Proteins

Protein test expression (12/19/2014)

Prep
- 15 mL LB broth per construct
- 1000x stock of antibiotics as necessary (e.g. 100 mg/mL Ampicillin)
- 100x (1M) IPTG stock
- Lysis Buffer + MgCl\textsubscript{2} (50 mL)
  - 50 mM Tris pH 7.0 (2.5 mL 1 M Tris pH 7.0)
  - 300 mM NaCl (6 mL 2.5 M NaCl)
  - 2.5 mM MgCl\textsubscript{2} (125 μL 1 M MgCl\textsubscript{2})
  - H\textsubscript{2}O to 50 mL
- Lysozyme (powder)
- DNaseI (6 mg/mL)

Day 1
- Start 5 mL overnight of each protein of interest.

Day 2
- Inoculate 10 mL LB + antibiotic with 100 μL from overnight
- monitor OD\textsubscript{600} until ~4-7 (1-3 hrs) then take 1mL sample (record OD\textsubscript{600}, pellet cells, discard supernatant and freeze), then induce with 10 μL 1M IPTG.
- Allow proteins to express for 4-5 hrs, then record final OD\textsubscript{600} and take 1 mL post induction sample. Discard supernatant and freeze.
- Pellet remaining 8 mL cells by centrifugation at 4000 RPM for 10 mins in tabletop centrifuge.
- Discard supernatant and resuspend pellet in 200 μL Lysis Buffer
- Lyse cells by adding 1 fleck of lysozyme and mix by inverting several times. Mixture should become very viscous.
- Add 4 μL 6mg/mL DNaseI and incubate at 37°C for 30 mins. Mixture should become less viscous.
- Take 30 μL sample and pellet for 10 mins at 14k RPM in microcentrifuge.
- Separate lysate from lysis pellet, but keep both for gel.

Run gel
- Resuspend pellets from 1 mL pre and post induction samples in 100 μL*(OD\textsubscript{600}) 2x SDS loading buffer for each sample.
- Resuspend lysis pellet in 80 μL 2x SDS loading buffer for each sample.
- Add 80 μL 2x SDS loading buffer to each lysate sample.
- Boil for 5 mins and run gel per manufacturer’s instructions.

Protein Expression (12/17/2012)
Prep (typical)
- 50 mL LB in 250 mL flask
- 2 x 1 L LB in 2L flask (25 g LB/L)
- 1000x Antibiotic stocks as necessary (e.g. 100 mg/mL Ampicillin)
- 1000x (1M) IPTG stocks

Overnight
- 50 mL LB (100 ug/mL Ampicillin or other antibiotic as necessary (see Antibiotics under Cell Bio)
- 1 colony of BL21 or Rosetta cells (CM resistant) transformed with plasmid of interest

Standard Expression
- Add 10 mL from overnight to each 1 L of LB for expression (usually 2 L total)
- 1 mL of 100 mg/mL Amp per 1 L culture (or other necessary antibiotic)
- Allow cells to grow to an optical density of ~0.5 measured at 600nm, then induce with 1 mM IPTG (1 mL of 1 M stock per 1L culture) (take 1 mL sample immediately before induction, centrifuge at 14 k RPM for 5 mins, discard supernatant, and freeze)
- Allow induction to proceed for 4 to 6 hours, take 1 mL sample and treat as above, then centrifuge at 7 k RPM for 10 mins in big centrifuge.

16°C expression
Seems to increase total expression and improve solubility for most proteins.
- Follow steps above until cells are at OD 0.4, then transfer to 16°C incubator and induce at OD 0.5 (about 25mins)
- Allow expression to proceed for at least 16hrs (overnight) remove 1 mL sample, then centrifuge at 7k for 10mins as above.

Expression in Minimal Media (06/06/12)
Prep
- 10X buffer base
  - For 1L of 10x concentrate:
    | Component                      | amount | FW  |
    |--------------------------------|--------|-----|
    | KH$_2$PO$_4$ (potassium phosphate monobasic) | 130g   | 136.09 |
    | K$_2$HPO$_4$ (potassium phosphate dibasic) | 100g   | 174.18 |
    | Na$_2$HPO$_4$ (sodium phosphate dibasic)    | 90g    | 141.96 |
    | K$_2$SO$_4$                                    | 24g    | 174.27 |
- Trace element solution
  (Starts out greenish-brown, and turns yellowish with overnight stirring)
<pre><code>| Component          | Amt for 100mL | Amt For 1L (g) |
|--------------------|---------------|----------------|
| CaCl$_2$.2H$_2$O   | 0.6g          | 6.00           |
| FeSO$_4$.7H$_2$O   | 0.6g          | 6.00           |
| MnCl$_2$.4H$_2$O   | 0.115g        | 1.15           |
</code></pre>
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
<td>0.08g</td>
<td>0.80</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.07g</td>
<td>0.70</td>
</tr>
<tr>
<td>CuCl$_2$.2H$_2$O</td>
<td>0.03g</td>
<td>0.30</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
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<td>0.020</td>
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<tr>
<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$.4H$_2$O</td>
<td>0.025g</td>
<td>0.25</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5g</td>
<td>5.00</td>
</tr>
</tbody>
</table>

- Autoclaved 1 M MgCl$_2$ solution
- Filter-sterilized 20% weight/volume glucose
- Antibiotic(s) as necessary
- Vitamin mix if desired, 5 mL/L

For 1L of M9 minimal media:
- Autoclave 100 mL of 10X Buffer Base + 870 mL H$_2$O
- Cool to room temperature, then add additional filter-sterilized components:
  - 1.1 g NH$_4$Cl (typically $^{15}$NH$_4$Cl for labeled protein) in 10 mL H$_2$O
  - 5 mL 1 M MgCl$_2$
  - 5 mL trace element solution
  - 2 g of glucose in water (10 mL 20% weight/volume glucose)
  - Antibiotic(s) as necessary (Typically 100 μg/mL Ampicillin)

**Growth**
- Grow 5 mL culture of desired strain in LB to density.
- Transfer 200 μL of LB culture into 25 mL minimal media and grow overnight.
- Inoculate remaining 975 mL minimal media with all 25 mLs of O/N culture.
- Follow same induction protocol for LB growth for optimal expression in minimal media.

**Protein Purification**

**Co$^{2+}$ Affinity Purification (03/13/2014)**

**Prep**

- Lysis Buffer (500mL)
  - 50mM Tris pH 7.0
  - 300mM NaCl
  - 415mL ddH$_2$O
- Wash Buffer (50mL)
  - 50mL Lysis buffer
  - 10mM Imidazole
- Elution Buffer (50mL)
  - 50mM Tris pH 7.0
  - 300mM NaCl
  - 150mM Imidazole

(25mL 1M Tris pH 7.0)  
(60mL 2.5M NaCl)  
(500μL 1M Imidazole pH 7.0)  
(2.5mL 1M Tris pH 7.0)  
(6mL 2.5M NaCl)  
(7.5mL 1M Imidazole pH 7.0)
-34mL H₂O

*Note: Phosphate buffers can also be used in place of Tris buffers if desired:
- 50mM NaPO₄ pH 7.0 (25mL 1M Na₂PO₄ pH 7.0 (make stock with about 5:1 mono:dibasic for pH 7))
- 300mM NaCl (60mL 2.5M NaCl)
- 415mL ddH₂O

-Cool 50 mL of each buffer to 4°C before beginning purification.

Resuspend Cells from expression in Lysis Buffer.
- 20mL buffer, 200µL PIC, 200µL 0.2M PMSF/1L culture

Sonicate with medium tip.
- 50% duty cycle, pulse for 1.5min on, 30s off, 4 times.
- Take 30µL sample, centrifuge and separate supernatant and pellet for gel analysis

Centrifuge
- Pour lysate into small centrifuge bottles (fill with max 20mL), centrifuge at 15k RPM for 40mins at 4°C

Equilibrate Co²⁺ resin (about 5 mL Talon™ resin [10mL of suspended resin] per 2L culture)
- 5 column volumes H₂O
- 5 column volumes Lysis Buffer

Bind
- Mix equilibrated resin and supernatant in 50mL Falcon tube and put on rotisserie at 4°C for at least 10mins.

Purify
- Reload resin onto column
- Collect flow through in 50 mL Fisher tube
- Wash with 5 CV lysis buffer; stirring resin occasionally
- Wash with 5 CV wash buffer (lysis buffer + 10 mM imidazole); stirring resin occasionally
- Elute protein with 50 mL lysis buffer + imidazole. Collect 10 mL fractions.
- Take 10µL sample from each wash and elution fraction for gel analysis.

Run gel
- Dissolve pellet from 1 mL post induction sample in 100 µL*(OD₆₀₀) 2x SDS loading buffer for each sample.
- Dissolve lysis pellet in 80 µL 2x SDS loading buffer.
- Add 80 µL 2x SDS loading buffer to lysate sample.

Regenerate Resin
- Wash resin with 5 CV H2O
- Wash with 5 CV 20mM MES buffer (pH 5.0) 0.1M NaCl
- Wash resin with 5 CV H2O
- Store at 4°C in 20% EtOH with 0.1% NaN3

Stringent Wash
- Wash resin with 4 CV 6M GdmHCl pH 5.0, 1% nonionic detergent.
- Wash with 5 CV H2O

Complete Regeneration (typically not necessary)
- Strip Co²⁺ by washing with 10 CV of 0.2M EDTA (pH 7.0) ➔ collect as hazardous waste
- Wash out EDTA with 10 CV H2O
- Recharge resin with 10 CV of 50 mM CoCl₂ solution ➔ collect to be reused.
- Wash out excess Co²⁺ ions with 10 CV H2O ➔ collect as hazardous waste.

ULPI protease cleavage (07/12/12)

Cleave
- Concentrate protein and buffer exchange back into lysis buffer (this can be shortened by running 15 mL of buffer through your protein in the concentrator, and then performing the remainder of this reaction in a dialysis bag and leaving it in 2 L of lysis buffer at 4°C with constant stirring O/N)
- Determine protein concentration (by UV is fine)
- Add 1 mg ULPI per 50 mg protein. Make sure final glycerol concentration is <10% (usually ULPI is in 50% glycerol)
- Allow reaction to proceed for 2hrs at room temp (or O/N at 4°C)

Purify
- Equilibrate CO²⁺ column in lysis buffer as per Co²⁺ Affinity Purification protocol above.
- Bind His6-SUMO tag to resin by combining protein and resin as above.
- Place protein-resin mixture in a gravity column, and collect flowthrough (your protein will be in the flowthrough!)
- Wash 5 – 10 times with lysis buffer, collecting 10 mL fractions (10 times is safe for the first time with any protein, most proteins will be completely washed out before the 8th fraction, but you can calibrate how many washes you will need for each individual protein)
- Elute His6-SUMO tag with at least 50 mL of elution buffer, as above.
- Regenerate resin as above, and store in 20% EtOH, 0.1% NaN₃

**SP Column Purification (7/15/2008-)**

**Prep**

- **SP Buffer A (5L)**
  - 50mM NaPO₄ pH 6.0 (1.31g dibasic, 5.78g monobasic)
  - 5mM EDTA (1.86g)
  - 5mM DTT (0.385g)

- **SP Buffer B (1L)**
  - 1M NaCl (53.5g)
  - 50mM NaPO₄ pH 6.0 (1.31g dibasic, 5.78g monobasic)
  - 5mM EDTA (1.86g)
  - 5mM DTT (0.385g)

**Procedure**

- Dialyze necessary fractions in 4L SP Buffer A overnight
- Equilibrate SP column with SP Buffer A and SP Buffer B overnight
- Run Column at 0.5mL/min and collect 5mL fractions. Protein generally elutes around fractions 34-41
- Run fractions on SDS PAGE gel, dialyze and concentrate as needed.

**Protein Gel Electrophoresis (12/04/2013)**

**Prep**

2x LDS Loading Buffer
- 4x LDS Sample Buffer
- 10 mM DTT

Mix protein sample and LDS buffer 1:1 and boil for 3 mins (pellets from 1 mL bacterial growth can be dissolved in 100 µL 2x LDS, Lysis pellet in 120 µL LDS, and lysate in 80 µL). Briefly centrifuge and then load 15 µL onto gel. Run at 165V for 35 mins.

**Protein Concentration Determination**

**UV (12/5/2011)**

Prepare 3 x 60 µL dilutions of sample. For typical 2L prep, at 1mL assume between 300 and 1500µM and dilute to around 50 µM
- 10x (60 µL total V)
- 50x (100 µL total V)
-100x (100 µL total V)

-Multiply A280 by dilution factor, then by your protein’s extinction coefficient from Expasy protparam site.

Useful Extinction coefficients: \((\text{M}^{-1}\text{cm}^{-1} \text{ at } 280\text{nm}) / \text{MW (g/mol)}\)

- \(\text{SaA:} \quad 14440\)
- \(\text{BaA:} \quad 14440\)
- \(\text{BaC:} \quad 22920\)
- \(\text{SaA-V168C:} \quad 14440\)
- \(\text{SrtB:} \quad 23380\)

**BCA Assay (reduced volume and concentration) (5/5/2014)**

(affected positively by Trp, Tyr, and Cys. Also, check buffer compatibility: http://www.piercenet.com/Objects/View.cfm?type=Page&ID=B49EC38D-254A-400D-BACA-F0C564454B72)

(compatible with HEPES to 100mM, TRIS to 250mM, Imidazole to 50mM)

- 1:20 ratio of sample:working reagent (WR)
- \(\text{WR} = 1:50 \text{ ratio of B:A}\)

- Make 1mL of WR for each sample and standard + blank.

Concentration needs to be between 5 and 250ug/mL
- Dilute BSA standards 10x (12.5ug/mL - 200ug/mL):
  - 5µL each std + 45µL H2O

- Dilute sample so it should fall in this range. (prepare 3 dilutions to cover all possibilities)
  - 50x
  - 100x
  - 200x

Prepare
- 1mL WR
- 50µL Sample

Procedure
- Incubate for 30 mins at 60°C in water bath
- Cool to room temperature
- Measure absorbance of all samples at 562 nm within 10 mins.
Bradford Assay (12/02/11)
(reacts primarily with Arg, His, Lys)

Mix Bradford dye (Coomasie) thoroughly before beginning, to dissociate dye-dye complexes.
- Add 1mL dye
- 20 µL sample

Sample should be diluted to fall in range of standards, 0.125 to 2 mg/mL. For typical 2L prep:
- 10x
- 50x
- 100x

Procedure
- Mix samples and dye, and allow to equilibrate for 10 minutes.
- Measure absorbance at 595nm

Sortase Transpeptidation Assay (12/14/2012)

Prep
Assay Buffer:
300 mM TRIS-HCl pH 7.5
150 mM NaCl
(5 mM CaCl if SrtA)

Reasonable sortase concentrations:
SrtA = 10 µM
SrtB = 100 µM (for kinetics, see max v_o around 10 mM for SrtB)

Reasonable substrate concentrations:
[GGG] = 2 mM
[peptide substrate] = 200 µM

Reaction
- Combine all reagents listed above, adding sortase last. (100 µL total V is standard)
  Example reaction:
  -10 µL SrtB (1 mM)
  -10 µL Peptide substrate (2 mM)
  -10 µL GGGGG (20 mM)
  -70 µL assay buffer
- Incubate at 37 °C for 24 hrs (1-3hrs if looking for v_o)
- Quench reaction with 50 µL of 1 M HCl/100 µL reaction mixture
- Analyze results by reversed phase analytical HPLC (quantitative) or MALDI-TOF MS (qualitative/for identification)

**For 100 µL reactions:**
- 10 µL SrtB (1 mM)
- 10 µL peptide (2 mM)
- 10 µL GGGGG (20 mM)
- 70 µL Buffer

**Sortase B NPQT* Modification Reaction (11/13/2012)**

**Prep**
SrtB Xtal Buffer:
- 10 mM Tris-HCl pH 7.0
- 20 mM NaCl

Purified Sortase B  1 mM
CuCl₂  100 mM    (0.0170 g/mL)
DTT  10 mM    (0.00154 g/mL)
NPQT* in 75% DMSO  10 mM
Proline in Xtal Buffer  4 M    (0.4605 g/mL)

**Procedure**
*Prepare a 100 µL test reaction before trying any larger scale preps so you don't waste material.*

In 1 mL microcentrifuge tube (DO NOT USE FALCON TUBE, impurities in plastic will make your protein precipitate!):

<table>
<thead>
<tr>
<th>reagent</th>
<th>final concentration</th>
<th>V for 100 µL reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SrtB</td>
<td>100 µM</td>
<td>(10 µL)</td>
</tr>
<tr>
<td>NPQT*</td>
<td>1 mM</td>
<td>(10 µL)</td>
</tr>
<tr>
<td>DTT</td>
<td>1 mM</td>
<td>(10 µL)</td>
</tr>
<tr>
<td>Proline</td>
<td>1 M</td>
<td>(25 µL)</td>
</tr>
<tr>
<td>Xtal Buffer</td>
<td></td>
<td>(45 µL)</td>
</tr>
</tbody>
</table>

- Wait 4 hrs, (or monitor reduction of NPQT*S-S*TQPN dimers by MALDI until only monomer at 595 m/z is present) and then add CuCl₂ to 10 mM (10 µL)
- Monitor progress of reaction by MALDI every 24 hrs, for 1 week, by removing 1 µL sample, diluting with xtal buffer to 10 µL, zip-tipping and plating. Reaction should hit ~50% modification after 1-2 days and then max-out at around 80% modification after ~6 days.

**DNA**

**DNA/RNA Concentration Determination (4/09/2012)**

**Prep:**
- dilute samples 20x (3 µL sample into 60 µL water) then A260*1000 will give [DNA] in ng/µL

**Procedure**
- Blank spectrophotometer at A260 with water in 100µL UVette using metal adapter
- Measure A260 for all samples
- [sample](ng/µL) = 50ng/µL*A260*(dilution factor)
- 1kb of dsDNA is about 6.5x10^5g/mol so nM = ng/µl*(1/0.65/kb)

**Reconstituting 1KB Plus DNA ladder from Invitrogen (8/07/2013)**
- 150 µL ladder
- 600 µL water
- 125 µL 6x DNA Dye

**Designing Primers for inserts (7/15/2008-)**

**Essentials:**
- Your gene of interest (with start codon)
- Ribosome binding site (if not already present upstream of restriction site on plasmid: 5’-AGGAGGACAGCTATG-3’)
- Stop codon at end of gene unless adding a C-terminal tag from vector.
- Restriction sites on both ends of gene for integration into plasmid
- Always make sure to translate new DNA constructs before ordering the primers.

**PCR (General) (04/16/12)**

*same protocol for phusion or Q5

**Prep: (50µL)**
- 10 µL 5x Phusion Buffer
- 2.5 µL FW'D primer (10uM)
- 2.5 µL REV primer (10uM)
- 0.1-0.2ug plasmid template (~1 µL)
- 1 µL dNTP mix (10mM each)
- 0.5 µL Phusion polymerase
- 32.5 µL H₂O (up to 50µL)
Prep: (25 µL)
- 5 µL 5x Phusion Buffer
- 1.25 µL FWD primer (10uM)
- 1.25 µL REV primer (10uM)
- 0.5 µL plasmid template
- 0.5 µL dNTP mix (10mM each)
- 0.3 µL Phusion polymerase
- 16.2 µL H₂O (up to 25µL)

Procedure:
- Initial Denature: 95°C, 2 mins
- 30 Cycles:
  1. Denature 95°C, 30s
  2. Anneal 55°C, 30s (or 5°C below Tm)
  3. Elongate 72°C, (30s/kb) *This temp and time are variable based on poly.
- Final extension 10 mins at 72°C
- Hold at 4°C indefinitely

Quikchange Mutagenesis (12/17/12)
2 Separate reactions: 1 for bottom primer, and 1 for top. (Primer pairs should have melting temperatures somewhere around 60-70°C)

Prep (each 25µL reaction):
-2.5µL 10x Pfu Buffer
-1µL primer (10µM)
-0.1-0.2ug plasmid template (usually ~1µL)
-0.5µL dNTP mix (10mM)
-0.5µL Pfu µLtra II polymerase
-H₂O up to 25µL (~19.5µL)

Procedure:
1. Initial Denaturation: 95°C, 2mins
2. 95°C, 1min
3. 55°C, 1min (or 1°C below lowest primer Tm)
4. 68°C, 30 s/kb up to 10kb plasmid (check booklet for plasmids >10kB)

Repeat 2-4 for 6 cycles, hold at 4°C. (should take ~2-4hrs)

- Combine both reactions from above, add 0.5 µL Pfu poly, treat as above, but repeat 2-4 for 18 cycles. (should take ~10hrs)

Add 0.5µL DPN1 enzyme. Mix and incubate at 37°C for 1 hour.
Transform 5-10µL of DNA into XL2blue cells (or XL10s for plasmids greater than 10kb) and plate. (Mutagenesis frequency should be ~80%, so usually send 4 cols for sequencing for 99.8% chance of a positive. If they’re all wrong then your QC reaction probably failed and should be repeated.)

**Colony PCR (12/17/2012)**
- Used to screen for the presence of your gene of interest.
- Usually want to PCR a fragment that is somewhere around 500 bases for best results.
- To minimize false positives, its best to use 1 primer (fwd) that anneals to the vector and 1 (rev) that anneals to your gene.

**Prep**
- 90µL 2x APEX Red polymerase master mix
- 9 µL FWD primer (10uM)
- 9 µL REV primer (10uM)
- H₂O up to 180µL (72µL)

**Procedure**
- Prepare above mixture and pipet 20 µL aliquots into 8 PCR microtubes.
- Remove a colony from plate, and shake tip vigorously in PCR buffer in microtube, and then immediately streak onto new plate, labeling plate and tube identically.
- Put plate in incubator at 37°C
- PCR
  - Initial Denature: 95°C, 5 mins
  - 30 Cycles:
    1. Denature 95°C, 1 min
    2. Anneal 55°C, 1 min (or 5°C below Tm)
    3. Elongate 68°C, (1min/kb)
  - Final extension 4 mins at 68°C
  - Hold at 4°C indefinitely

- Prepare 1% agarose gel and run all samples to detect the presence of your gene of interest.

**Recursive PCR (9/25/2013)**

**Prep**
- Purchase primers of 50-60 bp with at least 17-20bp of overlap between each primer or try gBlocks from IDT. (Consult Recursive PCR: a novel technique for total gene synthesis)
- Prepare PCR reaction mix as per General PCR except:
- Add 0.2-0.3 pmol of each internal primer, and 20 to 30 pmol of outer primers (that’s 2-3 µL of a 0.1 µM stock (a 100x dilution of your 10 µM stock) for the inner primers)
- Be prepared to sequence about twice as many colonies as you would for a normal cloning venture, as this protocol can sometimes lead to single nt insertions/deletions, or other weird anomalies. These can often be easily fixed by a post-ligation quickchange.

Procedure:
- Initial Denature: 95°C, 2 mins
- 30 Cycles:
  1. Denature 95°C, 2 mins
  2. Anneal 55°C, 2 mins (or 5°C below lowest Tₘ)
  3. Elongate 72°C, (30 s/kb)
- Final extension 10 mins at 68°C
- Hold at 4°C indefinitely

Restriction Endonuclease Digestion (12/04/2013)
*check enzyme/buffer compatibility on NEB website,
double digest buffer chart - Enzymes can display significant star activity (cleavage site promiscuity) when used in alternative buffers.

Prep (30 µL rxn)
- 3 µL 10x Buffer (based on chart above)
- 23 µL DNA
- 2 µL each enzyme (high enzyme concentration, esp. higher than 10% of total volume, can lead to increased star activity)

Procedure
- Incubate at 37°C for 1 hour. (extended reaction time can lead to increased star activity)
  (-if sequential digestion is required, PCR purify, or heat deactivate and adjust buffer conditions, and then digest with second enzyme in new buffer conditions)
- Gel Extract products
- Proceed to ligation
  (-To determine efficiency of an individual endonuclease, single digest plasmid. Or, (this only works when each end has a different restriction site) cut with a single enzyme, PCR purify (or heat inactivate) and ligate. Run a gel of ligated and unligated, and you should see a dimer if your cut and ligation were successful.)
Ligation (01/09/12)

Prep
- 2 µL 5x T4 ligase buffer
- 6:1* molar ratio insert:vector (100 ng vector) (2:1 or 4:1 if large insert (<700b)) *use 2:1 or 3:1 if insert is 1 kb or larger.
- 0.5µL T4 DNA ligase
- H₂O to 10 µL

Procedure
- Leave at room temperature for 1hr (up to 3hrs for difficult insertions) at room temp or O/N at 4°C
- Heat to 65°C for 10 mins to denature enzyme
- Transform entire reaction into XL2 blue cells (XL10 if plasmid is 8kb or larger), and plate on appropriate antibiotic.

Antarctic Phosphatase treatment (9/25/2013)
Dephosphorylates 5’ end of vector to prevent self-ligation. (Does NOT require additional purification before ligation)

Prep
- 2 µL 10x Antarctic Phosphatase buffer
- 1 µL Antarctic Phosphatase
- 17 µL vector

Procedure
- Incubate at 37°C for 30 mins
- Heat deactivate at 70°C for 5 mins
- Proceed directly to ligation.

Agarose Gel Electrophoresis (7/15/2008-)
(Lower limit of detection is about 10 ng DNA)

Prep:
TAE Buffer (Tris-acetate-EDTA)
-40 mM Tris-Acetate
-1 mM EDTA
-0.571 mL/L Acetic Acid

Agarose, 1% (for separating 0.4 to 7 kb DNA, use up to 2% for small MW, or 0.8% for large/genomic)
- 1 g agarose
- 100 mL TBE or TAE buffer

Procedure
- Microwave agarose gel mixture until all agarose is dissolved (or until agarose is liquefied if using stock solution)
- Pour warm gel into mold.
- Add desired comb, and allow 20 mins to cool.
- Transfer to gel running box.
- Fill reservoir with TAE or TBE buffer to cover gel
- Mix samples with loading dye, and load ~12 µL into each well (significantly more can be loaded if bands are to be extracted)
- Run at 90V for 1.25 hours. (DNA will migrate towards positive (red) terminal)
- Stain gel for 15 to 30 mins in 0.5 µg/mL ethidium bromide (EtBr)
- Submerge gel in wash buffer briefly to remove excess EtBr
- Visualize with UV light (~360 nm) (Minimize UV exposure if bands are to be extracted, especially plasmids)

Resuspending Stock DNA ladder (4/16/2012)
- Add 500 µL H₂O
- Add 100 µL DNA dye

Radiolabeling RNA

Prep
Rnase free T4 PNK buffer:
70 mM Tris-HCl, pH 7.6
10 mM MgCl₂
5 mM DTT
T4 polynucleotide kinase (PNK)
γ-[³²P] ATP
ssRNA

Protocol
Reaction Conditions for 50 µL reaction:
1-50 pmol of RNA
1X T4 PNK buffer
50 pmol γ-[³²P] ATP
20 units of T4 PNK

Transfer γ-[\textsuperscript{32}P]PO\textsubscript{4} to 5' end of RNA substrate - incubate at 37°C for 30 mins
Inactivate enzyme – 65°C for 20 mins
Store at 4 °C

Cell BIO

**Antibiotic working concentrations (12/17/12)**

*E. coli*
- Ampicillin: 100 µg/ml
- Kanamycin: 50 µg/ml

*E. coli* - Rosetta
- Chloramphenicol 34 µg/ml

*B. subtilis*
- Ampicillin: 100 µg/ml (in water)
- Chloramphenicol: 5 µg/ml (in 100% EtOH)
- Erythromycin: 1 µg/ml (in 100% EtOH)
- Spectinomycin 100 µg/ml (in water)

**E. coli Transformation (12/04/2013)**

<table>
<thead>
<tr>
<th>From pure plasmid</th>
<th>From QC reaction</th>
<th>From Ligation Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 3 µL plasmid</td>
<td>- 5 µL plasmid</td>
<td>- 10 µL plasmid</td>
</tr>
<tr>
<td>- 125 µL Cells</td>
<td>- 125 µL Cells</td>
<td>- 125 µL Cells</td>
</tr>
<tr>
<td>- 25 µL 5x KCM</td>
<td>- 25 µL 5x KCM</td>
<td>- 25 µL 5x KCM</td>
</tr>
<tr>
<td>- 47 µL H\textsubscript{2}O</td>
<td>- 45 µL H\textsubscript{2}O</td>
<td>- 40 µL H\textsubscript{2}O</td>
</tr>
</tbody>
</table>

Procedure:
- Incubate on ice 10 mins
- Heat shock 35 s at 42°C
- Incubate on ice 10 min
- Add 150 µL SOC media and rescue at 37°C for 1hr in shaker. (unless antibiotic resistance is AMP, then skip)
- Plate 150 µL Cells on appropriate antibiotic.

**Agar Plates (7/15/2008-)**

500 mL LB/Agar:
- 12.5 g LB
- 7.5 g Agar
- 500 mL H₂O

**Procedure**
- Autoclave
  - Easy cycle, liquid, 20 mins
- Allow to cool until it can be handled easily
- Add antibiotic (100ug/mL Amp, or other as necessary)
- Pour into plates, utilizing sterile technique.
- Quickly flame the top of each plate to sterilize and remove bubbles

**KCM competent E. coli (3/12/2014)**

**Prep**

TSS buffer (100 mL)
- 10g PEG 3350
- 5mL DMSO
- 2mL 1M MgCl₂
- LB up to 100 mL
- Filter sterilize.

**Procedure**

**Day 1**
- Grow O/N culture of strain of interest in 10 mL LB (+antibiotics if necessary, i.e. Rosettas)
- Label 100 0.5 mL tubes
- Autoclave 500 mL LB (liquid cycle)
- Autoclave the labeled 0.5 mL tubes (dry cycle)
- Chill in Freezer: 25 mL serological pipets (3-4), labeled/autoclaved 0.5 mL tubes, purple rack for holding the tubes, 1 mL pipet tips, 1-2 centrifuge bottles.

**Day 2**
- Add 5 mL of your O/N culture to 500 mL LB and grow to an OD₆₀₀ of 0.4-0.5
- Chill centrifuge rotor and bottles
- Chill cells on ice to stop growth
- Pellet cells at 3500 rpm for 15 mins. Discard Supernatant.
- Resuspend pellet in 25 mL TSS
- Place chilled tubes in chilled rack, and keep everything on ice
- Aliquot 300 uL cells into each tube using aseptic technique, and drop aliquots into liquid nitrogen (and leave them there until you’re done aliquoting), then freeze at -80.

**Bacillus subtilis Protocols**

**Quick Competent B. subtilis**
*only for use with strains that don’t have a deficiency in competence.*

10x T-base  
-0.15 M \((NH_4)_2SO_4\)  
-0.8 M \(K_2HPO_4\) (Potassium Phosphate dibasic)  
-0.64 M \(KH_2PO_4\) (Potassium Phosphate monobasic)  
-34 mM \(NaC_3H_5O(COO^-)_3\) (Sodium Citrate)

SpC media (100mLs):  
-10 mL 10x T-base  
-2 mL 10% yeast extract  
-1 mL 50% glucose  
-0.4 mL amino acids as required (10 mg/mL Trp, Phe, Thr)  
-0.25 mL 10% casamino acids  
-0.1 mL 1 M MgSO_4  
-86.5 mL H_2O

SpII  
-10 mL 10x T-base  
-1 mL 50% Glucose  
-1 mL 10% yeast extract  
-0.4 mL amino acids as required (10 mg/mL Trp, Phe, Thr)  
-0.35 mL 1 M MgSO_4  
-0.1 mL casamino acids  
-87.15 mL H_2O

**Procedure**  
- Streak out strain required on LB agar and incubate at 37°C overnight.  
- Incubate 1 colony (or ~3 µL from glycerol stock) in 2 mL SpC medium in a 50 mL Falcon tube in shaker, at 37°C for 5-6 hours until cells are saturated.
- Put 0.5 mL of the SpC culture into 5 mL SpII (you can make 4 of these), and incubate at 37°C for 90 mins.
- Pellet cells at 3,500 RPM at room temp.
- For each 5 mL culture, retain 0.1 mL of the culture supernatant, and use to resuspend the pellet from all 5 mLs of cells. (Do NOT resuspend in fresh media)
- Use immediately, or add glycerol to 10% (14 µL of 80% glycerol per 100 µL of cells) and freeze at -80°C

**B. subtilis Transformation**

In each of 2 tubes:
- 100 µL competent cells
- ~500 ng plasmid DNA (~5 – 10 µL)

**Procedure**
- Shake at 37°C for 20 mins
- Plate on LB agar + antibiotic, and grow at 37°C overnight
- Verify presence of gene by colony PCR

**Electroporation**

Notes:
- Transformation efficiency reported to increase after 2 freeze thaw cycles
- Multiple pulses reportedly yields fewer transformants

**Prep:**
Wash buffer
- 1 mM HEPES pH 7.0

Electroporation buffer
- 1 mM HEPES pH 7.0
- 10% glycerol

**Procedure:**
- Grow cells (10 mL/transformation you intend to do) to OD₆₀₀ of ~0.6 (mid log phase. As cells approach stationary phase, transformation efficiency decreases)
- Chill on ice 30 mins, pellet
- Wash twice in wash buffer (wash steps are necessary to remove all salt and prevent arcing)
- Wash twice in cold electroporation buffer
- Resuspend cells in 1/200th of the original culture volume (10^9-10^10 CFU)
- Mix 50 µL cells with 1 µL DNA (~100-500 ng)
- Chill on ice 1 min
- Transfer to cold 0.2 cm cuvette
- Electroporate: 25 µF capacitance; 7,500 V/cm; 200 Ω.
- Dilute 10x into LB, incubate at 30˚C for 1 hr.
- Plate on selective media

**Cellulase Display Assay (12/17/2012)**

**Prep:**
- 50% xylose
- 1 M IPTG
- CMC buffer – 20mM Tris-HCl, pH 6.0
- 0.5% carboxymethylcellulose in CMC buffer (allow to dissolve overnight)
- DNS detection buffer 100mLs:
  - 1% 3,5-dinitrosalicylic acid
  - 1% NaOH
  - 0.2% phenol (in -20˚C)
  - 0.5% Na_2SO_3

**Procedure**
- Start 5mL overnight of B. subtilis strain of interest in LB with 1 µg/mL erythromycin, or other antibiotic as necessary using 1 colony from a plate, or chunk of ice from glycerol stock.
- Next morning, dilute 100x (100 µL into 10 mL LB + antibiotic)
- Grow to OD 0.1, (about 1:20 – 1:30 hrs) then induce sortase with 0.5% xylose* (100 µL of 50%), and induce CelA with 1 mM IPTG (10 µL of 1 M). *Not necessary with new IPTG inducible sortases.
- Allow cells to grow for 4 hours. Record final OD. (if multiple cultures, stop around same OD)
- Pellet all 10 mL cells at 3500 RPM for 10 mins in big tabletop centrifuge
- Wash pellets with 1 mL CMC buffer by resuspending in buffer and pelleting again (6000 rpm 2 mins in microfuge). Decant Supe, repeat.
- Resuspend in 1 mL 0.5% carboxymethylcellulose (CMC)
- Incubate at 37˚C for 60 mins (no shaking)
- Pellet samples (6000 rpm 2 mins in microfuge), and mix 0.5 mL of supernatant with 0.5 mL DNS.
- Boil samples, and CMC blank + DNS for 10mins, utilizing eppie cap holders. (be careful when removing these from heat! Wear goggles in case the caps pop off!)
- Detect reducing sugars by measuring ABS at 575 nm

**Variable Reporter Dockerin Binding Assay (01/09/12)**
Prep
- Dock Buffer
  - 50 mM Tris-HCl pH 7.0
  - 250 mM NaCl
  - 30 mM CaCl$_2$

Procedure
- Start 5mL overnight of B. subtilis strain of interest in LB with 1 µg/mL erythromycin, or other antibiotic as necessary.
- Next morning, dilute 100x (100 µL into 10 mL LB + antibiotic)
- Grow to OD 0.1, (about 1 – 2 hrs) then induce sortase with 0.5% xylose* (100 µL of 50%), and induce COH with 1 mM IPTG (10 µL of 1 M). *Not necessary with new IPTG inducible sortases.
- Allow cells to grow for 3 to 4 hours (if multiple cultures, stop around same OD)
- Pellet 3 mL cells (if 10 mL starter culture, you will have 3 samples).
- Wash by resuspending and repelleting in 1 mL Dock Buffer. Decant supe.
- Resuspend pellet in 20 µL of 100 µM ReporterX-Dock protein in Dock Buffer, and incubate on ice for 1 hr. (This step needs to be optimized. Can likely be done in less time.)
- Wash by resuspending and repelleting in 1 mL Dock Buffer, 3x. Decant supe each time.
- Resuspend in 1 mL of whatever buffer is necessary for your detection assay of choice, and proceed with detection.

Easy Cell Fractionation (03/01/2012)
*Adjusted from Tim’s protocol

Prep:
STM buffer:
- 25% sucrose
- 50 mM TRIS-HCl pH 8.0
- 5 mM MgCl$_2$

Procedure
- Grow 5 mL cultures and induce with 1 mM IPTG O/N (to saturation)
- Pellet at 3k x G for 5 mins. Separate, and retain supernatant.

Secreted proteins:
- Precipitate secreted proteins from growth media by adding Trichloroacetic acid (TCA) to the supernatant to a final concentration of 10% w/v (0.45 g/tube).
- Incubate at RT for ~1 hr mixing occasionally until all TCA is dissolved and solution is somewhat cloudy
- Pellet at 20k x G for 10 mins and discard supernatant.
- Resuspend pellet in ~100 µL H₂O (adjusted for cell density), making sure to also resuspend film on walls of tube. Pellet will contain secreted proteins.

**Wall bound proteins:**
- Wash cell pellet by resuspending in 1mL STM buffer, and pelleting again.
- Resuspend pellet in ~100 µL (adjusted for cell density) STM + 500µg/mL lysozyme and incubate at 37˚C for 30 mins.
- Pellet protoplasts at 20k x G for 10 mins. Supernatant will contain solubilized cell wall proteins, and membrane associated proteins.

**Transmembrane and cytoplasmic proteins:**
- Release protoplast membrane proteins by resuspending pellet in ~100 µL (adjusted for cell density) of 0.1M NaOH. By first gently resuspending pellet in 90% of total volume as H₂O, and then adding final 10% volume as 1M NaOH.
- Mix by vortexing and inverting tube, DO NOT PIPET UP AND DOWN. Allow reaction to proceed for ~5mins
- Neutralize with 3 µL of 3M HCl, and mix by vortexing and inverting tube.
- Add 2 µL DNaseI (6 mg/mL) and incubate at 37˚C for 30 mins
- Load gel with 15 µL of each sample after mixing with 2x SDS and boiling for 3 mins as usual. (If sample is still too viscous to pipet accurately, boil for an additional 5-10 mins in SDS)

**Western Blotting (04/16/2014)**
- Keep a 10x stock of Towbin buffer (without MeOH) at pH 8.3
- Keep a 5x stock of TBST buffer

**Prep**

Transfer Buffer (Towbin buffer)
- 0.025 M Tris Base (pH 8.3)
- 0.192 M Glycine
- 20% MeOH

TBST buffer
- 20 mM Tris-HCl pH 7.5
- 500 mM NaCl
- 0.05% Tween

Phosphate buffered saline (PBS) (only necessary if stripping antibodies from membrane with buffers below)
- 8g of NaCl
- 0.2g of KCl
- 1.44g of Na₂HPO₄ pH 7.4
- 0.24g of KH2PO4
- H₂O to 1L

Stripping buffer (only necessary if blotting with two different primary antibodies)

For 1L:
- 15 g glycine
- 1 g SDS
- 10 ml Tween20
- Adjust pH to 2.2
- Bring volume up to 1 L w/ water

Harsh stripping buffer (only necessary if stripping buffer doesn’t work)

**Prepare buffer and strip membranes under a fumehood.

- Buffer, 0.1 liters
- 20 ml SDS 10%
- 12.5 ml Tris HCl pH 6.8 0.5M
- 67.5 ml ultra pure water
- Add 0.8 ml β-mercaptoethanol under the fumehood

Procedure

- Separate proteins by SDS PAGE using standard procedures (outlined above) making sure to use an appropriate ladder to be detected by your antibodies (usually 3 µL of Magic Mark ladder)

Transfer Proteins to polyvinylidene difluoride (PVDF) membrane

- Soak the gel in transfer buffer for 15 mins with gentle agitation.
- Remove any floppy parts from the gel with a razor blade.
- Cut the membrane to the size of the gel, and remove the top right corner of the membrane to mark which side of the blot will contain protein, and to determine the orientation of the samples after transfer.
- Wet the membrane in 100% MeOH for < 15 s.
- Equilibrate the membrane, 2 scotch bright pads, and 4 membrane size pieces of filter paper in transfer buffer.
- Build a “blotting sandwich” by placing, in order: 1 scotch bright pad, 2 filter papers, the membrane, the gel, 2 filter papers, and then the final scotch bright pad, from + to – in the transfer assembly. (Proteins will flow towards the ++ anode)
- Close and lock the sandwich inside the cassette, and place the cassette into the transfer box, making sure to align the cassette properly.
- Fill the chamber with transfer buffer, attach the leads, and run the blot at about 400mA for 2 hrs.

Stain with antibodies (procedure here is for secondary antibody detection)
- Remove membrane from transfer box and IMMEDIATELY place in TBST buffer. (Must do this very quickly so membrane doesn’t dry out. If any part of the membrane turns a chalky white color, rewet in MeOH and then quickly transfer to TBST)
- Allow membrane to equilibrate in TBST for 5 mins on belly dancer to remove residual MeOH.
- Block membrane by soaking in 20mL TBST + 5% BSA (1 g) for 1.5 hrs at RT or O/N at 4°C on belly dancer.
- Incubate membrane in 10 mL of first antibody in TBST + 1% BSA (0.1 g) on belly dancer for 1 hr (usually 1:100 dilution of ABGENT purified mouse anti-His6 IgG antibody)
- Wash 3x in TBST: 15 mins, 5 mins, 5 mins
- Incubate in 10 mL of secondary antibody in TBST + 1% BSA (0.1 g) on belly dancer for 30 mins - 1 hr. (usually HRP conjugated rabbit anti-mouse IgG; 1:5,000 dilution)
- Wash 3x in TBST: 15 mins, 5 mins, 5 mins
- Incubate with Pierce ECL Western blotting substrate, 0.125 ml/cm² of membrane (~ 6 mL of each for full sized membrane), for 1 min then place membrane between two sheets of plastic wrap, being careful to remove all bubbles.
- Cut away any significant excess of plastic wrap so that the plastic covered blot can be more easily manipulated.
- Take the blot, a closed box of autoradiography film, the silver clampy box thing, a timer, tweezers, clean gloves, some Kim Wipes, a pen, and the darkroom key, to the darkroom on the first floor. (you will need to be trained before you can use the machine there to develop your film)
- Make sure the red “Ready” light is on, on the film developer. If not, follow procedure on the wall (will take abt 15-20 mins to warm up.)
- Expose autoradiography film with your blot for between 10 s and 15 mins (Usually expose the same film several times for various lengths of time to be sure one will work, e.g. 30s, 1 min, 5 mins) in dark room with only the red light on
- Without turning the light on, put your film in the machine via the entry port on the right to develop. Once the film has entered the machine you can turn the lights back on and wait for your film to flop out of the slot on the top right.

*Stripping Antibodies for Reblotting*
*this protocol will reduce S/N and should not be used to compare signal from the same membrane to itself*
- Incubate membrane with a volume of stripping buffer that will cover the membrane at room temperature for 5-10 minutes, then discard buffer and repeat.
- Incubate for 10 minutes in PBS, discard buffer and repeat.
- Incubate for 5 minutes in TBST, discard buffer and repeat.
- Return to blocking stage of above protocol and continue with your new antibodies.

*If harsh stripping is necessary:*
- Heat harsh stripping buffer to 50°C
- Place membrane in a small box with a tight lid and add harsh stripping buffer to cover it. Incubate at 50°C for up to 45 mins with agitation. Discard buffer as hazardous waste.
- Rinse membrane under running tap water for 1-2 hrs to remove all traces of β-mercaptoethanol which can damage antibodies.
- Wash for 5 mins in TBST
- Return to blocking stage of above blotting protocol.

**Instruments**

**AKTA HPLC (09/25/2012)**
Make sure to read through and understand this protocol before hooking up your column and trying to start a run to prevent damaging your column, and the system

Pressure max for mechanical components (pumps, valves, mixer, etc) = 25 MPa
- Plastic connectors handle much less than this, ~5 MPa before they start leaking
- Your column may be made to handle significantly less pressure than this

Take care not to mix organic and aqueous buffers to prevent precipitation of salts and clogging of the column or the instrument

**Prep**
- Prepare at least 1 L of each of your necessary buffers and filter them by vacuum filtration (this step also serves to degas the buffers) (Make sure to monitor filtration of organic solvents, as they may dissolve the normal cellulose membranes. You may want to use nylon filters instead)

**BEFORE CONNECTING YOUR COLUMN:**
- Close the system by placing the female-female adapter in place of any column attached to the system.
- In manual control mode, flush the system with >/= 50mL of filtered nanopure water through both pumps to remove any unknown buffers from previous runs. (10mL/min; 50% B)
- Run PumpWashBasic for both pumps (this will take a few minutes)
- Rinse the loop with >/= 10 mL nanopure water by switching inlet valve to “inject”
- Place the inlet tubes from pumps A and B into your buffers A and B, respectively (making sure to rinse/wipe them off between buffers)
- Switch inlet valve back to “load”
- Run 50 mL of 100% B through the system to equilibrate pump B
- Switch inlet position to “loop” and flush the loop with ~10 mL of buffer A
- Run 50 mL of 100% A through the system to equilibrate pump A and prime the system for your column

**Equilibrate the column**
- Attach your column in place of the female-female adapter, making sure the arrows on the column align with the system’s direction of flow.
- Equilibrate with your buffers (usually >/=10 CV of buffer A) as necessary for your column and protocol

**Run Method**
- Set up parameters for gradient and fractionation in unicorn
- Manually inject your sample (at least 1 mL) into injection port. DO NOT remove the syringe from the injection port until your sample has been injected onto the column, or the system will flush your sample to waste.
- Right-click method and select “run,” then click next through all menus and “Start”

**Clarke Lab HPLC (1/10/2013)**

**Prep**

**Typical Buffers:**
- MiliQ water (2x 500 mL)
- 100% ACN (500 mL)
- MiliQ water +0.1% trifluoroacetic acid (TFA) (500 mL)
- ACN +0.1% TFA (500 mL)
- Filter all buffers through 0.4 µm or better filter to remove any particulate matter, and degas.
  (Water based buffers can be filtered through standard cellulose filters, but ACN needs to be filtered through a nylon filter)
- Transfer buffers to 500 mL Erlenmeyer flasks, or equivalent container that will fit in the HPLC, and keep covered with parafilm.

**Procedure**
*IMPORTANT – make sure to remove any columns, and flush the system with at least 20 mL of pure water before attaching your column to prevent precipitation of salts or running an incompatible buffer through your column. (instructions below)*
*Check to make sure waste container is not full.*
*And for God’s sake, please don’t save over any of my methods.*

*Wash the system (AlexWash.M)*
- Open the door at the front of the machine and remove any columns from the system, then cap both ends.
- Close the system by attaching both lines to a female/female adapter.
- Remove any buffers that are currently attached, rinse the inlet hoses with MQ water, and wipe with Kim Wipes, then attach your buffers (Buffer A goes on the left), using foam Eppendorf floaties to adjust the height of your flask if necessary.
- In computer click:
  - Instrument > Set up pump
  - Fill in 50% A and 50% B in the top section, flow rate of 1 mL/min, and pressure limit of 200 bar. (ignore the bottom “gradient” section for now)
- Turn on the HPLC pumps and detector using physical buttons on the front of the machine, and allow pumps to run for 10 mins. This will flush the entire system, including both pumps, with water.
- Turn off the pumps with the physical button on the front of the machine.

Set up system
- Make sure pumps are off, then attach your column, making sure to properly orient it so the direction of flow is from left to right.
- Remove water flasks and replace with your buffers. (typically A is 0.1% TFA in water, and B is 0.1% TFA in ACN)

Flush pump B (Alex C18B.M)
- In computer click:
  - Instrument > Set up pump
  - Fill in 0% A and 100% B in the top section, flow rate of 1 mL/min, and pressure limit of 200 bar. (ignore the bottom “gradient” section for now)
- Turn pump on using physical button, and allow it to run for ~10 mins to flush pump B with buffer B, and to elute anything that was stuck on the column.

Flush pump A (this step can be skipped by replacing your first sample with a water sample)
- In computer click:
  - Instrument > Set up pump
  - Fill in 100% A and 0% B in the top section, flow rate of 1 mL/min, and pressure limit of 200 bar. (ignore the bottom “gradient” section for now)
  - Turn pump on using physical button, and allow it to run for ~10 mins to flush pump A with buffer A, and to equilibrate the column.

Set Up Run Method
- In computer click:
  - Instrument > Set Up Pump
    - Fill in default conditions for the pump (i.e. what you want the pump to do as soon as you turn it on) on top and conditions for your gradient (i.e. what you want the pumps to do after you click inject) on the bottom.
  - File > Save Method As
- Instrument > Set Up Injector
  - Injector Volume = 100 µL (at least 10 µL less than total sample V)
  - Draw Speed = 104 µL/min (always)
- Instrument > Set Up DAD Signals
  - add whatever wavelengths you want to record a chromatogram for. (Only A will be shown in real time)
- Sequence > Sequence Parameters
  - Operator Name: ‘yourName’
  - change “Datafile” to desired output filename
  - Post Sequence = x (and choose desired option)
- Sequence > Sequence Table
  - Location = Vial0 (adding new lines with additional vial locations will automatically run samples in series)
  - Sample Name = ‘yourSample’
  - Method Name = ‘yourMethod’
  - Injection Volume = 100 µL

Run method
- Manually set up fraction collector with physical buttons on front if desired.
- Make sure everything on screen is green (i.e. no red lights)
- Click Start
- Wait until inject, then transfer outlet line to fraction collector input and press “Ready” then “Run” on the front of the fraction collector.

Clean
- Fill your column with whatever storage buffer is appropriate (probably 100% ACN)
- Remove column, capping both ends
- Replace female/female adapter
- Flush the system, including both pumps with MiliQ water.

**Waters Reversed Phase C\textsubscript{18} HPLC Column (9/25/2012)**
Pressure max = 26 MPa
Flow Rates
- Our AKTA: 0.4 mL/min (set pressure limit to 5 MPa)
- Clarke lab HPLC: 1mL/min
- (recommended flow rate is 4mL/min and runs should take ~5 mins)

Typical Buffer A = H\textsubscript{2}O + 0.1% TFA (vacuum filtered through cellulose filter)
Typical Buffer B = HPLC grade ACN + 0.1% TFA (vacuum filtered through nylon filter)
Equilibrate with $\geq 10$ CV (3.5 mL) of Buffer A (Use $\geq 100$ CV if adding or removing salts)

Typical gradient is from 0 to 45% B over $\sim 60$ CV

If mobile phase contained a salt, flush with 100 CV (35 mL) of filtered nanopure water before storing

Store column in 100% ACN

**MALDI mass spectrometry (04/17/2012)**

Matrix solvents:
- A: 50% EtOH, 0.1% TFA
- B: 50% ACN, 0.1% TFA
- C: 33% ACN, 0.1% TFA
- D: 16.5% ACN, 0.05% TFA (C diluted 50% with water)

Matrices:
- DHB – For both peptides and proteins, usually seems to work best in buffer A
- Sinapinic acid – For proteins, usually works best in buffer B