MBP-FUSION PROTEIN PURIFICATION PROTOCOL v2.0.0

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1.0 Outline
2.0 Protocol

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(i. Cell storage conditions: see "Cell Growth and Storage" protocol)

(1) Preparation of E. coli soluble fraction for chromatography:

a. Thawing of samples
b. Sonication
c. Centrifugation

(2) First capture of 6 MPB-fusion proteins for TEV cleavage by IMAC:

a. Chromatographic system
b. Column running solutions
c. Column running procedure
d. IMAC
e. Desalting for TEV cleavage

(3) TEV cleavage of the MBP-fusion proteins and IMAC batch cleanup:

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(4) Purification of cleaved protein:

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c. Column running procedure
d. Desalting into appropriate buffer A for ion exchange
e. Ion exchange on MonoQ or MonoS
f. Desalting into appropriate buffer for structural studies

(5) Initial Concentration for hand off to crystallization and NMR screening:

a. Test to determine if detergent is necessary
b. Concentration to 5 mg/ml

(Final concentration for crystallization trials -- first part of crystallization trial protocol)

Addenda:

a. Setups on the AKTA purifier and FPLC
b. Short instructions for running the AKTA purifier and FPLC
c. Preparation of the IMAC resin for batch capture
d. Buffer recipes

2.0 Protocol

(1) Preparation of E. coli soluble fraction for chromatography from 10 g of cells.

(Sample storage -- 6 different proteins with at least 10 g of cells for each protein.)

About 10 to 20 grams of cells per sample are suspended in IMAC-A buffer to give a total volume of 60 ml per sample. The cells are resuspended in IMAC-A before freezing overnight at -20 degrees C.)

a. Thawing of samples -- obtained thawed cells resuspended in cell lysis buffer quickly.

(1) The appropriate amount of the SIGMA cocktail of protease inhibitors with EDTA is added to each tube.

(2) The sample is thawed by placing the container into a beaker of room temperature water on ice.

b. Sonication -- breaking of cells and shearing of DNA at low temperature.

(1) The cells are placed in aluminum tubes mounted in a beaker containing ice water. The sample in each tube is sonicated 8 times for 15 seconds each with 15 seconds cooling in between sonications. After sonication, the 60 ml sample is transferred to two 40 ml centrifuge tubes and placed on ice. All 6 cell samples are sonicated in this way.

(2) A 5 ul sample diluted with 20 ul of water is taken for analysis by SDS-PAGE.

N.B. At this point the sample should have the expected appearance of well-sonicated cells -- non-viscous liquid with a somewhat darker color than before sonication.

c. Centrifugation -- clarification of soluble fraction for chromatography.

The well-sonicated sample is transferred to two 40 ml centrifuge tubes which are balanced using a pan balance. The samples are centrifuged at 25,000 rpm (74,000xG) for 25 minutes. The supernatants are transferred to two new 40 ml centrifuge tubes and centrifuged again for 25 minutes. At this point the samples should be a yellow brown, rather translucent and non-viscous liquid. It is very important that no particles are observed in the samples in order to protect the chromatographic instrument and columns

(1) A 5 ul sample diluted with 20 ul water is taken for analysis by SDS-PAGE. The six samples are ready for the "First capture..." protocol.

(2) First capture of 6 MBP-fusion proteins for TEV cleavage.

Chromatographic System

AKTA purifier equipped with 2D options including a sample pump with air detector to monitor sample loading and FRAC950 fraction collector.

Columns -- 6 IMAC Columns Plus 1 Desalting Column

The six Immobilized Metal Affinity Chromatography (IMAC) columns are each composed of two serially connected 5 ml HiTrap nickel-loaded metal chelating columns (Amersham Pharmacia) equilibrated in 15% IMAC buffer B.

The one desalting column is a 26/10 HP desalting column (Amersham Pharmacia) equilibrated in 3% IMAC-B and capable of desalting 15 ml of applied sample.

Column Running Solutions

Sample buffer (from sample preparation protocol)

IMAC buffer A:

50 mM NaPhosphate, pH 7.7
500 mM NaCl

IMAC buffer B:

50 mM NaPhosphate, pH 7.7
500 mM NaCl
350 mM Imidazole

Desalting buffer (also TEV-cleavage buffer)
97% IMAC buffer A/3% IMAC buffer B

Column Running Procedure -- IMAC Followed by Desalting

(1) Loading onto IMAC column plus initial 40 ml wash is done sequentially to minimize proteolysis. The six approx. 55 ml samples are loaded sequentially onto the IMAC columns via a separate sample pump onto the column at a flow rate of 2.5 ml/minute.An installed air sensor detects the end of each sample. The column is washed with 40 ml of 15% IMACB at 2.5 ml/minute. In between the loading plus 40 ml wash of each sample and that of the next sample, and after that of the sixth sample, the sample pump and associated tubing are washed with 30 ml of IMAC-A buffer at 10 ml/minute to minimize cross-contamination between samples.

(2) Washing and eluting and desalting are repeated 6 times, once for each sample.

a. The column is washed with 40 ml of 15% IMAC-B at 2.5 ml/minute.

b. The bound MBP-fusion protein is eluted with 40 ml of 100% IMAC-B at 2.5 ml/minute. The first 15 ml of the eluted protein is collected by a peak detection method and directed to a 25 ml sample loop.

c. The 15 ml in the sample loop is desalted using the desalting column and 80 ml of 3% IMAC-B as the column running buffer at 8.0 ml/minute. The desalted protein is collected by a peak detection method and the approx. 25 ml peak is directed to a 50 ml Falcon tube. "Desalted" in this procedure really refers to removal of 97% of the imidazole as the NaCl remains the same - 500 mM.

(3) A 25 ul sample is taken for analysis by SDS-page.

(4) A 200 ul sample is diluted to 1 ml using MQ water. The A280 nm of this 1 ml of diluted sample of MBP-fusion protein is recorded by measuring the UV/VIS spectrum from 850 to 250 nm (1 ml disposable cuvette, Cary 50). This 1 ml sample is recovered and saved for ICP metal and mass spectroscopic analysis.

The approx. 25 ml of desalted protein is ready for the "TEV-Cleavage" protocol.

(3) TEV-cleavage of MBP-fusion proteins and removal of the 6 His-tagged proteins from the cleavage mixture and SDS-PAGE analysis of the results.

TEV Proteolysis of MBP-Fusion Protein

(1) To each approx. 25 ml sample is added 1 ml of TEV-protease (1 mg/ml or greater). The samples are placed in a cold box for overnight cleavage.

(2) After overnight cleavage, a 25 ul sample of each cleavage mixture is taken for analysis by SDS-PAGE. Also, the state of "cloudiness" of the mixture is recorded.

IMAC Batch Capture of 6 His-tagged Proteins Remaining in the Cleavage Mixture

(1) Each tube of cleavage mixture is decanted into a 50 ml Falcon tube containing approx. 6 ml of packed, nickel-loaded metal chelating resin. (Here we could use the "better" tetra-chelating resin to reduce nickel contamination after this point.) The cleavage mixture and the resin are gently resuspended.

(2) Each tube is placed onto a roller drum in the cold box and incubated for 20 minutes and then centrifuged at 750 g in a table top centrifuge for 10 minutes. A 25 ul sample of each supernatant is taken for SDS-PAGE analysis. Each supernatant which is judged by SDS-PAGE analysis to contain a reasonable amount of an A. thaliana protein is filtered into a new 50 ml falcon tube. The protein sample is now ready for the "Purification of Cleaved Protein" protocol, or may be suitable already for the "Concentration for NMR and Crystallization" protocol.

SDS-Analysis of Results

(1) The five 25 ul samples taken of each protein prep (1--whole cell, 2--soluble fraction, 3--pre-cleavage MBP-fusion protein, 4--post-cleavage mixture, and 5--after IMAC batch) are analyzed by SDS-PAGE.

(2) A decision is made about the suitability of each protein for NMR or crystallization, for further purification or banishment to "triage". The decision consists of deciding if there is enough of the cleavage product, i.e., the A. thaliana protein, to merit further work. The cleavage should appear as a single band on the Coomassie-stained gel, or at most two distinct bands, should have the correct molecular weight and should be roughly estimated to total 20 mg (amount here is negotiable!).

N.B. The SDS-PAGE analysis is also useful for other diagnostic purposes such as percentage of "soluble" protein and also the percentage of stable, non-aggregated AT protein produced by the MPB-fusion system. For example, aggregation of the AT protein can be diagnosed if it appears in SDS-PAGE post-cleavage sample but does not appear in the "after IMAC batch capture sample. Also, the two A280 nm readings recorded pre-cleavage and after the IMAC batch step will tell if the AT protein disappears.

(4) Purification of cleaved protein and initial concentration for crystallography and NMR pipelines.

N.B. For this polishing ion-exchange step, the proteins can be chosen to all lie in a certain range of calculated pI's so as to make the process more efficient.. In this way, the same desalting and column running buffers can be used for a group of proteins. Or else, several

desalting columns can be installed on the AKTA FPLC and each could be equilibrated with a particular desalting buffer.

Chromatographic system- AKTA FPLC with FRAC950 fraction collector equipped with 2D options including sample pump and air detector to monitor sample loading and a FRAC-950 fraction collector.

Columns

10/10 MonoS -- for proteins with a calculated $pI > 7$

10/10 MonoQ -- for protein with a calculated $pI < 7$

2 HP 26/10 desalting columns connected in series (one may suffice)

Column Running Solutions

MonoS Buffers:

For proteins with $7.0 < pI < 8.5$
MonoS pH6 bufferA 50 mM MES, pH 6.0
MonoS pH6 bufferB " " + 1M NaCl

For proteins with $pI > 8.5$
MonoS pH7 bufferA 50 mM NaPhosphate, pH 7.0
MonoS pH7 bufferB " "+ 1M NaCl

MonoQ Buffers:

For proteins with $pI < 5.5$

MonoQ pH7 bufferA 20 mM bis-Tris propane, pH 7.0
MonoQ pH7 bufferB " " + 1 M NaCl

For proteins with $5.5 < pI < 7.0$

MonoQ pH8 bufferA 20 mM Tris, pH 8.0
MonoQ pH8 bufferB " " + 1 M NaCl

Desalting Buffers

Pre-ion exchange:

Composed of 5% bufferB, 95% bufferA chosen for the ion exchange step.

Post-ion exchange:

For crystallography --three choices for pH based on calculate pI:

(1) pH 6.0 for (pI <5) or (7.0 < pI < 8.0)
10 mM MES, pH 6.0 + 100 mM NaCl

(2) pH 7.0 for (5.0 < pI < 6.0) or (8.0 < pI < 9.0)
10 mM HEPES, pH 7.0 + 100 mM NaCl

(3) pH 8.0 for (6.0 <pI < 7.0) or (pI > 9.0)
10 mM TRIS, pH 8.0 + 100 mM NaCl

(4) For NMR -- always NaPhosphate but need input on pH.

Desalting for Ion Exchange followed by Ion Exchange

The desalting and ion exchange are done in 1 run using the 2-D capabilities of the AKTA FPLC system.

Desalting for Ion Exchange:

The column is run at 8 ml/minute. The sample from the IMAC batch capture step is in approximately 28 ml. About 14 ml of the sample is loaded onto the desalting column via a sample pump with air sensor to detect the end of the sample. The sample is desalted using 80 ml of the appropriate 95%bufferA/5%bufferB for the chosen ion exchange column. The desalted protein is collected by a timed method and directed into a 30 ml sample loop. The total mg of protein is estimated from the chromatogram.

Ion Exchange:

The column is run at 5 ml/minute. The appropriate 10/10 Mono column is equilibrated with 40 ml of 5% buffer B followed by injection of the desalted sample in the 30 ml loop. The column is washed with 30 ml of 5% buffer B. A gradient elution up to 50% buffer B of 160 ml duration (20 column volumes) is performed followed by 20 ml wash of 50% buffer B. This is followed by 40 ml of 100% buffer B and 40 ml of 5% buffer B. There are 30 4 ml fractions during the elution step.

Examination of the Ion Exchange Chromatogram-- Is there a peak?

The chromatography is examined to see if there is one major peak of protein eluted during the column run. The purity of the loaded sample is known from the previous SDS-

PAGE analysis, therefore it should be relatively easy to identify the AT protein peak. If not, SDS-PAGE analysis should be performed.

Final Desalting for Crystallization or NMR:

The column is run at 8 ml/minute. The chosen ion-exchange elution peak or post-IMAC batch capture sample of a pure A. thaliana protein is loaded using the sample pump and desalted using the appropriate buffer from the list above. The desalting column is equilibrated with 80 ml of the appropriate buffer. The sample is injected onto the column and eluted with 50 ml of buffer. The desalted protein is collected by a timed method and directed to a 50 ml falcon tube.

(5) First Concentration for crystallography and NMR.

(1) Test to determine if detergent is necessary for concentrating a protein.This tests uses Microcon concentrators to attempt to assay how well a protein will concentrate. About 200 ul is loaded into a Microcon concentration unit with a nominal MWCO of 10 K (or lower if necessary, 3 K) which was washed one time with the appropriate buffer. The concentrator is loaded into a refrigerated microfuge and spun for 10 minutes at the appropriate g-force. The Microcon is removed and the eluate and retentate are examined. A positive result is an easily concentrated protein with no visible precipitant. A negative result is little concentration and/or visible precipitant. Of course there will be intermediate results which are hard to judge. The detergent LDAO is added to those samples with a negative result to the concentration of 0.005% using a stock solution in MQ H20 of 2%. This is a dilution factor of 1:400.

(2) Concentration to 5 mg/ml. Samples are loaded into an appropriately sized AmiconUltra concentration unit with a nominal MWCO of 10 K (lower if necessary, 5 K) and concentrated until a concentration of approximately 5 mg/ml is reached. Concentration is assayed by diluting 10 ul of the concentrate into a 1 ml solution of the same buffer and doing a wavelength scan from 310 nm to 260 nm and using the calculated absorption extinction coefficient at 280 nm to estimate the protein concentration. The 1 ml of 100-fold diluted protein used to measure the spectrum is discarded. When the 5 mg/ml target is reached, the sample is now ready for the NMR protocol or the crystallization protocol.

Setup on AKTA Purifier for IMAC Capture Step

Column Valve:

1 Bypass
2 10 ml IMAC
3 10 ml IMAC
4 10 ml IMAC

5 10 ml IMAC
6 10 ml IMAC
7 10 ml IMAC
8 HPdesalting 26/10

Buffers:

S1-S6: Cell supernatants in IMAC-A for the "Run6sample" program or IMAC low pH "Strip" buffer for the "Stripandcharge6" program (see below); 0 mM MES, 1 M NaCl, 50 mM EDTA, 0.5% NaN3, pH 5.5

S7

S8 Sample buffer for washing sample pump

A1-1 IMAC-A
A1-2
A1-3
A1-4
A1-5
A1-6
A1-7 0.5M NaOH for more thorough cleaning of IMAC columns
A1-8 MilliQ H2O
A2 nothing
B1 IMAC-B
B2 100 mM Ni+2-containing "Charge" solution for IMAC columns

Setup on AKTA FPLC for Polishing Step

Column Valve:

1 Bypass
2: 8 ml monoQ, anion exchange
3 8 ml monoS, cation exchange
4 Superdex200 10/30 plus Superdex5 10/30, gel filtration
5 HPdesalting 26/10, buffer exchange
6 HiTrapdesalting 10 ml, buffer exchange
7 free for users
8 free for users

Buffer Pairs for Anion Exchange:

A1: 20 mM BTP, pH 7.0
B1: 20 mM BTP + 1 M NaCl, pH 7.0

A2: 20 mM Tris, pH 8.0
B2: 20 mM Tris + 1 M NaCl, pH 8.0

Buffer Pairs for Cation Exchange:

A3: 50 mM MES, pH 6.0
B3: 50 mM MES + 1 M NaCl, pH 6.0

A4: 20 mM NaPi, pH 7.0
B4: 20 mM NaPi + 1 M NaCl, pH 7.0

Buffers for Final Desalting:

A5: 10 mM MES, 100 mM NaCl, pH 6.0
A6: 10 mM HEPES, 100 mM NaCl, pH 7.0
B5: 10 mM Tris +100 mM NaCl, pH 8.0

Buffer Slots Not Assigned:

A7: Free for users
A8: H2O
B6: Free for users
B7: Free for users
B8: H2O

Short Instructions for Running the AKTA Purifier Programs

2/28/03 Three programs are available to facilitate:

(1) Stripping and recharging the 6 IMAC columns.

(2) Equilibrating the 6 IMAC columns with 15% IMAC-B.

(3) Loading, washing, eluting, and desalting 6 samples.

At this date, manual intervention is required in between running the three programs. Each day empty the two 4 L graduated cylinders which catch waste liquid from the fraction collector and the various waste lines.

Before the "Stripandcharge6" program:

(1) Place sample lines 1 to 6 in a 500 ml bottle containing MQ water and manually run MQ water through each sample line until the Air Sensor reads "no air."

(2) Place sample line 1 in a container of 100% ethanol and run 100% ethanol through sample line 1 at 20 ml/min until the sample pressure stabilizes (approx. 40 ml).

(3) Place sample line 1 back in the bottle of MQ water and run MQ water through sample line 1 at 20 ml/min for about 2 minutes.

(4) Place sample lines 1 to 6 in a 500 ml bottle containing "Strip" buffer up to the very top (the programs requires 480 ml of "Strip" buffer).

(5) Ensure that buffer bottle "A1-8" contains at least 500 ml of MQ water.

(6) Ensure that buffer bottle "B2" contains at least 500 ml of "Charge" solution.

Now, run the "Stripandcharge6" program. The 6 IMAC columns should be a pale blue. After running "Stripandcharge6" and before running "Equilibrate6":

(1) Place sample lines 1 to 6 in a 500 ml bottle containing IMAC-A.

(2) Place sample line 8 in buffer bottle "S8 Wash" containing at least 500 ml of IMAC-A buffer.

(3) Ensure that buffer bottle "A1-1" is full of IMAC-A buffer(2l).

(4) Ensure that buffer bottle "B1" contains at least 1 L of IMAC-B buffer.

Now, run the "Equilibrate6" program. The 6 IMAC columns should be a medium blue which is somewhat darker than before.

After running "Equilibrate6" and before running "Run6samples":

(1) Place sample lines 1 to 6 in the 100 ml graduated cylinders which contain the samples.

(2) Ensure that buffer bottle "A1-1" is completely full of IMAC-A buffer to the very top.

(3) Ensure that buffer bottle "B1" contains at least 1 L of IMAC-B buffer.

(4) Put 15 ml tubes in the first 6 rows of the fraction collector and 50 ml tubes in all 8 slots of the fraction collector.

Now, you can run the "Un6samples" program and hope for the best. After the fifth or sixth sample is loaded, click on "Pause" to suspend the program. Fill the buffer bottle "A1-1" with IMAC-A. Click on "Continue" to allow the program to finish. Start the cycle again with the "Before the "Stripandcharge6" program" section.

Short instructions for running the AKTA FPLC (2/28/03):

Two types of programs are used on the FPLC for the CESG protein purification pipeline operations. These programs are run after the triage step which requires a decision about the purity of the TEV-cleaved Arabidopsis thaliana protein.

If it is decided that the protein is very pure, then a "Final Desalting" program is run to place the protein in the buffer used for crystallization. If, on the other hand, the protein is judged to require further purification, then a "Desalting Plus Mono" program is run. This type of program will first change the buffer of the protein sample and then run the appropriate ion exchange column to purify the protein.

At this date, manual intervention is required before running the either of the two types of programs.

Thus, before running either a "Final desalting" or a "Desaltplusmono" program:

(1) Place sample line 1 in MQ water and manually run MQ water through sample line 1 until the Air Sensor reads no air.

(2) Place sample line 1 in a container of 100% ethanol and run 100% ethanol through sample line 1 at 20 ml/min until the sample pressure stabilizes (approx. 40 ml).

(3) Place sample line 1 back in the bottle of MQ water and run MQ water through sample line 1 at 20 ml/min for about 2 minutes.

(4) Click on "End" to reset the system parameters.

(5) Ensure that the appropriate buffer bottles contain the required volume of buffer. See the list below to ascertain which buffers are used by which programs and how much of each buffer is needed.

(6) Place sample line 1 in a 100 ml graduated cylinder which contains the protein sample which much consist of at least 6 ml.

(7) Place one 50 ml falcon tube in the X1 slot on the fraction collector.

(9) Place 15 ml test tubes in the A through F rows on the fraction collector.

Now, either the "Final Desalting" or a "Desaltplusmono" program may be run. The "Final Desalting" program will take about 25 minutes to run and the "Desaltplusmono" program will take about 95 minutes. A maximum of 14 ml of sample can be loaded in each run of either program, and at least 6 ml of sample is required. If the same sample is run a second time and no air bubbles have entered sample line 1, then it is not necessary to perform steps 1 through 3 above.

FPLC Programs:

These programs are found inside the "CESG2d" folder in the program area. Follow the directory tree to find the appropriate program based on the calculated isoelectric point, the "pI", of the A. thaliana protein in the sample.

"Final Desalting" type programs require that the buffer bottle which is used contain at least 500 ml of buffer.

(1) FinaldesaltpIlt5 uses the buffer bottle labeled "A5 Final Desalt pH6".
(2) FinaldesaltpI5to6 uses the buffer bottle labeled "A6 Final Desalt pH7".
(3) FinaldesaltpI6to7 uses the buffer bottle labeled "A7 Final Desalt pH8".
(4) FinaldesaltpI7to8 uses the buffer bottle labeled "A5 Final Desalt pH6"
(5) FinaldesaltpI8to9 uses the buffer bottle labeled "A6 Final Desalt pH7".
(6) FinaldesaltpIgt9 uses the buffer bottle labeled "A7 Final Desalt pH8".

Each of the "Desalt plus mono" type programs uses a pair of buffers. The "A" buffer has no salt and the "B" buffer has 1 M salt. The "A" buffer bottle must contain at least 1 L of buffer and the "B" buffer bottle must contain at least 500 ml of buffer.

(1) pIlt5p5monoQ uses the buffer bottles labeled "A1 MonoQ pH7" and "B1 MonoQ pH7 1M NaCl".

(2) pI5p5to7monoQ uses the buffer bottles labeled "A2 MonoQ pH8" and "B2 MonoQ pH8 1M NaCl".

(3) pI7to8p5monoS uses the buffer bottles labeled "A3 MonoS pH6" and "B3 MonoS pH6 1M NaCl".

(4) pIgt8p5monoS uses the buffer bottles labeled "A4 MonoS pH7" and "B4 MonoS pH7 1M NaCl".

Preparation of IMAC Batch Capture Resin

This procedure prepares about 50 ml of Ni2+-loaded IMAC resin for the batch capture step. The procedure takes several hours because there are ten "settling" steps which require each at least 30 minutes. Thus, this procedure should be started early in the morning the day before the batch capture step is performed.

Several of the steps call for decanting the supernatant into a 1 L beaker. These decanted supernatants should be allowed to settle and the settled resin recovered for future use.

Newly purchased IMAC resin comes in a 20% ethanol solution and should be prepared starting at step 5 with a water wash.

(1) Recover the approx. 50 ml of resin from the six 50 ml falcon tubes used to perform the batch capture step into a 250 ml bottle.

(2) Add MQ water up to the 250 ml mark and resuspend the resin. Allow the resin to settle completely: about 30 minutes. Decant the supernatant carefully into a 1 L beaker trying not to lost very much of the settled resin.

(3) Repeat step 2.

(4) Add "Strip" buffer up to the 250 ml mark and resuspend the resin. Allow the resin to settle completely. Decant the supernatant carefully into the same 1 L beaker.

(5) Repeat step 4. At this point the resin should be white.

(6) Repeat step 2.

(7) Repeat step 2.

(8) Add "Charge" buffer up to the 250 ml mark and resuspend the resin. Allow the resin to settle completely. Pour off and save the supernatant into a 1 L bottle again carefully.

(9) Repeat step 2.

(10) Repeat step 2. The resin should be a light blue at this point.

(11) Add a solution of 97% IMAC-A/3% IMAC-B up to the 250 ml mark and resuspend the resin. Distribute the approx. 250 ml of suspended resin into 6 new 50 ml falcon tubes. Centrifuge the 6 tubes at 1500 rpm for 10 minutes in the table top centrifuge. Decant the supernatant into a 1 L beaker. At this point the resin should be a somewhat darker blue than after step 10.

The six 50 ml falcon tubes with approx. 6 ml of Ni2+-loaded IMAC resin each are now ready for the batch capture step.

Protocol for SDS-Gel Sample Prep for Polishing Step

The peak heights on peaks in the salt gradients performed on the Mono columns are hard to predict from one run to the next. The volume of sample loaded on the SDS-gels will therefore vary from run to run. Very dilute samples resulting from small peaks will require trichloroacetic acid precipitation of the protein in the sample in order to reduce the volume of sample to an amount which is reasonable to load on the SDS-gel, <=15ul. Use this protocol to first decide if TCA precipitation is required and then to determine how to perform the TCA precipitation.

(1) If the peak height is 250 mAU or more, then no TCA precipitation is required. For each fraction in the peak, simply take 15 ul, dilute with 15 ul of loading buffer, heat and load as normal.

(2) For peak heights(PH) less than 250 mAU, use the following formula to determine the sample volume (SV): SV=(250/PH)*15 ul. So, for example a peak height (PH) = 50 requires a SV=75 ul.

(3) Add to each sample an amount of 70% TCA according to the following formula: TCA=SV/5. Thus a 75 ul sample requires 15 ul of 70% TCA.

(4) Mix the samples and TCA thoroughly by vortexing and allow to sit for 10 minutes or more on ice.

(5) Centrifuge the samples for 10 minutes at the highest speed in the microfuge at 4 degrees C.

(6) Remove the supernatant carefully using a pipetteman and turn the eppendorfs upside down on a paper towel to dry a bit.

(7) Add 10 ul of water to each tube and use the pipette tip to resuspend the precipitated protein. This is done by scraping the bottom of the tube and pipetting in and out of the pipette tip the 10 ul of water. You won"t always be able to see the precipitated protein, but you still need do the resuspension.

(8) Add 15 ul of loading buffer and mix. Some samples will be yellow at this point.

(9) To the yellow samples, add 1 ul of 1.5 M Tris, pH 8.5 and mix the sample. Check that the sample is now blue. Repeat the 1 ul of Tris addition until the sample is blue.

(10) At this point, the sample can be treated by boiling for 5 minutes before loading onto the SDS-gel.

IMAC-A and IMAC-B Buffer Preparation

(1) Eight liters of IMAC-A and four liters of IMAC-B are made at the same time using three 4 L graduated cylinders to mix and adjust the pH of the solutions. The solutions will be stored in a total of six 2 L bottles.

(2) All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

(3) Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

(4) The IMAC-A is filtered first and then the IMAC-B using the same 500 ml bottle-top filter unit.

For IMAC-A (Make 8 liters total using two 4 L graduated cylinders):

Chemical: NaPhosphate (anhydrous monobasic)
MW: 119.96
g per 4 liters: 24.0

Chemical: NaCl
MW: 58.44
g per 4 liters: 116.9

Chemical: NaOH
MW: 40.00
g per 4 liters: 7.5

(1) Weigh out the three chemicals and add to a 4 L graduated cylinder.

(2) Add MQ water to the 4 l mark and add a large stir bar.

(3) Stir until dissolved and measure the pH.

(4) The pH should be near to pH 7.7. Adjust if necessary using either 10 M NaOH or 50% HCl.

(5) Filter directly into dry, previously rinsed 2 L bottles.

(6) Label the bottle "A1-1 IMAC-A" using a printed label and store at 4 degrees C.

For IMAC-B:

Make 4 liters using one 4 L graduated cylinder.

Chemical: NaPhosphate (anhydrous monobasic)
MW: 119.96
g per 4 liters: 24.0

Chemical: NaCl
MW: 58.44
g per 4 liters: 116.9

Chemical: Imidazole
MW: 68.08
g per 4 liters: 95.3

(1) Weigh out the three chemicals and add to a 4 L graduated cylinder.
(2) Add MQ water to the 3.8 l mark and add a large stir bar.
(3) Stir until dissolved and measure the pH.
(4) The pH start near pH 8.0 and should be adjusted to pH 7.7 using 50% HCl.
(5) Filter directly into dry, previously rinsed 2 L bottles.
(6) Label the bottles "B1 IMAC-B" using a printed label and store at 4 degrees.

Strip Buffer for IMAC Columns:

Four liters of the strip buffer are made using a 4 L graduated cylinders to mix and adjust the pH of the solution. The solution will be stored in two 2 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

Strip Buffer:

Make 4 liters total using one 4 L graduated cylinder:

Chemical: MES monohydrate
MW: 213.3
g per 4 liters: 42.7

Chemical: NaCl
MW: 58.44
g per 4 liters: 233.8

Chemical: EDTA
MW: 292.25
g per 4 liters: 58.4

Chemical: NaAzide
MW:
g per 4 liters: 20.0

(1) Put a large stir bar in the 4 L graduated cylinder. Weigh out the four chemicals and add them to the graduated cylinder.

(2) Add MQ water to the 3.8 l mark.

(3) Stir until dissolved and measure the pH.

(4) The pH should be adjusted to pH 5.5. Adjust using 50% HCl. Top up to the 4 l mark with MQ water and stir.

(5) Filter directly into dry, previously rinsed 2 L bottles.

(6) Label the bottles "Strip" using a printed label and store at 4 degrees C.

Charge Buffer for IMAC Columns:

Four liters of the charge buffer are made using a 4 L graduated cylinder to mix and adjust the pH of the solution. The solution will be stored in two 2 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

Charge Buffer:

Make 4 liters total using one 4 L graduated cylinder.

Chemical: Nickel chloride
MW: 237.7
g per 4 liters: 95.1

OR:

Chemical: Nickel sulfate
MW: 262.86
g per 4 liters: 104.8

(1) Put a large stir bar in the 4 L graduated cylinder. Weigh out the one chemical and add it to the graduated cylinder.

(2) Add MQ water to the 4.0 l mark.

(3) Stir until dissolved.

(4) Filter directly into dry, previously rinsed 2 L bottles.

(5) Label the bottles "Charge" using a printed label and store at 4 degrees C.

Ion Exchange Buffer Pair A1 and B1:

Four liters of a buffer A1 and two liters of the buffer B1 are made at the same time using one 4 L graduated cylinder and one 2 L graduated cylinder to mix and adjust the pH of the solutions. The solutions will be stored in a total of two 2 L bottles and two 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

The 4 liters of buffer A1 is filtered first and then 2 liters of buffer B1 using the same 500 ml bottle-top filter unit.

Buffer: A1
Chemical: Bistrispropane
MW: 282.3
g per 4 liters: 22.6
pH: 7.0

(1) Weigh out the one chemical and add it to a 4 L graduated cylinder.

(2) Add MQ water to the 3.8 l mark and add a large stir bar.

(3) Stir until dissolved and measure the pH.

(4) The pH begins at about pH 10.6 and should be adjusted to pH 7.0 using 50% HCl.

(5) Filter directly into dry, previously rinsed 2 L bottles.

(6) Label the bottles "A1 MonoQ pH7" using a printed label and store at 4 degrees C.

Buffer: B1
Chemical: Bistrispropane
MW: 282.3
g per 2 liters: 11.3
pH: 7.0

Buffer: B1
Chemical: NaCl
MW: 58.44
g per 2 liters: 116.9
pH:

(1) Add a large stir bar to the 2 L graduated cylinder. Weigh out the two chemicals and add to the 2 L graduated cylinder.

(2) Add MQ water to the 1.9 l mark.

(3) Stir until dissolved and measure the pH.

(4) The pH starts near pH 10.6 and should be adjusted to pH 7.0 using 50% HCl.

(5) Filter directly into dry, previously rinsed 1 L bottles.

(6) Label the bottles "B1 MonoQ pH7 + 1M NaCl" using a printed label and store at 4 degrees C.

Ion Exchange Buffer Pair A2 and B2:

Four liters of a buffer A2 and two liters of the buffer B2 are made at the same time using one 4 L graduated cylinder and one 2 L graduated cylinder to mix and adjust the pH of the solutions. The solutions will be stored in a total of two 2 L bottles and two 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

The 4 liters of buffer A2 is filtered first and then two liters of buffer B2 using the same 500 ml bottle-top filter unit.

Ion Exchange Buffer Pair A2 and B2:

Buffer: A2
Chemical: Tris
MW: 121.1
g per 4 liters: 9.7
pH: 8.0

(1) Weigh out the one chemical and add it to a 4 L graduated cylinder.

(2) Add MQ water to the 3.8 l mark and add a large stir bar.

(3) Stir until dissolved and measure the pH.

(4) The pH begins at about pH 10.4 and should be adjusted to pH 8.0 using 50% HCl. Top up with MQ water to the 4 l mark, and stir.

(5) Filter directly into two dry, previously rinsed 2 L bottles.

(6) Label the bottles "A2 MonoQ pH8" using a printed label and store at 4 degrees C.

Buffer: B2
Chemical: Tris
MW: 121.1
g per 4 liters: 4.8
pH: 8.0

Buffer: B2
Chemical: NaCl
MW: 58.44
g per 4 liters: 116.9
pH:

(1) Add a large stir bar to the 2 L graduated cylinder. Weigh out the two chemicals and add to the 2 L graduated cylinder.

(2) Add MQ water to the 1.9 l mark.

(3) Stir until dissolved and measure the pH.

(4) The pH starts near pH 10.1 and should be adjusted to pH 8.0 using 50% HCl. Top up with MQ water to the 2 l mark and stir.

(5) Filter directly into two dry, previously rinsed 1 L bottles.

(6) Label the bottles "B2 MonoQ pH8 + 1 M NaCl" using a printed label and store at 4 degrees C.

Ion Exchange Buffer Pair A3 and B3:

Four liters of a buffer A3 and two liters of the buffer B3 are made at the same time using one 4 L graduated cylinder and one 2 L graduated cylinder to mix and adjust the pH of the solutions. The solutions will be stored in a total of two 2 L bottles and two 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

The 4 liters of buffer A3 is filtered first and then two liters of buffer B3 using the same 500 ml bottle-top filter unit.

Ion Exchange Buffer Pair A3 and B3:

Buffer: A3
Chemical: MES
MW: 213.3
g per 4 liters: 42.6
pH: 6.0

(1) Weigh out the one chemical and add it to a 4 L graduated cylinder.

(2) Add MQ water to the 3.8 l mark and add a large stir bar.

(3) Stir until dissolved and measure the pH.

(4) The pH begins at about pH 4.0 and should be adjusted to pH 6.0 using 50% HCl. Top up with MQ water to the 4 l mark and stir.

(5) Filter directly into dry, previously rinsed 2 L bottles.

(6) Label the bottles "A3 MonoS pH6" using a printed label and store at 4 degrees C.

Buffer: B3
Chemical: MES
MW: 213.3
g per 4 liters: 21.3
pH: 6.0

Buffer: B3
Chemical: NaCl
MW: 58.44
g per 4 liters: 116.9
pH:

(1) Add a large stir bar to the 2 L graduated cylinder. Weigh out the two chemicals and add to the 2 L graduated cylinder.

(2) Add MQ water to the 1.9 l mark.

(3) Stir until dissolved and measure the pH.

(4) The pH starts near pH 3.6 and should be adjusted to pH 6.0 using 50% HCl. Top up with MQ water to the 2 l mark and stir.

(5) Filter directly into dry, previously rinsed 1 L bottles.

Label the bottles "B3 MonoS pH6 + 1 M NaCl" using a printed label and store at 4 degrees C.

Ion Exchange Buffer Pair A4 and B4:

Four liters of a buffer A4 and two liters of the buffer B4 are made at the same time using one 4 L graduated cylinder and one 2 L graduated cylinder to mix and adjust the pH of the solutions. The solutions will be stored in a total of two 2 L bottles and two 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

The 4 L of buffer A4 is filtered first and then 2 L of buffer B4 using the same 500 ml bottle-top filter unit.

Buffer: A4
Chemical: NaPhosphate anhydrous
MW: 119.96
g per 4 liters: 9.6
pH: 7.0

(1) Weigh out the one chemical and add it to a 4 L graduated cylinder.
(2) Add MQ water to the 3.8 l mark and add a large stir bar.
(3) Stir until dissolved and measure the pH.
(4) The pH begins at about pH 4.1 and should be adjusted to pH 7.0 using 10 M NaOH. Top up with MQ water to the 4 l mark and stir.
(5) Filter directly into dry, previously rinsed 2 L bottles.
(6) Label the bottles "A4 MonoS pH7" using a printed label and store at 4 degrees C.

Buffer: B4
Chemical: NaPhosphate (monobasic anhydrous)
MW: 119.96
g per 2 liters: 4.8
pH: 7.0

Buffer: B4
Chemical: NaCl
MW: 58.44
g per 2 liters: 116.9
pH: 7.0

(1) Add a large stir bar to the 2 L graduated cylinder. Weigh out the two chemicals and add to the 2 L graduated cylinder.
(2) Add MQ water to the 1.9 l mark.
(3) Stir until dissolved and measure the pH.
(4) The pH starts near pH 4.0 and should be adjusted to pH 7.0 using 10 M NaOH. Top up with MQ water to the 2 l mark, and stir.
(5) Filter directly into dry, previously rinsed 1 L bottles.
(6) Label the bottles "B4 MonoS pH7 + 1 M NaCl" using a printed label and store at 4 degrees C.

Final Desalting Buffer A5:

Four liters of a buffer A5 are made using one 4 L graduated cylinder to mix and adjust the pH of the solution. The solutions will be stored in a total of four 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

Buffer: A5
Chemical: MES
MW: 213.3
g per 4 liters: 8.5
pH: 6.0

Buffer: A5
Chemical: NaCl
MW: 58.44
g per 4 liters: 23.4
pH:

(1) Weigh out the two chemicals and add them to a 4 L graduated cylinder.
(2) Add MQ water to the 3.8 l mark and add a large stir bar.
(3) Stir until dissolved and measure the pH.
(4) The pH should be adjusted to pH 6.0 using 10 M NaOH or 50% HCl. Top up with MQ water to the 4 l mark and stir.

(5) Filter directly into dry, previously rinsed 1 L bottles.
(6) Label the bottles "A5 Final Desalt pH6" using a printed label and store at 4 degrees C.

Final Desalting Buffer A6:

Four liters of a buffer A6 are made using one 4 L graduated cylinder to mix and adjust the pH of the solution. The solution will be stored in a total of four 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

Buffer: A6
Chemical: HEPES
MW: 238.3
g per 4 liters: 9.6
pH: 7.0

Buffer: A6
Chemical: NaCl
MW: 58.44
g per 4 liters: 23.4
pH:

(1) Weigh out the two chemicals and add them to a 4 L graduated cylinder.
(2) Add MQ water to the 3.8 l mark and add a large stir bar.
(3) Stir until dissolved and measure the pH.
(4) The pH should be adjusted to pH 6.0 using 10 M NaOH or 50% HCl. Top up with MQ water to the 4 l mark and stir.
(5) Filter directly into dry, previously rinsed 1 L bottles.
(6) Label the bottles "A6 Final Desalt pH7" using a printed label and store at 4 degrees C.

Final Desalting Buffer B5:

Four liters of a buffer B5 are made using one 4 L graduated cylinder to mix and adjust the pH of the solution. The solutions will be stored in a total of four 1 L bottles.

All of the glass- and plasticware is rinsed in MQ water. The bottles are turned upside down on paper towels in order to dry.

Please check that the MW's on the chemical bottles correspond to those in the recipe. Make a note if a different MW form of a chemical is used and how many grams were used. The MW/grams ratio should be identical.

Buffer: B5
Chemical: Tris
MW: 121.1
g per 4 liters: 4.8
pH: 8.0

Buffer: B5
Chemical: NaCl
MW: 58.44
g per 4 liters: 23.4
pH:

(1) Weigh out the two chemicals and add them to a 4 L graduated cylinder.
(2) Add MQ water to the 3.8 l mark and add a large stir bar.
(3) Stir until dissolved and measure the pH.
(4) The pH should be adjusted to pH 8.0 using 10 M NaOH. Top up with MQ water to the 4 l mark and stir.
(5) Filter directly into dry, previously rinsed 1 L bottles.
(6) Label the bottles "B5 Final Desalt pH8" using a printed label and store at 4 degrees C.