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# NESG Protein Production Standard Operations Protocol

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CLONING
Author: Dongyan Wang
Last Revision: December 2005

Summary

This is the SOP for high throughput cloning and expression for NESG targets in Montelione lab at Rutgers University. The target information is obtained from the bioinformatics team of the lab and primers synthesized by Operon. After the target sequences are cloned into vectors and screened for expression and solubility, the right clones are passed on to the fermentation team for larger scale production for NMR studies. Data is entered into SPINE construct and expression database and reagents are archived.

1. Material Checklist:

1.1 Reagents.

<table>
<thead>
<tr>
<th>Name</th>
<th>Source/Vendor</th>
<th>Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Primer Plates</td>
<td>Operon</td>
<td></td>
</tr>
<tr>
<td>Advantage HF-2 PCR Kit</td>
<td>BD Biosciences</td>
<td>639123</td>
</tr>
<tr>
<td>Advantage-GC Genomic PCR Kit</td>
<td>BD Biosciences</td>
<td>639117</td>
</tr>
<tr>
<td>Template Genomic DNA</td>
<td>ATCC</td>
<td></td>
</tr>
<tr>
<td>Vectors</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>Qiaquick 96 PCR BioRobot Kit</td>
<td>Qiagen</td>
<td>963141</td>
</tr>
<tr>
<td>Qiaprep 96 Turbo BioRobot Kit</td>
<td>Qiagen</td>
<td>962141</td>
</tr>
<tr>
<td>Restriction Enzymes/Buffers/BSA</td>
<td>NEB</td>
<td></td>
</tr>
<tr>
<td>T4 Ligase/Buffer</td>
<td>NEB</td>
<td>M0202L</td>
</tr>
<tr>
<td>dNTP Set</td>
<td>Amersham</td>
<td>27-2035-02</td>
</tr>
<tr>
<td>Taq Polymerase for Colony PCR</td>
<td>Made in the Lab, Li &amp; Rong</td>
<td></td>
</tr>
<tr>
<td>T7 Forward and Reverse Primers</td>
<td>Operon</td>
<td></td>
</tr>
<tr>
<td>80% Glycerol, Sterile</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>Competent Cells, XL-Gold</td>
<td>Stratagene</td>
<td>2003315</td>
</tr>
<tr>
<td>Competent Cells, Magik</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>10X DNA Loading Dye</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>100 bp DNA Ladder</td>
<td>NEB</td>
<td>N3231L</td>
</tr>
<tr>
<td>3 M NaOAc</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>LB,SOC / Superbroth,MJ9</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>LB-Amp plates</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>LB-Amp-Kan-Glu 24-well Plates</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>1000X Amp, Kan and Trace Elements</td>
<td>Made in the Lab</td>
<td></td>
</tr>
<tr>
<td>1000X Vitamin</td>
<td>Gibco</td>
<td>11120-052</td>
</tr>
</tbody>
</table>

1
1.2 Disposables and Other Materials:

<table>
<thead>
<tr>
<th>Name</th>
<th>Source/Vendor</th>
<th>Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Plates</td>
<td>Denvil</td>
<td>C-18080</td>
</tr>
<tr>
<td>S-blocks</td>
<td>Qiagen</td>
<td>19585</td>
</tr>
<tr>
<td>Elusion Microtubes</td>
<td>Qiagen</td>
<td>120008</td>
</tr>
<tr>
<td>48-well blocks</td>
<td>Qiagen</td>
<td>19577</td>
</tr>
<tr>
<td>24-well blocks</td>
<td>Qiagen</td>
<td>19583</td>
</tr>
<tr>
<td>Centri-Sep 96</td>
<td>Princeton Separations</td>
<td>CS-961</td>
</tr>
<tr>
<td>96-well Round Bottom Plates</td>
<td>VWR</td>
<td>82050-626</td>
</tr>
<tr>
<td>Sterile Glass Beads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1100 ul Robot Tips</td>
<td>CLP</td>
<td>BTR1100Q.B</td>
</tr>
<tr>
<td>300 ul Robot Tips</td>
<td>CLP</td>
<td>BTR300Q.B</td>
</tr>
<tr>
<td>24-well plate</td>
<td>BD Falcon</td>
<td>353047</td>
</tr>
<tr>
<td>60X15 mm Plate</td>
<td>BD Falcon</td>
<td>353002</td>
</tr>
<tr>
<td>Combitips Plus 0.1ml–50 ml</td>
<td>Eppendorf</td>
<td></td>
</tr>
<tr>
<td>Matrix Tips 30 ul/125 ul/1250ul</td>
<td>Matrix</td>
<td>7431/7441/8256</td>
</tr>
<tr>
<td>1100 ul Robot Tips</td>
<td>Qiagen</td>
<td>9012598</td>
</tr>
<tr>
<td>300 ul Robot Tips</td>
<td>Qiagen</td>
<td>9012599</td>
</tr>
<tr>
<td>Airpore Cover</td>
<td>Qiagen</td>
<td></td>
</tr>
<tr>
<td>Thermowell Sealing Tape</td>
<td>Corning Costar</td>
<td>6570</td>
</tr>
</tbody>
</table>

2. Equipments Checklist

Qiagen BioRobot 8000.
ABI PCR Machine
Speed Vac
Sonicator
Water Bath, 55 C and 70 C
Shakers, 37 C and 17 C
Centrifuge, Sorval, with swing rotor SH-3000
Gel apparatus for Agarose and SDS PAGE gels

3. Related SOPs

BioRobot 8000 User Manual
Qiasoft 4.0 Operating System User Manual
Qiasoft 4.1 Operating System User Manual

Reagents Preparation (For all the above “Made in the Lab”).
Archiving of Wet Reagent at NESG

4. Target Excel Spreadsheets

Each set of 96-well target has its own Excel file for recording experimental data generated when working with this protocol. The file contains 13 worksheets, each linked by Excel functions to other sheets, processing data to facilitate your next step. Many calculations and automatic fills are carried out by functions programmed into the sheets. Here is a summary of the functions of each worksheet:

- **Paste Primer Data**: Primer data generated by Zebaview and Primer prim’er is pasted starting from cell C2 of this sheet and the positions of commas in each line are shown from column L to S.
- **Primer Design Sheet**: Each line of primer data from previous sheet is parsed into useful fields according the positions of commas.
- **Set Summary**: Forward and Reverse primer information is put together to give the expected size, MW, restriction sites of the cloned products. PCR results are entered to get the ligation digestion sites.
- **Ligation Setup**: Mostly like Set Summary sheet, column “Well” is for the actual well of the ligation plate, column “PWell” is for what’s in that well----its corresponding well number in the Primer Design Sheet and Set Summary sheet.
- **Colony PCR (1) (2) (3)**: For entering colony PCR plate layout and results. Generally 2 to 3 colonies per plate are picked after XL-Gold transformation, the first set of colonies is labeled Colony PCR #1, 2nd #2, etc. These number 1, 2, or 3 are entered into column H “Clone #”. Once the column “Ligation well” and “clone #” are entered, the rest of the information about that target will be automatically filled in. After running the PCR products on gel, you will enter
“yes” or “no” as to whether they are of the right size and whether that colony is picked for miniprep.

- **Miniprep Setup**: Enter the Colony PCR # and Colony PCR Well into column B and C, other information about that clone is automatically filled in, giving you one or more plates for the miniprep. The first plate wells are labeled A1 to H12, second plate wells are labeled A1’ too H12’. Column M and N, “Expression” and “Solubility” are read automatically from SDS Gel Setup sheet.

- **SDS Gel Setup**: Once the Miniprep Setup sheet is finished, data is automatically entered into the SDS Gel Setup sheet to give you the gel # and layout of the SDS gels you are going to run after expression. It gives you the target name and expected size of each lane on the gel. You will enter the expression and solubility on a level of 0 to 5 of each target after staining. The targets with good expression and solubility levels will go through Competition analysis to determine whether to go ahead to fermentation.

- **Fermentation**: This sheet is for displaying target information on the clones given to fermentation. Enter the “Well” column, rest is auto-fill.

- **Spine Construct**: For organizing the construct data for uploading into the Spine database. Data is automatically read from the Miniprep Setup sheet.

- **Spine Expression**: For organizing the expression data for uploading into the Spine database. Data is automatically read from the Miniprep Setup sheet.

### 5. Cloning Procedures

#### 5.1 PCR of Open Reading Frame (ORF):

**NOTE:** For HR targets, set up 2 PCRs – one at low temperature and one at regular temperature.

Use RT PCR template (33ul of each for a total of 100ul for 96-well) for HR targets. Refer to SOPs_SUPT: Supporting protocols for Preparing Reagents for RT PCR protocol.

**NOTE:** Check the GC content of target organisms before deciding on the PCR kit and protocol to use. Go to [http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/) web site to search the genome database for the sequence information of that organism.

Use Advantage-GC Genomic PCR Kit and its protocol if the GC% is high (i.e. 60%).

Use Advantage HF-2 PCR Kit and its protocol if the GC% is low.

5.1.1 Thaw primer plates ahead of time. Spin down.

5.1.2 When the genomic DNA is used for the first time, add 50 ul EB, 37 C for 1 hr. Resuspend the DNA gently. Store in -20 C. Thaw on ice and spin down before each use. Use 10 ul per 96-well plate.
5.1.3 For each 96-well plate, make PCR mix for 105 reactions (excluding the primers) in a 14 ml falcon tube and keep on ice. Use a repeater to add mix to each well.

Advantage HF-2 PCR mix for 1 reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>10 ul per 96-well, add to whole mix.</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>5 ul</td>
</tr>
<tr>
<td>10X DNTPs</td>
<td>5 ul</td>
</tr>
<tr>
<td>Polymerase</td>
<td>1 ul</td>
</tr>
<tr>
<td>Sterile dH2O</td>
<td>35 ul</td>
</tr>
</tbody>
</table>

Primers added separately:
- Forward primer = 2 ul
- Reverse primer = 2 ul

Total = 50 ul

ABI PCR machine program name: hf-per.reg

Advantage-GC Genomic PCR mix for 1 reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>10 ul per 96-well, add to whole mix.</td>
</tr>
<tr>
<td>5X PCR buffer</td>
<td>10 ul</td>
</tr>
<tr>
<td>50X DNTPs</td>
<td>1 ul</td>
</tr>
<tr>
<td>GC Polymerase</td>
<td>1 ul</td>
</tr>
<tr>
<td>25mM Mg(OAc)2</td>
<td>2.2 ul</td>
</tr>
<tr>
<td>GC Melt</td>
<td>7.5 ul</td>
</tr>
<tr>
<td>Sterile dH2O</td>
<td>24 ul</td>
</tr>
</tbody>
</table>

Primers added separately:
- Forward primer = 2 ul
- Reverse primer = 2 ul

Total = 50 ul

ABI PCR machine program name: hf-per.gc-long
5.2 Gel Extraction of PCR Fragments:

5.2.1 Run 2% Agarose gel @ 120 V using 3 15-well combs with 100-bp DNA ladder, and cut out bands of correct size in S-Block. Push the gel slices into the bottom of the wells. They can be stored frozen at -20 C.

5.2.2 Enter PCR result into Excel sheet “Set Summary” and “Ligation Setup”. In column “PCR”, put “yes” in the cell if that target has a PCR product at the correct size. Leave it blank if it doesn’t.

5.2.3 Use robot program **96-well Gel Extraction** in MASTER FOLDER to purify the cut PCR gel bands. Choose the default elution volume of 80 ul. After buffer QG has been added by the Robot, manually transfer the S-block to 55 C waterbath. After the agarose is all melted, about 30-45 min, put it back onto the robot and resume the protocol.

5.3 RE Digest #1:

5.3.1 Make master mix for the 1st set of RE digests in a 14 ml falcon tube or 2 ml Qiagen collection tube and keep on ice

**Master Mix for RE digest #1:**

<table>
<thead>
<tr>
<th>NdeI</th>
<th>Xhol of BamHI, EcoRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA = 100 ul</td>
<td>DNA = 100 ul</td>
</tr>
<tr>
<td>10X NEB4 = 12 ul</td>
<td>10X NEB2 = 12 ul</td>
</tr>
<tr>
<td>NdeI = 5 ul</td>
<td>100X BSA = 1 ul</td>
</tr>
<tr>
<td>dH2O = 3 ul</td>
<td>Xhol = 5 ul</td>
</tr>
<tr>
<td>Total = 120 ul</td>
<td>Total = 120 ul</td>
</tr>
</tbody>
</table>

5.3.2 Incubate O/N @ 37C

5.4 RE Digest #2:

5.4.1 Make master mix for 2nd set of RE digests (Xhol, BamHI, RI, NotI) in a 14 ml falcon tube or in 2 ml Qiagen collection tubes and keep on ice

**Master Mix for RE digest #2:**

<table>
<thead>
<tr>
<th>Xhol of NdeI</th>
<th>BamHI/EcoRI</th>
<th>NotI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA = 120 ul</td>
<td>DNA = 120 ul</td>
<td>DNA = 120 ul</td>
</tr>
<tr>
<td>10X NEB2 = 15 ul</td>
<td>BamHI/RI buffer = 15 ul</td>
<td>10X NEB3 = 15 ul</td>
</tr>
<tr>
<td>100X BSA = 1.5 ul</td>
<td>100X BSA = 1.5 ul</td>
<td>100X BSA = 1.5 ul</td>
</tr>
<tr>
<td>Xhol = 6 ul</td>
<td>BamHI/RI = 5 ul</td>
<td>NotI = 6 ul</td>
</tr>
<tr>
<td>dH2O = 7.5 ul</td>
<td>dH2O = 8.5 ul</td>
<td>dH2O = 7.5 ul</td>
</tr>
<tr>
<td>Total = 150 ul</td>
<td>Total = 150 ul</td>
<td>Total = 150 ul</td>
</tr>
</tbody>
</table>
5.4.2 Incubate O/N @ 37°C

5.5 RE Digest Clean-up:

Use robot program Restriction Digest 96-well clean up in MASTER FOLDER to set up the purification. Choose the default 80 ul elution volume.

5.6 Centri-Sep 96 Filtration:

5.6.1 Lyophilize the above purified 96-well RB plate in speed vacuum for 2-3 hours. Use manual run till completely dry. Meanwhile allow the Centri-Sep plate to come to RT. Remove the foil from the bottom and the top of the plate.

5.6.2 Re-suspend the lyophilized samples in the 96-well RB plate in ~25 ul of dH2O using a multi-channel pipette, or use a repeater to add 25 ul of dH2O to each well.

5.6.3 Stack the Centri-Sep plate on top of a 96-well block (Centri-Sep base) and balance it with another 96-well block (Centri-Sep balance).

5.6.4 Centrifuge @ 1500 X g for 2 minutes. Discard the liquid in the Centri-Sep base.

5.6.5 Place the provided 96-well PCR plate in the Centri-Sep base and stack the Centri-Sep plate on top. Make sure the alpha-numeric indices on all plates are aligned.

5.6.7 Transfer samples from the 96-well RB plate to the Centri-Sep plate in the center of the gel matrix using a multichannel pipette.

5.6.8 Balance again and centrifuge @ 1500 X g for 1 min.

5.6.9 Seal the 96-well PCR plate and freeze until ready for ligation.

5.7 96-well Ligations:

5.7.1 Using the multi-channel pipette, transfer 1.2 ul of the Centri-Sep sample to a 96-well PCR plate. Keep the 96-well PCR plate on ice!

5.7.2 Make master mix and keep on ice!

**Ligation Mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>1.2 ul</td>
</tr>
<tr>
<td>Vector</td>
<td>1 ul</td>
</tr>
<tr>
<td>10X buffer</td>
<td>2 ul</td>
</tr>
<tr>
<td>dH2O</td>
<td>16 ul</td>
</tr>
<tr>
<td>Ligase</td>
<td>1 ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20 ul</strong></td>
</tr>
</tbody>
</table>

7
5.7.3 Add 20 ul of the mix to each well using the repeater
5.7.4 Use program **16 Ligation** in ABI machine for 17 hours. Put the plate in when the temperature has reached 16 C. The program will kill the Ligase for 10 min at 65 C at the end of the 16 C incubation.

**5.8 RE Digest of Ligations:**

5.8.1 Check for internal RE sites
5.8.2 Make master mix and keep on ice!

**Mix for RE digest of ligations:**

<table>
<thead>
<tr>
<th></th>
<th>EcoRI</th>
<th>HindIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lig samples</td>
<td>= 20 ul</td>
<td>Lig samples</td>
</tr>
<tr>
<td>EcoRI buffer</td>
<td>= 3 ul</td>
<td>10X NEB2</td>
</tr>
<tr>
<td>EcoRI</td>
<td>= 1 ul</td>
<td>HindIII</td>
</tr>
<tr>
<td>dH2O</td>
<td>= 6 ul</td>
<td>dH2O</td>
</tr>
<tr>
<td>Total</td>
<td>= 30 ul</td>
<td>Total</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>BamH1</th>
<th>SacI/SalI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lig samples</td>
<td>= 20 ul</td>
<td>Lig samples</td>
</tr>
<tr>
<td>BamHI buffer</td>
<td>= 3 ul</td>
<td>10X NEB1/NEB3</td>
</tr>
<tr>
<td>100X BSA</td>
<td>= 0.5 ul</td>
<td>100X BSA</td>
</tr>
<tr>
<td>BamH1</td>
<td>= 1 ul</td>
<td>SacI/SalI</td>
</tr>
<tr>
<td>dH2O</td>
<td>= 5.5 ul</td>
<td>dH2O</td>
</tr>
<tr>
<td>Total</td>
<td>= 30 ul</td>
<td>Total</td>
</tr>
</tbody>
</table>

5.8.3 Add mix to each well.
5.8.4 Incubate at 37C for ~1 hour using ABI PCR machine program **37C**.

**5.9 Transformations into XL-Gold Competent Cells:**

5.9.1 Add 1.2ul of the digested ligated samples into chilled 96-well PCR plates
5.9.2 Thaw competent cells on ice!
5.9.3 Add ~15ul to each of the wells using a repeater
5.9.4 Incubate on ice for 30 minutes
5.9.5 Heat shock @ 42C for 1 minute on ABI PCR machine. Use program **42 heatshock**. Put the plate into PCR machine when temperature has reached 42 C; take it out when the 1 min is over.
5.9.6 Incubate on ice for 2-5 minutes

8
5.9.7 Add ~100ul of SOC to each well using a repeater & incubate @ 37C for 1 hour
5.9.8 Meanwhile label LB/Amp plates and add glass beads
5.9.9 Plate the whole mix. Incubate O/N @ 37C

5.10 Screening colonies for right constructs:

5.10.1 Enter the number of colonies into Excel worksheet Colony PCR (1) (2) (3). Enter “20” if there are more than 20 colonies on the plate. Update the sheet with Colony PCR well layout.
5.10.2 Pick 2 colonies from each XL-Gold plate. Re-suspend each colony in 50 ul of dH2O in well positions determined by Colony PCR worksheets.
5.10.3 Setting up colony PCR:

5.10.3.1 Transfer 10ul of the colony resuspension into a 96 well plate using a 0.5 – 10 ul multichannel pipette
5.10.3.2 Make a PCR mix in an Eppendorf tube and keep on ice

**Master Mix for colony PCR:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony resuspension</td>
<td>10 ul</td>
</tr>
<tr>
<td>10X dNTPs</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.5 ul</td>
</tr>
<tr>
<td>T7 Forward primer</td>
<td>1 ul</td>
</tr>
<tr>
<td>T7 Reverse primer</td>
<td>1 ul</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>1 ul</td>
</tr>
<tr>
<td>dH2O</td>
<td>7 ul</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>25 ul</td>
</tr>
</tbody>
</table>

**NOTE:** If using 10X PCR buffer without Mg, add 1 ul 50 mM MgCl2 to each reaction.

T7 Forward and Reverse Primers: ordered from Operon at 250 pmol. Make stock solution with dH2O at 250 uM. Dilute 1:100 to working stock at 2.5 uM.
T7 Promoter primer 20mer: 5’-TAATACGACTCCTATAGGG-3’
T7 Terminator primer 19mer: 5’-GCTAGTTATTGCTCAGCGG-3’
5.10.3.3 Use a repeater, add 15 ul of the mix to each of the wells in the PCR plate
5.10.3.4 Use program colpcr on the ABI machine to run the PCR

5.10.4 Run 2% Agarose gel @ 120V and analyze the gel for the right constructs (Add 2ul of 10X DNA dye and load 15ul/well. Use 4 30-well combs).
5.10.5 Analyze gel. Enter result of colony PCR into Excel sheets. Repeat the process and pick more colonies if needed. When the number of right colonies has reached your planned number, enter their Colony PCR # and well # into the Miniprep Setup Excel sheet. Print out the sheet for inoculation reference. Generally miniprep plate 1 wells are labeled A1 to H12, plate 2 wells are labeled A1’ to H12’.

5.11 Mini-preps and Glycerol Stocks of the right constructs:

5.11.1 Inoculate ~10ul of the right construct in 2ml of LB/Amp in 2 Qiagen 48-well blocks according to the Miniprep Setup Excel sheet. Shake @ 37C O/N.
5.11.2 Following the SOP Archiving of Wet Reagent at NESG, open Platemater spreadsheets. Assign locations to GS and DNA plates for the target set and label plates for the next step.
5.11.3 Use robot program Gly Transfer from 48-well to 96 RB in MASTER FOLDER to do the glycerol stocks, which have 30 ul of 80% glycerol with 70 ul culture in each well. 2 duplicate glycerol plates for each pair of 48-well blocks. Flash freeze the glycerol stock plates in dry ice before transferring them to -80 C freezers later.
5.11.4 Spin for 10 min @ 3000 XG in Sorvall Evolution (SH 3000 Rotor). Decant the media.
5.11.5 Use robot program Qiaprep Turbo 96-elutionmicrotube in MASTER FOLDER on the robot to do mini-preps. Save an excel copy of GS and DNA positions to Platemater spreadsheets. DNA plates will be archived later.

6. Expression Procedures

6.1 Transformations in Expression Cells (Mgk):

6.1.1 Add 1 ul of mini-prep DNA into a 96-well PCR plate in the same order as the DNA plate
6.1.2 Thaw Magik competent cells on ice!
6.1.3 Add ~15 ul to each of the wells using a repeater
6.1.4 Incubate on ice for 30 minutes
6.1.5 Meanwhile label LB/Amp/Kan/glucose plates and add glass beads
6.1.6 Heat shock @ 42°C for 1 minute
6.1.7 Incubate on ice for 2-5 minutes
6.1.8 Plate the whole mix. Incubate O/N @ 37°C

6.2 Small Scale Expressions and Inductions:

6.2.1 Inoculate 500ul of fresh LB/Amp/Kan/0.5% glucose with 1 colony in S-block and grow all day @ 37°C
6.2.2 At the end of the day, use robot programs to do expression glycerol stock and inoculate into MJ9 media. Label plates according to the locations in Platemater spreadsheets. One stock plate per expression plate.

6.2.2.1 On robot A3251, use Glycerol stock of Expression + MJ9 Inoculation in MASTER FOLDER to do both the glycerol stock and the inoculation.

6.2.2.2 On robot A2576, use Gly Stock of expression-S-block to 96-well RB in MASTER FOLDER to do the glycerol stocks; S-Block to S-Block for ON transfer to MJ9 for the inoculation.

6.2.3 For overnight culture, inoculate 500ul of fresh MJ9 + 0.5% glucose, 0.5% MgSO₄, 1000X Amp, 1000X Kan, 1000X vitamin solution, 1000X trace elements with 10ul of the ‘all-day’ culture in S-block. Grow O/N @ 37°C.

6.2.4 Back dilutions for expressions:

6.2.4.1 Take a 1:10 dilution of the OD₆₀₀ of the O/N culture and calculate the amount needed for the starting OD₆₀₀ ~ 0.1 - 0.2. This amount is usually 200ul for a 2ml culture of MJ9 + 0.5% glucose, 0.5% (end concentration 1 mg/ml) MgSO₄, 1000X Amp, 1000X Kan, 1000X vitamin solution, 1000X trace elements

6.2.4.2 Make a master mix of MJ9 + 0.5% glucose, 0.5% MgSO₄, 1000X Amp, 1000X Kan, 1000X vitamin solution, 1000X trace elements

<table>
<thead>
<tr>
<th>Master Mix for 1000 ml culture:</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ9</td>
</tr>
<tr>
<td>20% glucose (40X)</td>
</tr>
<tr>
<td>MgSO₄ (200X)</td>
</tr>
<tr>
<td>Ampicillin (1000X)</td>
</tr>
<tr>
<td>Kanamycin (1000X)</td>
</tr>
</tbody>
</table>
Vitamin solution (1000X)  = 1 ml
Trace Elements (1000X)  = 1 ml

Bring up the volume to 1000 ml

6.2.4.3 Aliquot 2ml of the MJ9 media into 4 24-well blocks using a repeater.
6.2.4.4 Use robot program S-block to 24-well Inoculation for expression in MASTER FOLDER to back dilute to OD$_{600}$ ~ 0.1 - 0.2 for expressions

6.2.5 Grow @ 37C until OD$_{600}$ ~ 0.5 – 0.7 (usually ~ 2.5 – 3 hours)
6.2.6 Induce with 2ul of 1000X IPTG and grow O/N @ 17C

6.3 Sonications:

6.3.1 Use robot program 24-well to S-blocks after inductions in MASTER FOLDER to aliquot 1ml of induced samples into 2 S-blocks
6.3.2 Spin the S-blocks for 10 min @ 3000 XG (Sorvall Evolution with the SH 3000 rotor). Aspirate supernatant from both S-blocks and freeze 1 S-block
6.3.3 Add 100 ul of cold Native Lysis Buffer + 10mM BME to the other S-block using a repeater. Undiluted BME is 14.3 M. Keep the S-block on ice and re-suspend the pellets using a multi-channel pipette
6.3.4 Transfer the samples to a 96-well PCR plate. Seal with aluminum foil and keep on ice.
6.3.5 Make sure the power source of the Sonicator is connected to the output line labeled “Microplate”. Program the Sonicator for 40 minutes total run time with 5 minute pulse time and 1 minute wait time. Power output set to 7. Use Program 8. Re-suspend the samples using a multi-channel pipette after 20 minutes.
6.3.6 Fill a large beaker with ice water and use the variable flow pump to control the flow of water so that the plate is always chilled in ice water.
6.3.7 Place the plate with samples in the groove and press start to start sonicating.

6.4 Solubility analysis using SDS PAGE gels:

6.4.1 Prepping samples for SDS gels:

6.4.1.1 Transfer all samples from the PCR sonicating plate to a 96-well RB plate labeled ‘sonicated totals’ using a multichannel pipette.
6.4.1.2 Take out 30ul aliquots of ‘Total’ into another RB plate labeled ‘T’
6.4.1.3 Balance the ‘sonicated totals’ plate and spin in SH 3000 rotor @ 3000 XG for 10 minutes in Sorvall Evolution centrifuge
6.4.1.4 Carefully take out 30ul aliquots of ‘supe’ into a 3rd RB plate labeled ‘supe’. Make sure there are no pellets in the supe!
6.4.1.5 Thaw 1M DTT. Use 4/5 NuPage Buffer (4X) and 1/5 DTT to make the SDS loading buffer. You will need 10ul of the SDS loading buffer for a 30ul sample. So for 96 samples, make 1ml of loading buffer (800ul of NuPage buffer and 200ul of DTT)
6.4.1.6 Using a repeater, add 8ul of the loading buffer to each sample. Seal with foil and heat @ 72C for 10 minutes

6.4.2 Running SDS gels:

6.4.2.1 Update gel # on the “SDS Gel Setup” sheet.
6.4.2.2 While the samples are heating, make 1X SDS running buffer and add about 800ml to each protein gel box
6.4.2.3 Remove the seal and the comb from the gels, rinse and place them in the gel box with the groove facing inside
6.4.2.4 Load 15ul of the protein marker in the first well
6.4.2.5 Using the 30ul matrix pipette, load 15ul of sample in the wells (the order should be A1-H1, A2-H2, … A12-H12)

NOTE: Make sure the dispensing speed in the matrix multichannel pipette is set to only one square

6.4.2.6 Run @ 200V. Label gel boxes with gel #, initials and date for rinsing and staining

6.4.3 Staining of SDS gels:

6.4.3.1 Use the provided scapula to separate the gel and rinse them in respective gel-boxes 3 times – 10-15 minutes each time
6.4.3.2 Stain with 50-100 ml GelCode Blue Stain Reagent for at least an hour on the shaker. Can be longer or O/N
6.4.3.3 Destain with water.

6.4.4 Taking pictures/Scanning of the gels.

7. Data Management

7.1 Analysis of SDS Gels

1.1.1 In reference to the “SDS Gel Setup” sheet, look for bands of right size on the gel. Enter the expression and solubility data into the sheet.
1.1.2. Go to ZebaView for competition analysis. Choose target organism from the left column, the target list appears on the right frame. For the targets that have acceptable expression and solubility levels (according to Fermentation group), click on the TDB, PDBH, and PDB database icon on the left of their NESG id. Check to see if these targets already have a homolog structure already in PDBH and PDB. Enter the result into the spreadsheet. Those targets with no PBD and PDBH hits and those with E-value >0.01 are passed on to fermentation.

1.2. Transfer Targets to Fermentation Team

1.2.1. Fill in the “Fermentation” sheet and send it to members of the fermentation team.
1.2.2. The fermentation team can generally handle about 15 targets per week. Targets with MW<25 KD are counted as 2 because they are needed for both NMR and crystallography studies.

1.3. Data Upload into SPINE

http://spine.nesg.org/private/upload/
The user name is “genes”, password is “2find”.

If there are any mistakes in the data already uploaded, contact Jessica Lau in the lab or Michael Wilson of Yale at Michael.wilson@yale.edu to make corrections.

1.3.1. Construct upload

1.3.1.1. On sheet “Spine Construct”, delete the rows with uncloned targets. Change the “Researcher” name to your own, and “Cloned on date” to the date of the ligation was done. Copy all area from cell A2 to the end of column I.
1.3.1.2. On the above web page choose “Construct”. After logging in, paste the whole data set from the sheet “Spine Construct” into the blank area. Click “Submit” to get to the page asking for confirmation. If a target name is already in Spine, it will be labeled in green letter “Yes”. If not, in red “no”. Go back to the “Colony PCR” sheet of that target to change the “Clone #” to a number that is not in Spine, and repeat the uploading. When all the targets show the red “no”, complete the uploading.
1.3.1.3. If the target name ends with an “A”, it means is not full protein but a fragment. In that case change the “Insert Type” field to “Subsequence”.

1.3.2. Small scale expression upload

1.3.2.1. On sheet “Spine Expression”, delete the rows with uncloned targets. Change the “Researcher” name to your own, and “Expression date” to the date of the Magik cell transformation. Copy all area from cell A2 to the end of column I.

1.3.2.2. On the above web page choose “Small Scale Expression”. Same as 7.3.1.2.

1.3.3. Archive upload

1.3.3.1. Verify miniprep data has been pasted into Platemater spreadsheets and glycerol stock plates are in the freezers. Archive DNA plates according to the archiving SOP.

1.3.3.2. Copy the platemaster sheet of the target set into Archive Upload Template spreadsheet, starting from well#A2. Copy the location into well #M2. Delete the empty wells from column O to W and move cells up.

1.3.3.3. On the above web page choose “PST Archive”. Copy and paste the data for cloning GS and expression GS and submit.

1.3.3.4. Close the Archive Upload Template spreadsheet. Do not save changes.
FERMENTATION

Author: Kellie Cunningham
Last revision: December 2006

DAY 1

A. Select Targets
   a. Collect e-mails from Li, Tom, & Rong for *SPECIAL* requests
   b. Pick targets from Molecular Biology
      i. Targets >25 kD require one fermentation (SeMet labeled)
      ii. Targets <25 kD require two fermentations (SeMet and N/NC5 labeled)
   c. (Total capacity: Normally ~23 Fermentations per 5 day cycle)

B. Determine what media needs to be made
   a. *SPECIAL* requests will specify media type
   b. Mol Bio Targets >25 kD are SeMet labeled
   c. Mol Bio Targets >15 kD and <25 kD are SeMet labeled and N labeled
   d. Mol Bio Targets <15 kD are SeMet labeled and NC5 labeled

C. Set-up for Fermentation
   a. Determine an order for fermentations. (e.g. alpha-numeric) Set up OD Reading Sheet. Keep this order throughout entire cycle.
   b. Label colored tape (use multiple colors to avoid confusion)
      i. Fermentation ID [(Construct Name + Media Label) See Appendix], Date
      ii. 1 pair of the same color for each fermentation
         1. 2L baffled flask
         2. 250mL baffled flask
   c. Label 1 set of microfuge tubes for each fermentation
      i. Fermentation ID
         1. Clear tubes: mini-harvest
         2. Green tubes: Harvest Total (HT) Gel
         3. Yellow tubes: Harvest Supernatant (HS) Gel
   d. Label 3 identical sets of cuvettes for each fermentation
      i. Fermentation ID
         1. Before Induction OD
         2. Induction OD
         3. Final OD
   e. Label 1 set of 14mL Falcon Tubes: 1 tube per target
i. Construct Name

D. Transformation (if needed)
Determine if target needs to be transformed or if an expression glycerol stock is available

a. Use Archive Platemaster
   i. Select the Archive folder in Spins Webserver
   ii. Open Platemaster file
   iii. Press Control+F
   iv. Enter Target ID under “Find What” (i.e. ER1)
   v. Hit the “Options >>” button
   vi. Select Workbook in the dropdown menu under “Within”
   vii. Select Values in the dropdown menu under “Look In”
   viii. Click “Find Next”
   ix. Continue clicking “Find Next” until you have found the desired clone of your target
   x. Target location is the plate number on the worksheet tab (i.e. 5-1-A-1-1); Target position on the plate is noted by the Well Number the target corresponds to (i.e. A1)
   xi. Search Freezer Archive for 5-5 plates (expression glycerol stock plates); if a 5-5-A-1-1 plate is available, the target does not have to be transformed; if no such plate exists, a transformation must be done using the DNA from the 5-1 plate.
   xii. If target is not located in Archive Platemaster, search in boxes of personal DNA stocks located in Kellie’s freezer to perform transformation.

b. Perform transformations (if any) * See Mol. Bio SOP for transformation protocol*

DAY 2

A. Inoculations (A.M.)
   a. Aliquot 500 uL of LB Media + appropriate antibiotics into the pre-labeled 14mL Falcon Tubes
   b. If Target has expression glycerol stock available:
i. Obtain expression glycerol stock plates from the -80°C Archive and keep on dry ice

ii. Determine proper orientation of the plate so that position A1 is Upper Left

iii. Carefully puncture the a hole in the foil cover above the well corresponding to the target of interest with a pipet tip and scrape the surface of the sample until you have a small chunk on the tip of the pipet

iv. Carefully transfer the sample to the appropriate 14mL Falcon Tube. Pipet up and down a few times so the sample incorporates into the media. Release the pipet tip into the tube and cap tube

v. Re-seal the punctured well with a small square cut out of Thermo Well Sealing Tape

vi. Return the plate to its proper location in the -80°C Archive

c. If Target was transformed:
   i. Retrieve plates from the overnight incubation
   ii. Using a pipet tip, carefully select 2-3 isolated colonies from the plate
   iii. Carefully transfer the colonies to the appropriate 14mL Falcon Tube. Pipet up and down a few times so they are released from the tip into the media. Release the pipet tip into the tube and cap tube

d. Shake tubes at 37°C until media becomes turbid (~4-6 hours)

B. Media Preparation

   e. Prepare media in 5L graduated buckets

   f. Each media type must be separated and prepared in different buckets (MJ9 Media)
      i. SeMet/ 14N
      ii. N
      iii. NC5
      iv. NC

   g. Targets with antibiotic combinations other than the standard Amp/Kan must be separated and prepared in different buckets, as well as separated by media type

   h. Label buckets
      i. Media type
      ii. Antibiotic combination
      iii. Volume

   i. Add large stir bar to each bucket

   j. Prepare media according to attached recipe booklet

   k. pH media to 6.8
      i. calibrate pH meter
1. aliquot ~ 5mL of new calibration buffer (pH 4 and 7) into new 14mL Falcon Tubes
2. rinse pH meter with dH20 and wipe off with Kim Wipe
3. insert pH meter into pH 7 buffer
   (a) Press “CAL”
   (b) Press “CLEAR”
   (c) Press “ENTER”
4. wait for the pH value to stop flashing; the 1st calibration arrow will become solid
5. rinse pH meter with dH20 and wipe off with Kim Wipe
6. insert pH meter into pH 4 buffer
   (a) Press “CAL”
   (b) Press “ENTER”
7. wait for the pH value to stop flashing; the 2nd calibration arrow will become solid
8. rinse pH meter with dH20 and wipe off with Kim Wipe
   ii. insert meter into media while bucket is on a stir plate
   iii. adjust pH using HCl and NaOH
   iv. rinse off pH meter in between each bucket of media
   v. When finished, store pH meter in pH 7 buffer
l. Add Glucose and Supplements according to attached recipe booklet
m. Place each bucket on stir plate to ensure mixture is homogenized before filtering
n. Filter sterilize media
   i. Use Millipore 1000mL Funnel/ 1000mL Receiver 0.22um filter unit
   ii. Place one of the pre-labeled colored tapes from each pair on each 2L flask and on each 250mL flask
   iii. Use separate filter units for each antibiotic combination and also if using LB Media
   iv. All media possessing the same antibiotic combination can be filtered using the same filter unit as long as you follow the hierarchy rule:
      1. NC
      2. NC5
      3. N
      4. SeM/ 14N
   v. Measure and filter 1L at a time
   vi. Transfer filtered media to a 2L baffled flask with minimal interface with the environment and re-cover quickly with foil cap
   vii. Add 1mL Trace Elements to each liter of media EXCEPT LB Media
viii. Shake 2L flasks vigorously for a moment to incorporate Trace Elements

 ix. Transfer 40mL from the 2L flask to the corresponding 250mL flask
    1. the same pipet can be used for the same media type with the same antibiotic combination

 x. Place the 2L flasks in the 37°C shakers ordered according to the OD Reading sheet.
    1. Leave overnight with no shaking at 37°C; pre-warm flasks

C. 1st Dilution (P.M.)

 o. Retrieve 14mL Falcon Culture tubes from shaker; make sure the culture is turbid

 p. Transfer 40uL of culture growth for each Target to corresponding 40mL starter cultures in the 250mL flasks
    i. There may be multiple fermentations for each Target
    ii. i.e. ER1 has a SeM label and a NC5 label; 40uL of the ER1 culture growth should be transferred to both the ER1 SeM and ER1 NC5 250mL flasks

 q. Place 250mL flasks in 37°C shaker overnight; shaking

D. Expression Glycerol Stock

 Make expression glycerol stocks of Targets that were transformed for future repeats

 r. Label microfuge tube with Target name down the side of the tube and on a Microtube Dot Label; stick Dot to tube cover

 s. Add 70uL of culture growth from 14mL Falcon Tube to microfuge tube

 t. Add 30uL 80% glycerol to microfuge tube

 u. Vortex to mix

 v. Flash freeze in dry ice for 30 minutes

 w. Store in archive in -80°C freezer (location: 6-5-C)

DAY 3

A. 2nd Dilution

 a. Retrieve overnight starter cultures from 37°C shaker

 b. Make sure the culture is turbid

 c. Transfer the full contents of the 250mL flask to the corresponding 2L flasks that have been pre-warming overnight in the 37°C shaker
d. Turn on shaker and set to ~225 rpm

B. Monitoring ODs

- If using LB Media, let culture grow for ~60 mins before checking OD
- If using any form of MJ9 Media, let culture grow for ~90 mins before checking OD
- Using the Spectrophotometer to measure ODs
  - Flip the Spec switch to the “on” position
  - Open the program “Lab Power Jr” on the PC
  - Adjust the wavelength to 600nm
  - Blank the Spec using LB Media for LB growths and 14N MJ9 for MJ9 growths
  - Cuvettes should be inserted into the stand such that the smooth sides are perpendicular with the beam
- Begin monitoring ODs by taking one 1mL sample from any fermentation in the batch
  - Optimal OD should be between 0.6-0.8
  - If OD is lower than this range, allow the cultures to grow longer; estimate time based on OD reading
  - You can assume that all of your cultures are growing at approximately the same rate because they had the same dilution factor and they are the same volume
  - However, this is not always the case; some proteins will be “laggers” requiring more growth time
  - Continue monitoring your sample OD until it reaches the optimal range
  - Once your sample OD has reached 0.6-0.8, take a 1mL aliquot from every fermentation in the batch and measure the OD
  - Use the pre-labeled cuvettes (set 1)
  - If any are below the range, allow cultures to continue growing at 37°C and continue to monitor the OD until it is in range; those that are in range are deemed “ready” for induction.
  - Record the OD for each “ready” fermentation on the OD reading sheet under the appropriate fermentation ID (this is the first OD that is recorded on the sheet)

C. Induction

- Transfer the “ready” 2L flasks onto the platform shakers in the 17°C room (Induction Room)
- For SeMet fermentations
  - allow flasks to shake at 17°C for 5 mins
ii. add 10mL 10× *Amino Acid Solution* per liter to each flask

iii. add 1 vial (60mg) of Seleno-Methionine to each flask

iv. allow flasks to shake for 5 mins again

v. take 1mL aliquot for OD reading; use pre-labeled cuvettes (set 2)

vi. add 1mL 1M *IPTG* inducer to each liter

vii. Record OD on the OD reading sheet under the appropriate fermentation ID (this is the second OD that is recorded on the sheet)

k. For LB Media, N, NC5, NC, and 14N fermentations

i. allow flasks to shake at 17°C for 10 mins

ii. take 1mL aliquot for OD reading; use pre-labeled cuvettes (set 2)

iii. add 1mL 1M *IPTG* inducer to each liter

iv. Record OD on the OD reading sheet under the appropriate fermentation ID (this is the second OD that is recorded on the sheet)

l. Leave 2L flasks shaking at 17°C overnight

D. **Set up for Harvest**

m. On a cart, arrange 1L Sorvall Centrifuge Bottles with Cap Assembly (2 pieces); one for each fermentation

n. Set aside pre-labeled clear microfuge tubes for mini-harvest

---

**DAY 4**

A. **Harvest**

a. Take cart with bottles/cap assemblies and clear microfuge tubes into 17°C room

b. Add 5-6 drops of Antifoam to each 2L flask to remove any accumulating bubbles

c. Turn on the shaker to swirl in the Antifoam

d. If bubbles remain in any flasks, add more drops of Antifoam and mix

e. Take a sample from each fermentation

i. For SeMet and NC fermentations

1. Take 540uL sample; use pre-labeled clear microfuge tubes

(a) 140uL for glycerol stock

   (i) 70uL for Fermentation Group’s Archive

   (ii) 70uL for DNA Sequencing
(b) 100uL for final OD
(c) 300uL for mini-harvest

ii. For LB Media, N, NC5, and 14N fermentations
   1. Take 400uL sample; use pre-labeled clear microfuge tubes
      (a) 100uL for final OD
      (b) 300uL for mini-harvest

f. Leave rack with clear tubes on ice

g. For each fermentation, one at a time, remove tape label from 2L flask, stick it onto 1L Sorvall bottle, and transfer culture from flask to bottle
   i. Fill the bottle until the culture reaches the faint line on the neck of the bottle
   ii. Seal the bottle with the Sorvall Cap Assembly lightly (you will need to open it again to balance the bottles before centrifuging)

h. Centrifuge 1L bottles
   i. Use SLC-6000 rotor with Evolution Centrifuge
      1. Two Evolutions available
      2. SLC-6000 holds six 1L Sorvall bottles
   ii. Determine how many bottles you will centrifuge each round (twelve maximum); leave the remaining bottles in the 17°C room
   iii. Put SLC-6000 rotor into the Evolution centrifuge
   iv. Balance 1L bottles
      1. Zero the balance
      2. Balance two 1L bottles with cap assembly at a time using dH2O
      3. Once balanced, screw the cap assembly on tightly and insert the two bottles into the rotor on opposing sides.
   v. Cap the lid of the rotor on tightly by alternating screwing the large and small knobs
   vi. Close the hatch of the centrifuge lightly; locking it
   vii. Set the spin program
      1. Select the rotor type using the arrows; SLC-6000
      2. Set time using the arrows; 30 mins
      3. Set speed using the arrows; 7000 rpm
      4. Set temperature using the arrows; 4°C
      5. Push “Start”
   viii. When spin is over, remove bottles and make sure the supernatant is transparent
   ix. Decant the supernatant into the empty 2L flasks
   x. Seal bottle with a clear bottle top (Nalgene)
   xi. SeMet fermentations
      1. Store in -20°C in Room 314
   xii. N, NC5, NC, LB Media, 14N
1. store in -20°C across from 17°C room in Room 303

B. Preparation of Glycerol Stocks (For SeMet and NC Fermentations)
   a. Label clear microfuge tubes
      i. Fermentation ID
   b. Add 140uL of the sample into the newly labeled clear tubes
   c. Add 60uL of 80% Glycerol
   d. Vortex to mix
   e. Transfer 100uL into the current DNA Sequencing plate
   f. Flash freeze microfuge tubes and sequencing plate in dry ice for 30 minutes
   g. Store tubes in Fermentation Group’s Archive -80°C
   h. Store DNA Sequencing plate at -80°C

C. Read Final OD
   a. Add 900uL of 14N MJ9 to the third set of pre-labeled cuvettes for all MJ9 Media fermentations; add 900uL of LB Media to cuvettes for LB Media fermentations
   b. Add 100uL of the sample to each corresponding cuvette; pipette up and down to mix
   c. Set up Spectrophotometer
      i. Blank the Spec using LB Media for LB growths and 14N MJ9 for MJ9 growths
   d. Record OD on the OD reading sheet under the appropriate fermentation ID (this is the third OD that is recorded on the sheet)
   e. Multiply readings by 10 to account for the dilution factor

D. Mini-harvest/ Sonication/ Gel Preparation
   a. Centrifuge the remainder of the sample (300uL) in the tabletop microfuge
   b. Aspirate the supernatant
   c. Add 100uL Lysis Buffer / 10mM B-Me to each tube
   d. Vortex to re-suspend the pellet
   e. Arrange the tubes in the sonicator rack for sonication
   f. Sonocate
      i. Set up Flow Pump to keep plate sonicator full with water
      ii. Add ice to water to keep samples cold
      iii. Set the sonicator rack with tubes in the center of the plate sonicator
           1. make sure all tubes are touching the bottom
      iv. close lid
   v. Set program
      1. Press Prog/Data twice
      2. Set Total Process Time → 5:00
3. Press Enter
4. Set Pulse On Time \(\rightarrow 5:00\)
5. Press Enter
6. Set Pulse Off Time \(\rightarrow 0:05\)
7. Press Enter
8. Set Output Level \(\rightarrow 7.0\)
9. Press Enter
10. Select No Temperature Monitoring

vi. Press Start
vii. Repeat program twice for a total of three rounds of sonication; each time add ice to the water bath to keep the samples cold

g. Gel Preparation

i. Prepare *Sample Buffer* and aliquot 15\(\mu\)L into each of the green and yellow pre-labeled microfuge tubes

ii. Remove 30\(\mu\)L of the sonicated sample and add it to the appropriate pre-labeled green microfuge tube; this is your expressed portion

1. Pipette up and down to mix

iii. Spin down sonicated sample in a table top microfuge at top speed for \(~4\) mins

iv. Remove 30\(\mu\)L of the supernatant and it to the appropriate pre-labeled yellow microfuge tube; this is your soluble portion

1. Pipette up and down to mix

v. Heat all gel samples in a 72°C water batch for 10 mins

E. **Run Gels (Invitrogen SDS Polyacrylamide Gradient Gels)**

a. Fill gel box apparatus generously with 1\(\times\) SDS Buffer

b. Add 10\(\mu\)L of the BioRad Protein Standard to lane 1

c. Arrange the gels such that each fermentation’s HT and HS are next to each other on the gel

d. For a 15-well gel, load 14\(\mu\)L of the sample

e. For a 12-well gel, load 19\(\mu\)L of the sample

f. Set BioRad Power Source

1. 200v

2. 400mA

3. Set time

4. Press run

g. When gels are done running, crack the casing, place gel in a gel box, and wash with dH2O for \(~20\) mins on gel shaker

h. Pour out water and add 3 squirts (\(~30-40\)mL) of Pierce Gelcode Blue Stain; stain on gel shaker overnight
DAY 5

A. Gel Pictures
   a. De-stain gel
      i. Pour out stain and de-stain gel with dH2O for ~20 mins on gel shaker
   b. Take picture using Alpha Imager Instrument with bench top PC
      i. Take cover off of gel box and insert box with gel into picture case; make sure light table is pulled down; close door
      ii. Click “Acquire”
      iii. Select light source → Trans illuminator White for protein gels
      iv. Click “Expose Preview”
         1. Use buttons to perfect your picture
            (a) Exposure time
            (b) Aperture
            (c) Zoom
            (d) Focus
      v. Click “Acquire Image”
         1. Use Contrast Adjustment tools to adjust picture
         2. To enhance picture, use Enhancement Tools in the Tool Box
   vi. Save Image to PC
      1. File, Save, spins webserver, nmr-group, Kellie, GEL_FERMENTATION
      2. File Type → tif
      3. Save as Gel number; e.g. Gel 123.tif
      4. Click on ‘Image’ and scroll down to ‘Conversion’
      5. Convert picture to 8-bit scale
      6. File, Save, spins webserver, nmr-group, Kellie, GEL_FERMENTATION
      7. File Type → JPEG
      8. Save as Gel number; e.g. Gel 123.jpg
   vii. Print out pictures using Alpha Imager Printer on 2nd floor and paste them into Fermentation Gel Pictures Book

B. SPiNE Uploads
   a. Gel Picture Uploads
      i. Label gel images at desk PC
         1. Open Spins webserver, nmr-group, Kellie, GEL_FERMENTATION
2. Right click on gel of interest and open with Adobe Photoshop
3. Label gel wells
   (a) M for Marker
   (b) HT for Harvest Total
   (c) HS for Harvest Supernatant
4. Label gel with Fermentation IDs above corresponding HT and HS wells

ii. Save labeled gel
1. File, Save As, spins webserver, nmr-group, Kellie, GEL_FERMENTATION
2. File Type ➔ JPEG
3. Save As Gel number copy; e.g. Gel 123 copy.jpg

iii. Upload labeled gel picture into SPINE
1. web link: http://spine.nesg.org/private/upload/
   (a) Scroll down to “To upload a structure file or image file for one record”
   (b) File:
      (i) Browse for file name located in Drive F, KELLIE-GELS
      (ii) Select file
   (c) Record ID:
      (i) GEL_FERMENTATION
   (d) Name of file:
      (i) Gel name; e.g Gel 123
   (e) Click Submit

b. Fermentation Mass Uploads
i. Open Fermentation Upload Excel Template in spins
ii. Fill in the fields with the appropriate information
1. Expression ID
   (a) e.g ER1-21.1
2. Label
   (a) e.g SeMa (See Fermentation Naming Scheme in appendix)
3. Researchers
   (a) Scientists’ names
4. Expressed on
   (a) Date of expression; e.g 1/1/2006
5. Host Strain
   (a) e.g BL21 (DE3)+ Magic
6. Growth Temperature
   (a) 37°C
7. Induction Temperature
   (a) 17°C
8. Media
9. Expression Scale
   (a) Preparative
10. Expression Level
    (a) 0-5
11. Induction OD
12. Final OD
13. Fermentation Volume
    (a) 1L
14. Flask Volume
    (a) 2L
15. SDS Gel ID
    (a) This field is entered automatically after the appropriate data is entered in the ‘Gel #’ column
16. Comments
17. Phase
    (a) This field is entered automatically after the appropriate data is entered in the ‘Solubility’ column
18. Solubility
    (a) 0-5
19. Gel #

iii. Mass upload
1. Select and copy information on the Excel Mass Upload sheet
   (a) Select only from columns ‘Expression ID’ to ‘Solubility’
   (b) Do not include Row 1 with the column headings
2. Upload fermentations into SPiNE
   (a) Web link: [http://spine.nesg.org/private/bulk_ferm.pl](http://spine.nesg.org/private/bulk_ferm.pl)
   (b) Paste the information from Excel into the box
   (c) Click ‘Submit’
   (d) SPiNE will then tell you if there are errors with the upload or if they are o.k.
      (i) If there are errors, the Excel mass upload sheet needs to be fixed
   (e) Click “Insert”
(i) SPiNE will tell you if the entries have been inserted successfully.
APPENDIX for Fermentation SOP

• FERMENTATION IDs and FERMENTATION NAMING SCHEME

The Fermentation ID is the construct name + the media label.

The Fermentation Naming Scheme:

- The first time a construct is fermented in a specific media, the letter “a” follows the media label
  Ex: ER1-21.1-SeMa
  ER1-21.1-NC5a
  ER1-21.1-NCa
  ER1-21.1-Na
  ER1-21.1-14Na
  ER1-21.1-LBa

- The second time a construct is fermented (re-fermentation) in the same media, the letter “b” follows the media label
  Ex: ER1-21.1-SeMb
  Etc.

- The third time, a letter “c” would follow, the fourth time, a letter “d” would follow, and so on.

- **Exception** For OUT-OF-HOUSE SeMet re-fermentations (reproduction fermentations), an “-R#” follows the media label
  Ex: ER1-21.1-SeM-R1 (the 1 represents the first time this construct has had a SeMet reproduction fermentation)
  ER1-21.1-SeM-R2 (the 2 represents the second time this construct has had a SeMet reproduction fermentation)
  Etc.

• LB MEDIA

<table>
<thead>
<tr>
<th>1L</th>
<th>500mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 LB Media Capsules</td>
<td>12 Media Capsules</td>
</tr>
<tr>
<td>1L dH2O</td>
<td>500mL dH2O</td>
</tr>
</tbody>
</table>
1. Add stirbar to flask before autoclaving.
2. Autoclave 1L for 35 minutes.
3. Aliquot into bottles.

- **MJ9 MEDIA PREP**

  **PAY ATTENTION** Be sure to use the right supplement mix when preparing media. The supplement mix contains the antibiotics. The antibiotics MUST correspond to the resistance genes in BOTH the expression vector and the host that correspond with our construct.

  Most constructs contain Amp/Kan resistance. Thus, the media they are grown in must contain Amp/Kan Supplement Mix.

  **Below are the recipes for the stock solutions used to prepare media and the recipes for specific media types.**

  - **MONO-BASIC POTASSIUM PHOSPHATE/ NA CITRATE STOCK**

    4L

    KH2PO4  720g
    Na Citrate  40g

    * Add dH2O (Approx. Final Volume = 4L)

  - **DI-BASIC POTASSIUM PHOSPHATE STOCK  (0.5 g/mL)**

    1L

    K2HPO4  500g
    dH2O  Final Volume = 1L

  - **20% 12C GLUCOSE**

    1L

    12C D-Glucose  200g

    * Add dH2O (Final Volume = 1L)
○ 5% 13C GLUCOSE

1L

12C Glucose  190g
13C Glucose  10g

* Add dH2O  (Final Volume = 1L)

○ SUPPLEMENT MIX  Amp/ Kan

<table>
<thead>
<tr>
<th>IL of Media</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>(500X) MgSO4</td>
<td>2mL 100g</td>
</tr>
<tr>
<td>(1000X) Amp</td>
<td>1mL 10g</td>
</tr>
<tr>
<td>(1000X) Kan</td>
<td>1mL 1g</td>
</tr>
<tr>
<td>(1000X) Vitamins</td>
<td>1mL 100mL</td>
</tr>
</tbody>
</table>

*Add dH2O (Final Volume = 500mL)

○ SUPPLEMENT MIX  Kan

<table>
<thead>
<tr>
<th>IL of Media</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>(500X) MgSO4</td>
<td>2mL 100g</td>
</tr>
<tr>
<td>(1000X) Kan</td>
<td>1mL 1g</td>
</tr>
<tr>
<td>(1000X) Vitamins</td>
<td>1mL 100mL</td>
</tr>
</tbody>
</table>

*Add dH2O (Final Volume = 500mL)
SUPPLEMENT MIX  Amp

<table>
<thead>
<tr>
<th>1L of Media</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>(500X) MgSO4</td>
<td>2mL 100g</td>
</tr>
<tr>
<td>(1000X) Amp</td>
<td>1mL 10g</td>
</tr>
<tr>
<td>(1000X) Vitamins</td>
<td>1mL 100mL</td>
</tr>
</tbody>
</table>
*Add dH2O (Final Volume = 500mL)

**When you have constructs with Amp/Chlor resistance genes, use Amp Supplement mix and add 1mL 1000X Chloramphenicol/ 1L of media. When you have constructs with Kan/Chlor resistance genes, use Kan Supplement mix and add 1mL 1000X Chlorampehnicol/ 1L of media.

14N MJ9 (for SeM, 14N)

5L
- 250mL mono-PO4/ NA Citrate Stock
- 60mL di-PO4 Stock
- 10g 14N Ammonium Sulfate
- 4690mL dH2O
- pH to 6.8
- 100mL 20% Glucose
- 25mL Supplement Mix

4L
- 200mL mono-PO4/ NA Citrate Stock
- 48mL di-PO4 Stock
- 8g 14N Ammonium Sulfate
- 3752mL dH2O
- pH to 6.8
- 80mL 20% Glucose
- 20mL Supplement Mix

3L
- 150mL mono-PO4/ NA Citrate Stock
- 36mL di-PO4 Stock
- 6g 14N Ammonium Sulfate
- 2814mL dH2O
- pH to 6.8
- 60mL 20% Glucose
- 15mL Supplement Mix

2L
- 100mL mono-PO4/ NA Citrate Stock
- 24mL di-PO4 Stock
- 4g 14N Ammonium Sulfate
- 1876mL dH2O
- pH to 6.8
- 40mL 20% Glucose
- 10mL Supplement Mix
15N MJ9 (for NC, NC5, N)

5L
- 250mL mono-PO4/ NA Citrate Stock
- 60mL di-PO4 Stock
- 7.5g 15N Ammonium Sulfate
- 4690mL dH2O
- pH to 6.8

NC
- 25mL Supplement Mix
- 20g 13C Glucose

NC5
- 25mL Supplement Mix
- 100mL 5% 13C Glucose

N
- 25mL Supplement Mix
- 100mL 20% Glucose

4L
- 200mL mono-PO4/ NA Citrate Stock
- 48mL di-PO4 Stock
- 6g 15N Ammonium Sulfate
- 3752mL dH2O
- pH to 6.8

NC
- NC - 20mL Supplement Mix
  16g 13C Glucose

NC5
- 20mL Supplement Mix
- 80mL 5% 13C Glucose

N
- 20mL Supplement Mix
- 80mL 20% Glucose

3L
- 150mL mono-PO4/ NA Citrate Stock
- 36mL di-PO4 Stock
- 4.5g 15N Ammonium Sulfate
- 2814mL dH2O
- pH to 6.8

NC
- 15mL Supplement Mix
- 12g 13C Glucose

NC5

2L
- 100mL mono-PO4/ NA Citrate Stock
- 24mL di-PO4 Stock
- 3g 15N Ammonium Sulfate
- 1876mL dH2O
- pH to 6.8

NC
- 10mL Supplement Mix
- 8g 13C Glucose
• 15mL Supplement Mix
• 60mL 5% 13C Glucose

N
• 15mL Supplement Mix
• 60mL 20% Glucose

• 10mL Supplement Mix
• 40mL 5% 13C Glucose

N
• 10mL Supplement Mix
• 40mL 20% Glucose

○ TRACE ELEMENTS (1mL/ 1L MEDIA)

FeCl₃ x 6H₂O    16.2g
ZnSO₄ x 7H₂O    2.4g
CoCl₂ x 6H₂O    4.2g
Na₂MoO₄ x 2H₂O  4.2g
CuSO₄ x 5H₂O    4.8g
H₃BO₃        1.2g
MnSO₄         3.0g
HCl           30mL
dH₂O          570mL

• 10X AMINO ACID SOLUTION (10mL/ 1L SeM MEDIA)

Lysine    10g
Phenylalanine  10g
Threonine   10g
Isoleucine  5g
Leucine     5g
Valine      5g
• 1M IPTG (1mL/ 1L MEDIA)

\[ 1ML \quad Stock \]

| Component | Amount 
|-----------|---------
| IPTG      | 0.2g    
| dH2O      | Final Volume= 1mL 250mL |

*Aliquot into Orange Cap tubes

• LYSIS BUFFER + B-ME

Lysis Buffer 1mL
B-ME 0.7uL

Stock: (0.7uL B-ME) x (XmL Lysis Buffer)

• SDS-PAGE SAMPLE BUFFER

\[ 15uL \ (1 \ Sample) \quad 1mL \ (66 \ Samples) \]

dH2O 6uL 400uL
Sample Buffer 7.5uL 500uL
1M DTT 1.5uL 100uL

• LB AGAR PLATES (100mL = 8 plates)

\[ 1L \quad 500mL \]

25 LB Media Capsules 12 LB Media Capsules
15g Becto Agar 7.5g Becto Agar
1L dH2O 500mL dH2O
Add stirbar to flask before autoclaving. Autoclave 1L for 35 minutes. Add appropriate antibiotics after it has cooled (1mL of 1000X/ 1L)

- **1000X ANTIBIOTICS**

<table>
<thead>
<tr>
<th>Amp</th>
<th>Kan</th>
<th>Cam</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mg/ 1mL dH2O</td>
<td>10mg/ 1mL dH2O</td>
<td>34mg/ 1mL ethanol</td>
</tr>
</tbody>
</table>

  Filter sterilize using syringe.

- **LB AGAR PLATE COLOR CODE**

<table>
<thead>
<tr>
<th>Amp</th>
<th>Kan</th>
<th>Cam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>Blue</td>
<td>Black</td>
</tr>
</tbody>
</table>

- **LB MEDIA WITH ANTIBIOTICS**

<table>
<thead>
<tr>
<th>50mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Media</td>
</tr>
<tr>
<td>20% Glucose</td>
</tr>
<tr>
<td>1000X Antibiotic</td>
</tr>
</tbody>
</table>

- **AGAROSE GELS ( FOR DNA)**

<table>
<thead>
<tr>
<th>1%</th>
<th>2%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X TAE</td>
<td>200mL</td>
</tr>
<tr>
<td>Agarose Powder</td>
<td>2g</td>
</tr>
</tbody>
</table>
PURIFICATION OF SEMET LABELED PROTEINS

Two-Steps Purification of SeMet Labeled Protein by AKTApexress System
(HisTrap™ HP column/HiPrep™ Sephacryl S-100 column)

Author: Haleema Janjua
Last Revision: May 2006

Preparation

1. Obtain the detailed information of each protein from SPINE prior to starting the purification.
   1.1 pI
   1.2 Molecular Weight (kDa)
   1.3 Extinction Coefficient (mg/ml)

2. For each protein sample, create a record under the protein directory on the NMR lab server called nmrgroup on wetserver (Spins). Folder should include sub folders: Expression ID-GF and Aggregation.

3. Preparation of Buffers.
   - Binding Buffer: 50 mM Tris-HCl pH 7.5
     500 mM NaCl
     40 mM Imidazole
     1 mM TCEP
     0.02% NaN3
   - Elution Buffer: 50 mM Tris-HCl pH 7.5
     500 mM NaCl
     500 mM Imidazole
     1 mM TCEP
     0.02% NaN3
   - Low Salt Buffer: 10 mM Tris-HCl pH 7.5
     100 mM NaCl
     5 mM DTT
     0.02% NaN3

Low Salt Buffer needs to be filtered by 0.22 μm filter.
4. Equilibration of the AKTAxpress system.

4.1 Sign in to the PC using nmrgroup/nmrlab as user/password.

4.2 Sign in to UNICORN software by click the icon from the desktop.

4.3 Open System Control window.

4.4 Click the **Instant Run** button. *Result:* The Method Wizard opens

4.5 Select the method of **equi X columns and GF columns** from the directory of preparation (the number X is depended on how many protein you want to purify and how many system you are going to use).

4.6 Make the necessary entries and click the **Next** button to proceed.

4.7 Continue with the subsequent Method Wizard steps until you reach the Summary page.

4.8 The **Summary** page contains all the information needed to prepare the systems to perform the runs. Each system is displayed in an individual tab.

4.9 Verify that the systems are set up according to the information on the Summary page. Confirm that the waste bottles have enough room to collect the waste.

A1: Binding Buffer
A3: Elution Buffer
A4: Low Salt Buffer
S1: sample 1
S2: sample 2
S3: sample 3
S4: sample 4

***GF columns are connected to column position 5.

4.10 Click the **Run** button in the Summary page.

4.11 Click the **Continue all** button to start the run.

**Sample Preparation**

5. Obtain cell pellets from freezer (1 liter culture).

6. Resuspend pellet in 25-30 ml of Binding Buffer (pH 7.5) and place in shaker for 10 minutes or until pellet is resuspended.
7. Sonication.

7.1 Sonicate for 10 minutes in an ice water bath.

7.2 Choose output level 7.

7.3 Program 30 second on/off cycles.

7.4 Select No temperature monitoring.

7.5 After sonication, take 2μl from the protein sample and label ‘T’ (stands for total).

8. Preparation of crude protein samples.

8.1 Centrifuge at 15K rpm for 40 minutes at 4°C (use Sorvall SS-34 Rotor).

8.2 Filter the supernatant using a 50 ml Tube Top Filter (use prefilter).

8.3 Take a 2μl from the filtered sample and label ‘S’ (stands for soluble).

Purification of proteins

9. Purification of proteins

9.1 Put the protein samples at sample location (S1, S2, S3 and S4).

9.2 Open System Control window.

9.3 Click the Instant Run button.

9.4 Select the method of Affinity gel from the directory of purification.

9.5 Enter the necessary information and click the Next button to proceed.

9.6 Continue with the subsequent Method Wizard steps until you reach the Summary page.

9.7 Verify that the systems are set up according to the information on the Summary page. Confirm that the waste bottles have enough room to collect the waste, and set up the 96-well plate for fraction collector. A1: Binding Buffer; A3: Elution Buffer; A4: Low Salt Buffer pH 7.5 (NMR buffer pH 4.5 or pH 6.5);
S1: sample 1; S2: sample 2; S3: sample 3; S4: sample 4.

9.8 Click the Run button in the Summary page.

9.9 Click the Continue all button to start the run.

9.10 The proteins will be purified by two-step purification.

**Maintenance**

10. Clean sample tubes and recharge nickel columns

10.1 Wash outside of tube with dH$_2$O, and wipe with EtOH.

10.2 Replace the sample tubes with Binding Buffer tubes (>40 ml).

10.3 Open System Control window.

10.4 Click the Instant Run button.

10.5 Select the method of Wash sample tube from the directory of Maintain.

10.6 Enter the necessary information and click the Next button to proceed.

10.7 Verify that the systems are set up according to the information on the Summary page.

10.8 Click the Run button in the Summary page.

10.9 Click the Continue all button to start the run.

10.10 For Nickel Recharge click the Instant Run button and select method of column Ni Recharge.

10.11 Choose the number of columns needed to be recharged.

10.12 Verify that the systems are set up according to the information on the Summary page.

10.13 Click the Run button in the Summary page.

10.14 Click the Continue all button to start the run.
Data Analysis and Data Management

11. Analyze the results and pool the fractions of protein.

11.1 Copy the result files to your local computer.

11.2 Start UNICORN software from your computer. Open the results file using the Evaluation Window.

11.3 Choose the fractions for SDS-PAGE analysis. Take 15μl per well for gel. Add 5 μl 4X Sample Buffer. Heat for 10 minutes at +70-80°C.

11.4 Choose Operations:Pool to pool the fractions for each protein. The pooled fractions can be adjusted manually based on the SDS-PAGE result. UNICORN will provide the calculated protein concentration (mg/ml) and total volume (ml) for the pooled protein sample.

****Fractions in microplate are collected in a serpentine manner. For example: A1, A2, → A11, A12 and then B12, B11, →B2, B1, and so on.

12. Create purification records under the expression records in SPINE

12.1 Create a Bulk Purification Record Upload form for each protein and obtain PST IDs.

12.2 Fill out:
Expression ID -- BoR11-21.3-SM
New entry -- NI or GF
Researcher -- First Name Last Name
Date purified -- mm-dd-yyyy
Method -- Superdex 75
Purification Buffer -- Low salt
Final Buffer -- Low salt
Final conc. -- 0.85
Final Vol. -- 24.0
Oligomerization State -- Monomer, Dimer
Determined by -- Gel Filtration
Number of Sample Tubes -- 1, 2, or 3

12.2 PST IDs will be dispensed after purification record table for each protein is uploaded

Protein Shipment

13. Preparation of samples for Columbia and HWI; Analysis of Final Purity
13.1 Pool the fractions, and concentrate to a final concentration between 5.0-10.0 mg/ml by Amicon centrifugal device, 5000 MWCO.

13.2 Determine concentration of each protein sample at 280nm using 6M Guanadine + 10mM Tris, pH 7.5 at a ratio of 1:20 (protein:buffer)

13.3 Calculate final concentration: \( \frac{OD_{280} \times DF (20) \times MW}{EXTINCTION\ COEFFICIENT} \).

13.4 For Columbia: Aliquot 50 μl/tube in 8-well PCR strip (total of 1.6ml or more) cap and immediately freeze by submerging sample into liquid nitrogen. Store sample with assigned PST ID in designated box in -80C.

13.5 For HWI: Aliquot 0.4ml sample in a 1.7ml Eppendorf tube and immediately freeze by submerging sample into liquid nitrogen. Store sample with assigned PST ID in designated box in -80C.

13.6 For Aggregation Screening: Aliquot 0.1ml in HPLC vial and immediately freeze by submerging sample into liquid nitrogen. Place vial in box labeled for Aggregation screening in 4C.

13.7 Assign PST ID to any leftover amount and store in designated box in -80C.

13.8 For Gel and Mass Spec analysis: Save about 0.05ml in 1.7ml Eppendorf tube and place in 4C.

13.9 Run SDS-PAGE to confirm purity of the final sample.

13.10 Confirm Molecular weight is within 500 Daltons of MW obtained from Expression ID by Mass Spec analysis.

14. Create PST records under the protein purification record in SPINE

14.1 Create a Bulk PST Record Upload form for each protein sample.

14.2 Fill out:

14.2.1 Tube ID -- BoR11.001
14.2.2 Tube format -- 8-well PCR strip/1.7 ml Eppendorf
14.2.3 Volume (ml) -- 0.5
14.2.4 Concentration (mg/ml) -- 10.0
14.2.5 Buffer -- Low salt
14.2.6 Current Location -G7-A1-8

14.3 Send Aggregation Screening information to appropriate person.
Aggregation Screening

15. Aggregation Screening by light scattering

15.1 Place vial containing thawed protein in autosampler of HPLC and perform aggregation screening by light scattering using 100mM Tris-HCl, 100mM NaCl, 250 ppm NaN₃, pH 7.5.

15.2 Analyze the result of light scattering. Fill out:
   15.2.1 Prep ID -- BoR11GF
   15.2.2 Buffer -- Low salt
   15.2.3 Date purified -- mm/dd/yyyy
   15.2.4 Date concentrated -- mm/dd/yyyy
   15.2.5 Concentration (mg/ml) -- 7.5
   15.2.6 Analyze date -- mm/dd/yyyy
   15.2.7 L.S. Prim Species -- monomer, dimmer …
   15.2.8 L.S. Prim Species % -- 95
   15.2.9 L.S. Prim Species kDa -- 24.0
   15.2.10 % recovery -- 100
   15.2.11 Injected vol (μl) -- 30
   15.2.12 Calculated amount (μg) -- 250
   15.2.13 #F/T cycles -- 1
   15.2.14 Storage Temp -- -80
   15.2.15 Storage time (day) -- 3
   15.2.16

15.3 Upload the aggregation records into SPINE

16. Sending samples to HWI and Columbia

16.1 Send all samples (0.4ml each) to HWI.

16.2 Send monodispersed samples to Columbia University for crystallization trial. Other protein samples will be set up using 96-well plate with 24 buffer conditions (provided by Columbia) for further aggregation screening.
PURIFICATION OF NC LABELED PROTEINS

Two-Steps Purification of NC Labeled Protein by AKTAxpress System
(HisTrap™ HP column/HiPrep™ Sephacryl S-100 column)

Author: KarISHMA ShETTY
Last Revision: December 2005

Purification Workflow for N/NC5 enriched proteins

Automated (6 days)                  Manual (7 days)

Day One                            Day One
Two Step Purification              NTA Purification
using AKTAxpress

Day Two/Three
SDS-PAGE of fractions
collected by AKTA

Day Three/Four
Pool desired fractions
and Concentrate

Day Four/Five
Buffer Exchange
And Concentrate

Day Five/Six
Final SDS-PAGE
And Mass Spec

Day Six/Seven
Bulk Upload and
NMR Sample Prep
Two-Steps Purification of N/NC5 enriched Proteins by AKTAxpress System  
(HisTrap™ HP/HiLoad™ 26/60 Superdex75 columns)

Automated – Data Preparation

A. Obtain the detailed information of each protein from SPINE prior to starting the purification.
   a. pI
   b. Molecular Weight (kDa)
   c. Extinction Coefficient (M⁻¹ cm⁻¹)

B. For each protein sample, create a record under the protein directory on the NMR lab server called nmrgroup on wetserver (Spins). Folder should be the exact expression ID of the protein.

C. Prepare the Binding Buffer, Elution Buffer, and Gel Filtration Buffer.

D. Binding Buffer: 50 mM Tris-HCl pH 7.5
   500 mM NaCl
   40 mM Imidazole
   1 mM TCEP
   0.02% NaN3

E. Elution Buffer: 50 mM Tris-HCl pH 7.5
   500 mM NaCl
   500 mM Imidazole
   1 mM TCEP
   0.02% NaN3

F. Gel Filtration Buffer:

   NMR Buffer pH 6.5: 20 mM MES
   100 mM NaCl
   5 mM CaCl2
   10 mM DTT
   0.02% NaN3

   NMR Buffer pH 4.5: 20 mM Ammonium Acetate
   100 mM NaCl
   5 mM CaCl2
   10 mM DTT
0.02% NaN3
*All buffers need to be filtered by 0.22 μm filter.

G. Equilibration of the AKTAxpress system.

a. Sign in to the AKTA PC.

b. Sign in to UNICORN software by clicking the icon on the desktop.

c. Open the System Control window.

d. Click the Instant Run button. Result: The Method Wizard opens

e. Select the method of equi X columns and Superdex75 column from the preparation directory (the number X is dependant on how many protein you want to purify and how many system you are going to use).

f. Make the necessary entries and click the Next button to proceed.

g. Continue with the subsequent Method Wizard steps until you reach the Summary page.

h. The Summary page contains all the information needed to prepare the systems to perform the runs. Each system is displayed in an individual tab.

i. Verify that the systems are set up according to the information on the Summary page. Confirm that the waste bottles have enough room to collect the waste.
   i. A1: Binding Buffer
   ii. A3: Elution Buffer
   iii. A4: Gel Filtration Buffer (NMR buffer pH 6.5 or pH 4.5)
   iv. S1: sample 1
   v. S2: sample 2
   vi. S3: sample 3
   vii. S4: sample 4
   viii. ***Gel Filtration columns are connected to column position 5.

j. Click the Run button in the Summary page.

k. Click the Continue all button to start the run.

Automated – Sample Preparation

H. Obtain cell pellets from the -80°C freezer (1 liter culture).
I. Resuspend pellet in 25 ml of Binding Buffer (pH 7.5).

J. Sonicate for 10 minutes in an ice water bath.
   a. Choose output level 7.
   b. Program 30 second on/off cycles.
   c. Select No temperature monitoring.
   d. After sonication take 5μl from the protein sample and label ‘T’ (which stands for total).

K. Preparation of crude protein samples
   a. Centrifuge at 15K rpm for 40 minutes at 4°C.
   b. Filter the supernatant using a 50 ml Tube Top Filter.
   c. Take a 5 μl from the filtered sample and label ‘S’ (stands for soluble)

L. Proceed to **Purification of Proteins**.

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**Manual Purification of N/NC5 Labeled Protein by Ni-NTA Superflow**

**Manual – Data Preparation**

A. Find the detailed information from SPINE prior to starting purification.
   a. Molecular Weight
   b. pI
   c. Extinction Coefficient

B. For each protein create a folder in the SPINS local server. Folder should be the exact expression ID of the protein.

C. Generate data sheet for each protein.

D. Prepare the buffers for Protein Purification
a. Lysis Buffer  
50 mM NaPi  
300 mM NaCl  
5 mM Imidazole  
5 mM 2-ME. pH 8.0

b. Wash Buffer  
50 mM NaPi  
300 mM NaCl  
20 mM Imidazole  
5 mM 2-ME. pH 8.0

c. Elution Buffer  
50 mM NaPi  
300 mM NaCl  
250 mM Imidazole  
5 mM 2-ME. pH 8.0

**Manual – Sample Preparation**

E. Obtain cell pellets (from 1L culture).

F. Resuspend pellet in 25ml of Lysis Buffer (pH 8.0)

G. Sonicate for 10 minutes with ice water bath
   a. Choose output level 7
   b. Program 30s on/30s off
   c. Select No temperature monitoring
   d. At conclusion remove a 5 µl sample for SDS-PAGE and label the tube total

H. Centrifuge at 15 K rpm for 40 minutes.
   a. Collect Supernatant in a labeled beaker and keep at 4°C
   b. Place the centrifuge tubes with the insoluble pellets in -20°C freezer
   c. From the Supernatant take a 5 µl sample for SDS-PAGE and label the tube soluble

I. Prepare an Ni-NTA Superflow column
   a. Pack 6 ml of 50% syrup into a column
b. Bed volume should equal 3 ml

c. Equilibrated with 50 ml of Lysis buffer

J. Pour the Supernatant into the Ni-NTA Superflow column

   a. Collect the flow through into a 50 ml centrifuge tube.

   b. Remove a 15 µl sample for SDS-PAGE and label it flow through.

K. Wash the column with approximately 50 ml of Wash buffer

   a. Collect the wash in a 50ml centrifuge tube.

   b. Remove a 15 µl sample for SDS-PAGE and label it wash.

L. Elute the protein from the Ni-NTA column using 10ml of Elution Buffer

   a. Remove a 15 µl sample for SDS-PAGE and label it purified.

   b. Store the elution at 4°C.

M. Run the 15 µl samples in an SDS-PAGE in the order they were taken alongside a Broad Range Standard.

N. Create a purification record under the expression record in SPINE

   a. Set the purification ID by adding –NI after the Expression ID.

   b. Fill out:

      i. Date Purified

      ii. Method – Ni-NTA

      iii. Purification Buffer – Lysis Buffer

      iv. Final Buffer – Elution Buffer

      v. Final Concentration – in mg/ml

      vi. Final Volume

   c. Upload a picture of the SDS-PAGE for each record created.

O. Proceed to Purification of Proteins.

All NC Samples Continued…

Purification of Proteins
1) Purification of proteins
   
a) Put the protein samples at sample location (S1, S2, S3 and S4).

b) Open System Control window.

c) Click the **Instant Run** button.

d) Select the method of **Affinity Gel Filtration** from the purification directory.

e) Enter the necessary information and click the **Next** button to proceed.

f) Continue with the subsequent Method Wizard steps until you reach the Summary page.

g) Verify that the systems are set up according to the information on the Summary page. Confirm that the waste bottles have enough room to collect the waste and set up the 96-well plate for the fraction collector.
   
   A1: Binding Buffer; A3: Elution Buffer; A4: Gel Filtration Buffer (NMR buffer pH 6.5 or pH 4.5);
   
   S1: sample 1; S2: sample 2; S3: sample 3; S4: sample 4.

h) Click the **Run** button in the Summary page.

i) Click the **Continue all** button to start the run.

j) The proteins will be purified by two-step purification.

**Maintenance**

2) Clean the sample tunings

a) Replace the sample tubes with Binding Buffer tubes (>40 ml).

b) Open System Control window.

c) Click the **Instant Run** button.

d) Select the method of **Wash sample tube** from the Maintenance directory.

e) Enter the necessary information and click the **Next** button to proceed.

f) Verify that the systems are set up according to the information on the Summary page.
g) Click the **Run** button in the Summary page.

h) Click the **Continue all** button to start the run.

**Data Analysis and Data Management**

3) Analyze the results and pool the fractions of protein.

   a) Copy the result files to your local computer.

   b) Start UNICORN software from your computer. Open the results file using Evaluation window.

   c) Choose **Operations: Pool** to pool the fractions for each protein. The pooled fractions can be adjusted manually. The resulting fractions are shown in the chromatogram. UNICORN will provide the calculated protein concentration (mM) and total volume (ml) for the pooled protein fractions.

      ****The collection in the microplate is performed in a serpentine manner i.e. first in A1, A2, ..., A11, A12 and then in B12, B11, ..., B2, B1, and so on.

   d) Pool the fractions for each protein.

   e) Determine the concentration of all the samples using the Unicorn software.

4) Run a SDS-PAGE to confirm the final samples

5) Run a mass spec for each sample to confirm the molecular weight.

6) Create purification records under the expression records in SPINE

   a) Create a Bulk Purification Record Upload form for each protein.

   b) Fill out:

      i) Expression IN -- BoR11-21.3-NC5 (e.g.)
      ii) New entry -- GF
      iii) Researcher -- Karishma G. Shetty. Rong Xiao
      iv) Date purified -- mm/dd/yyyy
      v) Method -- Superdex 75
      vi) Purification Buffer -- Binding Buffer
      vii) Final Buffer -- NMR Buffer 6.5 / 4.5
      viii) Final conc. -- 0.85 (e.g.)
      ix) Final Vol. -- 0.5
      x) Oligomerization State -- monomer
      xi) Determined by -- gel filtration
Sample tubes Date Purified -- 1 or 2 or 3

c) Upload JPEG files of the SDS-PAGEs, Mass Spec and chrom for each record created (This might be automated upload to SPiNE?)

7) Decide how many NMR Samples are to be prepared (1,2 or 3…..)

8) Use a desalting column to perform the buffer exchange into the selected NMR Buffers.

9) Add $D_2O$ to the sample at a final concentration of 5% (~25µl of $D_2O$ to 500µl of sample).

10) Concentrate the sample to ~0.5ml using an ultrafree4 at a speed of 3000xg.

11) Calculate the concentration of the sample by measuring the A280 by diluting 5µl of sample with 95µl of 6M Guanidine solution. Optimal concentration is between 0.5-1mM.

   NOTE: If the OD reading is too high or too low, the dilution might be altered.

   (Enter absorbance into spreadsheet to compute the concentration. Back calculate the concentration of the original sample).

12) Create a PST record under the protein’s purification record in SPiNE.

   Fill in the following columns in an Excel sheet and then upload into SPiNE.

<table>
<thead>
<tr>
<th>Tube ID</th>
<th>Tube format</th>
<th>Volume (ml)</th>
<th>Conc. (mg/ml) (mM)</th>
<th>Buffer</th>
<th>Current location</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR374.004</td>
<td>NMR tube</td>
<td>0.5</td>
<td>1.107</td>
<td>mM NMR Buffer pH 6.5</td>
<td>Inbox</td>
</tr>
<tr>
<td>SR374.005</td>
<td>NMR tube</td>
<td>0.5</td>
<td>1.53</td>
<td>mM NMR Buffer pH 5.5</td>
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<tr>
<td>SR374.006</td>
<td>NMR tube</td>
<td>0.5</td>
<td>0.8932</td>
<td>mM NMR Buffer pH 4.5</td>
<td>Inbox</td>
</tr>
</tbody>
</table>

13) Update SPINS database by entering the PST ID (will discuss with Mike to have Bulk uploading).

**NMR Sample Preparation**

14) Add 10 µl of protease inhibitor to the sample.

15) Transfer the sample from a 1.7 ml Eppendorf tube to a NMR tube and label the NMR tube with the same PST ID.

16) Put the NMR tubes in the Rack labeled “Need to be screened”, and send an email to Swapna  
    gvts@cabm.rutgers.edu
NOTE: In the future, this will be done automatically from PLIMS.

17) Leave a hard copy of the notebook in excel format for Swapna’s reference.
**Protein Purification SDS-PAGE Protocol**

**Prepare Running Buffer**
- Add 100 mL of *Nu-Page MES SDS Running Buffer, (10X)* to 900 mL of distilled water.

**Prepare Sample Buffer**
- In a 1.7 mL tube add 1mL of *BIO-RAD XT Sample Buffer, 4X* to 250µL of 5M DTT.

**Prepare SDS-PAGE Apparatus**
- Insert SDS-PAGE gel into apparatus.
- Fill the SDS-PAGE apparatus with enough running buffer to cover the wells.
- Remove any air bubbles present in the wells.

**Protein Sample Preparation**
- In a 1.7 mL tube add 5 µL of sample buffer to 15 µL working volume. Gently mix prepared sample ensuring not to cause bubbles.
- Heat the sample at 72°C for 10 minutes. Allow sample to cool.
- Load 10 µL of the sample to each well. Don’t forget the standard.

**Run SDS-PAGE GEL**
- Set power supply parameters as indicated
  - Current = 400 mA
  - Voltage = 200 V
  - Time = 40 minutes
- Regulate for constant voltage
- Place lid on top of the apparatus ensuring electrodes are connected and press start button on the power supply.

**Stain and Destain Gel**
- Remove gel from cast and place in a clean gel box.
- Wash gel with ~100 mL of distilled water for 10 minutes.
- Decant water and add ~25 mL of Gel Code Blue Stain. Stain gel for 1 hour.
- Decant stain and destain gel using ~100mL of distilled water
SAMPLE PREPARATION FOR HSQC SCREENING

Author: Karishma Shetty
Last Revision: December 2005

Gel Filtration Buffers:

- **NMR Buffer pH 6.5:**
  - 20 mM MES
  - 100 mM NaCl
  - 5 mM CaCl2
  - 10 mM DTT
  - 0.02% NaN3

- **NMR Buffer pH 4.5:**
  - 20 mM Ammonium Acetate
  - 100 mM NaCl
  - 5 mM CaCl2
  - 10 mM DTT
  - 0.02% NaN3

*All buffers need to be filtered by 0.22 μm filter.

1. Use a desalting column to perform the buffer exchange into the selected NMR Buffers.

2. Add D\textsubscript{2}O to the sample at a final concentration of 5% (~25μl of D\textsubscript{2}O to 500μl of sample).

3. Concentrate the sample to ~0.5ml using an ultrafree4 at a speed of 3000xg.

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   - Tom      acton@cabm.rutgers.edu
   - Rong     rxiao@cabm.rutgers.edu
   - Karishma shetty@cabm.rutgers.edu

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