMRView program the other (2.4) is make each ps file and merge them in the Linux or Unix shell.
   - 2.3 Read one trosy dataset in to the windows and draw, then read in everything in the same window. Go to the miscellaneous and overlay and manipulate the option to adjust the color and contour levels. To plot it, follow 1.3-1.6.
   - 2.4 This is what Kirill wrote and attached without any modification.
     - 2.4.1 To display spectra I create a window(s) with inner dimensions 4" height and 3" length. I actually use a ruler to measure it on the screen. Sounds stupid, but it turns out that if window is not same size all the time, the size and position of spectra on paper changes slightly so that it is difficult to overlay them.
     - 2.4.2 All DAGK spectra are referenced in nmrview Dataset->Manage dataset->Reference so that Gly121 CS are same (see exact values in CS table for Super)

2.4.3 To display spectra I use the following ppm ranges: 10.6-6.005 H ppm and 103.1-131.3 N ppm (zoom out); 9.8-6.4 H ppm and 103.1-128.0 N ppm (zoom in). (if 6.0 is used instead of 6.005 then "6" appears on some spectra but not on others due to digital resolution error).

2.4.4 To display spectra in the same window I use overlay option in Attributes->Miscellaneous.

2.4.5 After displaying spectra I create postscript file using plot option in nmrview (right click on the window). Plot parameters are as follows: width is 10, height is 7, linewidth is 1. Labels can be added too.

B. Making strip plots for a publication.

1. Run NMRView.
2. Read in the dataset you want to display, for example HNCA.nv, in NMRView. Create the window for that dataset, adjust the parameters and hit the draw button. Then read in the peak file, for example HNCA.xpk, and integrate the file to the spectra (this may not be needed).
3. Then go to window on the main menu bar and select the strips
4. Create a window , for example HNCA, click param and select what you want but set the width 0.07 which might give a better-looking spectra and set display none or leave it blank. If you set the display in param as HNCA.xpk then it will show up in the strips and make problem when you manipulate the strips. Type the peak number or atom into the "Enter" section as many as you want.
5. After the display is done, do right click and select "plot". Then follow A.1.3-A.1.6.

C. Making chemical shift delta or CSI plots for a publication

1. Once the assignment is done, subtract the observed chemical shifts from random coil values in the "Excel" or equivalent.
2. Open a file called "CSI.xpf" using notepad or Word.
3. There are many things you have to make changes. Amino acid sequence, print direction (portrait or landscape), how many residues per lane, how many things to display in the left column (HA, CA, CB, CO, NOE and so on).
4. Put an exact amount of data of ∆HA, ∆CA, ∆CB, ∆CO, NOE into the blank after each data section.
5. After you put all data in, save the file (you don't need to close the file, so leave it open) and distill it with Adobe distiller which produces the same file with pdf extension, for example, CSI.xpf.pdf.
6. Open CSI.xpf.pdf and check whether everything is correct. If it is not correct, make changes on the CSI.xpf file and distill again. Before you distill your file, you probably delete previous CSI.xpf.pdf file which may not be overwritten.

Chuck's note: 1/7/03: To go from a nice pdf into Word: Open the pdf file in acrobat. Select all. Then choose copy. Go to Word and do copy special/enhance metafile.