Lab 1 - Boiling Lysis Plasmid Preparation

Objective: To purify plasmid DNA containing the \textit{rnb} gene sequence (pRNBEc) 
To purify pET28a expression vector for use in subsequent labs

Supplies and Equipment:

- Overnight DH5\text{\scriptsize{\textalpha}} culture with pET28a
- Overnight DH5\text{\scriptsize{\textalpha}} culture with pRNBEc
- Micropipettors
- Sterile 1.5 mL microcentrifuge tubes
- Microcentrifuges (RT)
- Boiling water bath w/ mct racks
- Ice
- Sterile \textit{d}^2\text{H}_2\text{O} (Optima)
- STET
- Lysozyme solution (10 mg/mL)
- 3.0M sodium acetate (pH 5.2)
- Isopropanol
- 70% Ethanol (ice cold)
- TE (pH 8.0) with 20 \mu g/mL RNase A

Procedure:

1. Aseptically transfer 1.5 mL of the overnight culture to a 1.5 mL microcentrifuge tube (MCT) and pellet the cells by centrifugation (2 min @ 13,000 rpm).

2. Discard as much of the supernatant as possible by decanting away from the cell pellet or by aspiration. Pulse the tube in a microcentrifuge and remove any remaining supernatant with a micropipette.

3. Rinse the cell pellet by washing 1.0 mL of sterile distilled water gently down the inside wall of the MCT. This removes any traces of the supernatant adhering to the MCT wall while minimizing the disturbance to the cell pellet.

   \textit{Cell wall components in the medium will inhibit many restriction endonucleases making it critical to remove all traces of the medium from the cell pellet.}

4. Resuspend the cell pellet in 350 \mu L of STET using a micropipette. Ensure that the cell pellet is completely resuspended before continuing.

5. Add 25 \mu L of the Lysozyme solution and mix by inversion.

6. Place the MCT in the boiling water bath for 35 sec, remove and incubate on ice for 5 min.

   \textit{It is critical not to exceed the recommended time for boiling your sample as you will denature supercoiled DNA irreversibly. If this occurs your DNA will be unusable for many downstream applications (including digestion by restriction endonucleases)}
7. Pellet the cellular debris by centrifugation at 13,000 rpm for 15 min.

8. Using a micropipettor, transfer the supernatant to a fresh MCT and discard the pellet. Ensure that you do not disturb the pellet of cellular debris while performing this step.

9. Precipitate the plasmid DNA by adding 40 μL of 3.0 M sodium acetate (pH 5.2) and 420 μL isopropanol. Mix well by inversion and incubate for 5 min at room temperature.

10. Pellet the plasmid DNA by centrifugation at 13,000 rpm for 10 min. A pellet of plasmid DNA should be visible at the base of the MCT when complete.

11. Being careful not to disturb the pellet, discard the supernatant and rinse the pellet with 500 μL of ice cold 70% ethanol.

12. Repeat the above step.

13. Invert and tap the open MCT several times against a piece of paper towel on your bench to remove as much ethanol as possible.

14. Store the open MCT at room temperature for approximately 10 min to allow all remaining traces of ethanol to evaporate.

15. Add 50 μL of sterile d²H₂O or TE (pH 8.0) containing RNase A and resuspend the plasmid DNA by flicking the base of the MCT with your finger. The plasmid DNA is ready for use or can be stored long term at -20 °C.

Protocol modified from:

Lab 2 – Restriction Enzyme Digestion

Objective: To cut the rnb gene sequence out from pRNBEc
To cut and dephosphorylate the expression vector (pET28a)

Supplies and Equipment:
- pET28a and pRNBEc DNA from Lab 1
- Sterile 1.5 mL microcentrifuge tubes
- Micropipettors
- Water bath (37 °C)
- Microcentrifuge
- Sterile d\textsuperscript{2}H\textsubscript{2}O (Optima)
- Shrimp alkaline phophatase (SAP)
- NheI restriction endonuclease
- NheI 10x buffer
- BSA 10x

Procedure:
1. In two 1.5 mL MCTs setup the restriction enzyme reactions according to Table 1. Reagents should be kept on ice at all times and are to be added, using a fresh tip for each, in the order that they appear in the table (i.e. d\textsuperscript{2}H\textsubscript{2}O first).

**Do NOT add the shrimp alkaline phosphatase (SAP) to the pRNBEc sample**

<table>
<thead>
<tr>
<th>Tube</th>
<th>d\textsuperscript{2}H\textsubscript{2}O</th>
<th>DNA</th>
<th>Buffer</th>
<th>BSA</th>
<th>SAP</th>
<th>NheI</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRNBEc</td>
<td>8 μL</td>
<td>15 μL</td>
<td>3 μL</td>
<td>3 μL</td>
<td>-</td>
<td>1 μL</td>
<td>30 μL</td>
</tr>
<tr>
<td>pET28a</td>
<td>6 μL</td>
<td>15 μL</td>
<td>3 μL</td>
<td>3 μL</td>
<td>2 μL</td>
<td>1 μL</td>
<td>30 μL</td>
</tr>
</tbody>
</table>

2. Mix the contents by flicking the MCTs with your finger and spin briefly in a microcentrifuge to pool the reagents to the bottom.

3. Incubate the tubes overnight at 37 °C in the water bath. The tubes will be transferred in the morning to -20 °C storage for you.
Lab 3 – Gel Electrophoresis and Purification of Digestion Products

Objective: To purify the desired DNA fragments via extraction from an agarose gel

Supplies and Equipment:

- Water bath (50 °C and 65 °C)
- Microwave
- Balance
- Gel rigs and power supply
- UV Transilluminator with short plate
- Microcentrifuge
- Gel Trays and 6 well combs
- 50 mL graduated cylinder
- Spatulas
- Razor blades
- Micropipettors
- Agarose
- 1x TAE
- 1x TBE
- Loading dye (10x)
- DNA Marker (2-log DNA ladder, NEB)
- Ethidium bromide bath
- Agarose gel extraction kit (Qiagen)

Procedure:

Morning

1. Obtain your digests from last day and incubate them at 65 °C for 20 min to ensure that both the restriction endonuclease and the SAP are denatured.

2. Cast a 1.0% agarose gel in TAE buffer. Use the combs with 6 large teeth to ensure the wells are sufficiently large to hold your entire sample.

3. Prepare your digests for loading by adding 3 µL of 10x loading buffer, mix well, and spin down briefly in a microcentrifuge.

4. Load the entire sample to the agarose gel. In addition to your digests be sure to load a marker lane and unrestricted aliquots of your DNA (both pRNBEc and pET28a) for comparison.

5. Run the gel at 80 V until the dye front is near the end of the gel (~1 hr).

Afternoon

6. Stain your gel in the ethidium bromide bath for approximately 10 min with gentle agitation.

7. Set the UV transilluminator to the long wavelength setting (if available) and cut around the appropriately sized bands of DNA using a scalpel or sharp blade. Trim as closely to the bands of DNA as possible but work quickly to minimize the UV exposure of your DNA samples.
Caution: Protect your eyes and any exposed skin from UV irradiation.

8. Transfer your agarose gel slices to labeled 1.5 mL MCTs and follow the protocol for the gel extraction kit as supplied by the manufacturer.

   Elute your DNA from the column in sterile $d^2H_2O$ instead of the buffer supplied with the gel extraction kit.

9. Label your eluted DNA and store at -20 °C until next day.

10. Prepare a 1.0% agarose gel in TBE using the 12 well combs and store in 1x TBE buffer until the next lab. Only 2 gels will be needed for the entire class.
Lab 4 – Quantification of Isolated DNA and Ligation

Objective: To determine the concentration of isolated DNA and to use these values to ligate the \( rnb \) sequence into pET28a

Supplies and Equipment:

- Gel rigs and power supply
- UV Transilluminator
- Microcentrifuge
- Micropipettors
- 1x TBE Loading dye (10x)
- DNA Marker (2-log DNA ladder, NEB)
- Ethidium bromide bath
- T4 DNA ligase with buffer
- Ice

Procedure:

1. Prepare your samples for the gel on a piece of parafilm using the volumes given in Table 2. Only one of each of the markers lanes is required for each gel and they should be loaded so that they flank all of the loaded samples.

<table>
<thead>
<tr>
<th>Table 2.</th>
<th>Preparation of samples to be loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
</tr>
<tr>
<td>DNA Samples</td>
<td>2 µL</td>
</tr>
<tr>
<td>Marker Lane #1</td>
<td>1 µL</td>
</tr>
<tr>
<td>Marker Lane #2</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

2. Run the gel at 80 V for approximately 1 hr.

3. Stain the gel for approximately 10 min in the ethidium bromide bath.

4. Visualize the gel on the UV transilluminator and estimate the amount of DNA present in your sample based on the relative intensity of your band as compared to the DNA mass ladder. Use this value to determine the concentration of your sample in ng/µL.

5. Use the values you obtained in the previous step to setup your ligation reactions in 0.6 mL MCTs as outlined by Table 3. You want a 3:1 (insert:vector) molar ratio for your reaction but do not exceed 200 ng of total DNA. The following formula may be of use to complete this step.

\[
\text{(ng Vector)} \times \frac{\text{(bp Insert)}}{\text{(bp Vector)}} \times \text{(desired ratio)} = \text{ng Insert}
\]
Table 3. Ligation reaction setup

<table>
<thead>
<tr>
<th>Sample</th>
<th>d²H₂O</th>
<th>5x Buffer</th>
<th>Insert</th>
<th>Vector</th>
<th>T4 DNA Ligase</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>As req'd for final vol.</td>
<td>4 µL</td>
<td>T.B.D.</td>
<td>T.B.D.</td>
<td>1.0 U (0.1 µL)</td>
<td>20 µL</td>
</tr>
<tr>
<td>Negative Control</td>
<td>As req'd for final vol.</td>
<td>4 µL</td>
<td>0 µL</td>
<td>Same as above</td>
<td>1.0 U (0.1 µL)</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

6. Once all the components have been added to the tube, flick several times with your finger to mix, and pulse the tube in a microcentrifuge prior to incubation.

7. The ligation reaction will be carried out at room temperature (21 °C) for 16 hrs and will subsequently be transferred to -20 °C storage for you.
Lab 5 – Transformation of DH5α

**Objective:** To transform *E. coli* DH5α with the ligation products from Lab 4

**Supplies and Equipment:**

| Ligation products (from Lab 4) | LB broth (5 mL aliquots) |
| Competent DH5α cells (Lab 6 of P1) | LB Kan+ plates |
| Micropipettors w/ sterile tips | Bunsen burners |
| Water baths (37°C & 42°C) | 70% Ethanol |
| Sterile d2H2O | Plate spreaders |
| Ice |

**Procedure:**

1. Obtain the appropriate number of 100 μL aliquots of previously prepared chemically competent DH5α cells and thaw completely on ice.
   
   *Cells should be kept on ice at all times unless otherwise specified!*

2. To your aliquots add the appropriate reagent(s) as outlined in Table 3 and mix the contents by flicking several times with your finger. **DO NOT** mix competent cells by using a vortex or a pipettor as they are very fragile.

<table>
<thead>
<tr>
<th>Table 4. Transformation setup</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aliquot</strong></td>
</tr>
<tr>
<td>Negative Control</td>
</tr>
<tr>
<td>Positive Control</td>
</tr>
<tr>
<td>Sample(s)</td>
</tr>
</tbody>
</table>

3. Incubate the mixtures on ice for 30 min.

4. Heat shock the mixtures by transferring them to the 42 °C water bath for exactly 1 min. Immediately return the mixtures to ice for 5 min.

5. Add 890 μL of LB broth (with no antibiotic) to each of the tubes, mix gently by inversion, and incubate at 37 °C for 40 min.

6. Spread plate 100 μL directly onto LB Kan+ media.

7. Spin the remaining volume in the microcentrifuge at 13,000 rpm for 1 min to pellet the cells. Withdraw and discard all but approximately 100 μL of the
supernatant and resuspend the cell pellet in the remaining liquid. Make a spread plate of this mixture on LB Kan\(^+\) media.

8. Incubate your plates over night at 37 °C. In the morning they will be moved to 4 °C storage for you.
Lab 6 – Preparation of Competent BL21 Cells

Objective: To prepare chemically competent *E. coli* strain BL21 cells for use in expression testing

Supplies and Equipment:

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ice (large buckets)</td>
<td></td>
</tr>
<tr>
<td>100 mM CaCl₂ (sterile)</td>
<td></td>
</tr>
<tr>
<td>80 mM MgCl₂ 20mM CaCl₂ (sterile)</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
</tr>
<tr>
<td>Mid-log phase <em>E. coli</em> BL21 cells</td>
<td>- 100 mL per bench</td>
</tr>
<tr>
<td>Micropipettors w/ 'chelex' tips</td>
<td></td>
</tr>
<tr>
<td>Microcentrifuge</td>
<td></td>
</tr>
<tr>
<td>40 mL sterile centrifuge tubes (not Falcon)</td>
<td></td>
</tr>
<tr>
<td>Sterile 50 mL graduated cylinders</td>
<td></td>
</tr>
<tr>
<td>Top loading balance w/ beaker</td>
<td></td>
</tr>
<tr>
<td>Waste beakers</td>
<td></td>
</tr>
</tbody>
</table>

Procedure:

1. Place all solutions on ice to chill.

2. Pour approximately 40 mL of mid-log phase cells into a sterile centrifuge tubes (fill the centrifuge tube to the base of the neck). Balance your tube to within 0.1 g against a tube from the other group of students at your bench.

3. Incubate on ice for 10 min.

4. Centrifuge the cells at 2,700 g for 10 min. This spin will throw the cells into a pellet at the bottom of the tube.

5. Remove the tubes from the centrifuge and pour off the supernatant into the original flask being careful not to disturb the pellet. Tap the tube gently over paper towel to remove excess supernatant and let the tube stand inverted on a piece of paper towel for 1 min.

6. Add 30 mL of ice cold 80 mM MgCl₂ 20 mM CaCl₂ to each tube using aseptic technique. Resuspend the pellet of cells by pipetting (slowly) the mixture up and down. Ensure that cells are completely resuspended before proceeding.

7. Balance the tubes as needed and centrifuge in the same manner as above to pellet the cells. Pour off the supernatant, being careful not to disturb the pellet.

8. Resuspend each pellet in 2 mL of ice cold 100 mM CaCl₂ by swirling gently.
9. Add 600 μL of sterile 100% glycerol and swirl gently until thoroughly mixed.

10. Transfer 100 μL aliquots of the competent cells into 1.5 mL MCTs on ice. Cells will be frozen at -80 °C until use.
Lab 7 – Restriction Analysis

Objective: To determine the orientation of the insert using restriction analysis

Supplies and Equipment:

- Transformation plates from Lab 5
- Ice
- Micropipettors
- Microcentrifuges (RT)
- Boiling water bath w/ mct racks
- Sterile d²H₂O (Optima)
- Lysozyme solution (10 mg/mL)
- 3.0M sodium acetate (pH 5.2)
- Isopropanol
- 70% Ethanol (ice cold)
- TE (pH 8.0) with 20 µg/mL RNase A
- Waste beakers
- STET

Procedure:

Part A

Previous Day

1. Inoculate multiple 5 mL aliquots of LB broth (with the appropriate antibiotic) from your transformation plates. The number of cultures to start will depend upon your results from last day but aim for 10 cultures.

In Lab

2. Obtain your overnight cultures from last day and isolate the plasmid DNA using the boiling lysis plasmid preparation as outlined in Lab 1.

3. Use the values given in Table 5 to setup your restriction enzyme digests. Be sure to mix each tube thoroughly and spin down briefly in the microcentrifuge prior to incubation.

   *It is good practice when setting up multiple reactions to create a master mix of all common components. Not only is this a more efficient method but it also increases pipetting accuracy and ensures that all the reactions are setup in an equivalent manner.*

Table 5. Reaction setup for restriction analysis

<table>
<thead>
<tr>
<th>Tube</th>
<th>d²H₂O</th>
<th>DNA</th>
<th>Buffer (10x)</th>
<th>BSA (100x)</th>
<th>Sal I</th>
<th>Final Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample(s)</td>
<td>5.8µL</td>
<td>3µL</td>
<td>1µL</td>
<td>0.1µL</td>
<td>0.1µL</td>
<td>10µL</td>
</tr>
<tr>
<td>pET28a (+ve)</td>
<td>5.8µL</td>
<td>3µL</td>
<td>1µL</td>
<td>0.1µL</td>
<td>0.1µL</td>
<td>10µL</td>
</tr>
</tbody>
</table>
4. Incubate the digests overnight at 37 °C in the water bath. The tubes will be transferred in the morning to -20 °C storage for you.

**Part B**

5. Cast a 1.0% agarose gel in TBE buffer with 24 wells (2 combs per a gel will be required).

6. Obtain your digested plasmid DNA from Part A, add the appropriate volume of 10x loading dye, and mix well.

7. On a piece of parafilm, mix 3 μL of each of your undigested plasmid DNA samples with loading dye. You will want to do this near the gel rig so that you do not have to attempt to move the piece of parafilm across the lab after having mixed your samples.

8. Load each of these samples to the gel as well as the appropriate DNA markers.

   You want to load the digested and undigested aliquots of each sample immediately adjacent to each other to allow for easy comparison between the two.

9. Run the gel at 80 V for approximately 1 hr.

10. Stain the gel in the ethidium bromide bath for ~10 min and view on the UV transilluminator. Determine which of your plasmids contain the *rnb* gene sequence in the correct orientation.
Lab 8 – Transformation of BL21

Objective: To transform BL21 with the recently created rnb::pET28a constructs

Supplies and Equipment:

Constructs as identified in Lab 7B) pET28a DNA (from Lab 1)
Competent BL21 cells (from Lab 6) Micropipettors
Water baths (37 °C & 42 °C) Sterile d2H2O
LB broth (5 mL aliquots) LB broth (5 mL aliquots)
LB Kan+ plates 70% Ethanol
Plate spreaders Ice

Procedure:

1. Obtain the plasmid constructs you have identified in Lab 7 as having the rnb sequence in the correct orientation and use these to transform your previously prepared competent E. coli stain BL21 cells.

2. Obtain the appropriate number of competent BL21 cell aliquots from the -80°C freezer and thaw them on ice for 5-10 min.

3. To your competent cell aliquots, add the appropriate reagent(s) as outlined in Table 6 and mix the contents by flicking several times with your finger.  

   **DO NOT mix competent cells by using a vortex or a pipettor as they are very fragile.**

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>d2H2O</th>
<th>pET28a</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>3 µL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive Control</td>
<td></td>
<td>3 µL</td>
<td>-</td>
</tr>
<tr>
<td>Sample(s)</td>
<td></td>
<td>-</td>
<td>3 µL</td>
</tr>
</tbody>
</table>

4. Incubate the mixtures on ice for 30 min.

5. Heat shock the mixtures by transferring them to the 42 °C water bath for exactly 1 min. Immediately return the mixtures to ice for 5 min.

6. Add 897 µL of LB broth (with no antibiotic) to each of the tubes, mix gently by inversion, and incubate at 37°C for 40 min.

7. Spread plate 100 µL directly onto LB Kan+ media.
8. Centrifuge the remaining volume in the microcentrifuge at 13,000 rpm for 1 min to pellet the cells. Withdraw and discard all but approximately 100 \( \mu \)L of the supernatant and resuspend the cell pellet in the remaining liquid. Make a spread plate of this mixture on LB Kan\(^+\) media.

9. Incubate your plates over night at 37 °C. In the morning they will be moved to 4 °C storage for you.
Lab 9 – Small Scale Expression Testing

Objective: To select the appropriate \textit{rnb}\textsuperscript{::}pET28a constructs to be used as templates in ATW PCR

Supplies and Equipment:

- Transformation plates from Lab 8
- LB broth (5 mL aliquots)
- Inoculating loops
- Bunsen burners
- Kanamycin stock (1000x)
- IPTG stock (1 M)
- Sterile culture tubes (w/ wire racks)
- 37 °C shaking incubator
- Micropipettors w/ gel loading tips
- Microcentrifuges (RT)
- SDS-PAGE apparatus w/ 15 well combs
- Power supply (200 V)
- Temed
- APS (100 mg/mL)
- Resolving gel premix
- Stacking gel premix
- Scintillation vials
- Pasteur pipets
- 95% EtOH
- Filter paper
- Running buffer (10x)
- Graduated cylinders (100 and 1000 mL)
- 2x Reducing sample buffer
- 95 °C water bath (or heat block)
- Coomassie stain and destain
- Orbital shaker
- White light box

Procedure:

Morning (early!)

1. Inoculate multiple 5 mL aliquots of LB broth (with the appropriate antibiotic) from your transformation plates. You should inoculate 2 cultures for each plasmid you used for transformation in Lab 8. In addition you should also inoculate at least one culture using a cfu from the positive transformation control (pET28a).

Afternoon

2. Transfer 2 mL of each of your cultures to sterile culture tubes and induce expression with 1 mM IPTG (final concentration). Return both sets of the cultures to the 37 °C incubator with vigorous shaking (~200 rpm) for at least 90 min.

Casting the SDS-PAGE Resolving Gel

\textit{Caution:} Gloves must be worn while working with unpolymerized acrylamide

3. Assemble the SDS-PAGE casting apparatus as shown by your instructor

4. In a scintillation vial (or small beaker), add 5 uL TEMED and 50 uL of 100 mg/mL ammonium persulfate (APS) to 10 mL of the 12% resolving gel premix and
mix by swirling. The volume is sufficient for 2 gels.

5. Using a pasteur pipet, dispense the mixture between the glass plates by placing the tip of the pipet to one side and allowing the solution to run down the edge of the spacer. Fill the space between the plates until the level of the solution is approximately 5 mm below where the teeth of the comb will sit when inserted (determine this level prior to mixing the resolving gel).

   Work quickly as you do not want the gel to polymerize while you are still casting it.

6. Use a pasteur pipet to overlay the top of the resolving with 95% ethanol (a couple of mm above the top of the gel is sufficient). The ethanol will disperse any air bubbles you may have introduced while pouring the resolving gel so do not worry about them prior to this point.

7. Wait ~20 min for the resolving gel to polymerize, do not disturb the casting stand while waiting.

   You will be able to tell when the gel is polymerized by examining the unused resolving gel in your pasteur pipet or scintillation vial.

Casting the SDS-PAGE Stacking Gel

8. Once the resolving gel has polymerized, invert the gel (casting stand and all) to pour off the ethanol. Blot away any remaining ethanol with a kimwipe or a piece of filter paper.

9. In another scintillation vial, add 5uL TEMED and 25uL of 100 mg/mL APS to 5 mL of the stacking gel premix and mix by swirling (again, this is enough for 2 gels).

10. Use a pasteur pipet to dispense this solution on top of the polymerized resolving gel until you just slightly overflow the short plate (this helps to flush out any residual ethanol and air bubbles).

11. If no air bubbles are present, gently insert the 15 well comb ensuring that the top of the comb sits flush with the top of the long plate (aka spacer plate).

12. Wait as before for the stacking gel to polymerize. Once polymerized, assemble the gel rig (as shown by your instructor), submerge the assembled rig in the buffer chamber and flush out the wells gently with running buffer using a micropipettor or a syringe. The gel is now ready to be loaded with samples.
SDS-PAGE Sample Preparation

13. Retrieve both your original and induced cultures and aseptically transfer 1 mL from each to a sterile 1.5 mL MCT.

14. Pellet the cells in a microcentrifuge for 3 min at 13,000 rpm and discard as much of the supernatant as possible.

15. To each of your cell pellets add 35 μL of 2x reducing sample buffer (RSB) and incubate at 95 °C for 10 min.

   **DO NOT resuspend your cell pellet in the 2x RSB**

16. Once the samples have cooled to room temperature, centrifuge them at 13,000 rpm for 1 min.

17. Using a micropipettor with a gel loading tip, load 15 μL from each sample to the gel previously prepared. Ensure that each gel is loaded with an appropriate protein marker in addition to your samples.

18. Run the gel at 200 V until the dye front, provided by the 2x RSB, reaches the bottom of the gel (~1 hr).

Staining and Destaining

19. Disassemble the gel rig and split the long and short plates apart using a plastic spatula. Work carefully so as not to tear your gel.

   **Make a unique nick in your gel so as to be able to identify it during staining and destaining**

20. Place your gel in the coomassie stain and shake gently on the orbital shaker for 10 min.

21. Remove the gel from the stain and rinse excess stain away with water.

22. Place the gel into the destain and return to the orbital shaker. Blue bands of stained protein should be weakly visible within 15-30 min on a light box but complete destaining typically requires several hours (typically overnight).

23. Pictures of the gels will be taken for you and distributed via email according to the nicking patterns.
**SDS-PAGE Solutions used:**

<table>
<thead>
<tr>
<th><strong>12% Resolving Gel Premix (100mL)</strong></th>
<th><strong>5%Stacking Gel Premix (50mL)</strong></th>
<th><strong>2x Reducing Sample Buffer (500uL)</strong></th>
<th><strong>Coomassie Stain (100mL)</strong></th>
<th><strong>Destain (1L)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>29% Acrylamide/1%Bis-Acrylamide</td>
<td>29% Acrylamide/1%Bis-Acrylamide</td>
<td>2-mercaptoethanol (BME)</td>
<td>R250 Coomassie Brilliant Blue</td>
<td>Acetic Acid</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>2x Sample Buffer (above)</td>
<td></td>
<td>Methanol</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10% SDS</td>
<td></td>
<td></td>
<td>d\textsuperscript{2}H\textsubscript{2}O</td>
</tr>
<tr>
<td>d\textsuperscript{2}H\textsubscript{2}O</td>
<td>d\textsuperscript{2}H\textsubscript{2}O</td>
<td></td>
<td></td>
<td>d\textsuperscript{2}H\textsubscript{2}O</td>
</tr>
<tr>
<td>40 mL</td>
<td>6.6 mL</td>
<td>100 uL</td>
<td>0.25 g</td>
<td>100 mL</td>
</tr>
<tr>
<td>25 mL</td>
<td>12.6 mL</td>
<td></td>
<td>Acetic Acid</td>
<td>40 mL</td>
</tr>
<tr>
<td>1.0 mL</td>
<td>0.5 mL</td>
<td></td>
<td>Methanol</td>
<td>d\textsuperscript{2}H\textsubscript{2}O</td>
</tr>
<tr>
<td>34 mL</td>
<td>30.3 mL</td>
<td></td>
<td></td>
<td>50 mL</td>
</tr>
<tr>
<td><strong>2x Sample Buffer (10mL)</strong></td>
<td><strong>2x Reducing Sample Buffer (500uL)</strong></td>
<td><strong>Coomassie Stain (100mL)</strong></td>
<td><strong>Destain (1L)</strong></td>
<td></td>
</tr>
<tr>
<td>0.1% w/v Bromophenol blue dye</td>
<td>2-mercaptoethanol (BME)</td>
<td>R250 Coomassie Brilliant Blue</td>
<td>Acetic Acid</td>
<td></td>
</tr>
<tr>
<td>1.0 mL</td>
<td>2-mercaptoethanol (BME)</td>
<td></td>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>4.0 mL</td>
<td>2x Sample Buffer (above)</td>
<td></td>
<td></td>
<td>d\textsuperscript{2}H\textsubscript{2}O</td>
</tr>
<tr>
<td>2.0 mL</td>
<td>2x Sample Buffer (above)</td>
<td></td>
<td></td>
<td>500 mL</td>
</tr>
<tr>
<td>2.4 mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6 mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

19
Lab 10 – 'Around the World' PCR

**Objective:** To delete the desired domain from the *rnb* sequence as determined in Assignment #2

**Supplies and Equipment:**

**Procedure:**

*This portion of the lab manual has been intentionally left blank.*
Lab 11 – Purification and Intramolecular Ligation of PCR Products

**Objective:** To recircularize the linear plasmid DNA modified by PCR

**Supplies and Equipment:**

- PCR products from Lab 10
- Micropipettors
- Agarose
- TAE buffer (1x)
- Loading Dye (10x)
- DNA Marker (2-log DNA ladder, NEB)
- Gel rigs, 6 well combs
- Microwave
- Balance
- UV Transilluminator with short plate
- Microcentrifuge
- Spatulas
- Razor blades
- Ethidium bromide bath
- Agarose gel extraction kit (Qiagen)
- Water bath (50 °C)
- Sterile d²H₂O (Optima)
- T4 DNA ligase with buffer

**Procedure:**

1. Cast a 1% agarose gel in TAE using the 6 well combs.

2. Load 25 μL of your PCR mixture (with the appropriate amount of loading dye) and an appropriate DNA marker.

3. Run the gel at 80 V for ~1 hr.

4. Stain your gel in the ethidium bromide bath for approximately 10 min with gentle agitation.

5. Set the UV transilluminator to the long wavelength setting (if available) and cut around the appropriately sized bands of DNA using a scalpel or sharp blade. Trim as closely to the bands of DNA as possible but work quickly to minimize the UV exposure of your DNA samples.

   **Caution:** Protect your eyes and any exposed skin from UV irradiation.

6. Transfer your agarose gel slices to labeled 1.5 mL MCTs and follow the protocol for the gel extraction kit as supplied by the manufacturer.

   *Elute your DNA from the column in sterile d²H₂O instead of the buffer supplied with the gel extraction kit.*

7. Use your purified DNA to setup the intramolecular ligation as outlined in Table 7.
Table 7. Intramolecular ligation setup.

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>d$^2$H$_2$O</th>
<th>Purified DNA</th>
<th>5x Buffer</th>
<th>T4 DNA Ligase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligation #1</td>
<td>5.9 $\mu$L</td>
<td>2 $\mu$L</td>
<td>2 $\mu$L</td>
<td>1.0 U (0.1 $\mu$L)</td>
</tr>
<tr>
<td>Ligation #2</td>
<td>3.9 $\mu$L</td>
<td>4 $\mu$L</td>
<td>2 $\mu$L</td>
<td>1.0 U (0.1 $\mu$L)</td>
</tr>
<tr>
<td>Negative Control</td>
<td>6.0 $\mu$L</td>
<td>2 $\mu$L</td>
<td>2 $\mu$L</td>
<td>-</td>
</tr>
</tbody>
</table>

8. Once all the components have been added to the tube, flick several times with your finger to mix, and pulse the tube in a microcentrifuge prior to incubation.

9. The ligation reaction will be carried out at room temperature (21 °C) for 16 hrs and will subsequently be transferred to -20 °C storage for you.
Lab 12 – Transformation of BL21

Objective: To transform BL21 with the ligation products from Lab 11

Supplies and Equipment:

<table>
<thead>
<tr>
<th>Ligation mixtures from Lab 11</th>
<th>Sterile d²H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28a DNA (from Lab 1)</td>
<td>LB broth (5 mL aliquots)</td>
</tr>
<tr>
<td>Competent BL21 cells (from Lab 6)</td>
<td>LB Kan⁺ plates</td>
</tr>
<tr>
<td>Micropipettors</td>
<td>70% Ethanol</td>
</tr>
<tr>
<td>Water baths (37 °C &amp; 42 °C)</td>
<td>Plate spreaders</td>
</tr>
<tr>
<td></td>
<td>Ice</td>
</tr>
</tbody>
</table>

Procedure:

Use the procedure as outlined in Lab 8 making the appropriate changes as required. Following the overnight incubation at 37 °C, the transformation spread plates will be moved to 4 °C storage.
Lab 13 – Small Scale Expression Testing of The Putative Mutants

Objective: To determine if the appropriate deletion mutation of the \textit{rnb} sequence was performed successfully.

Supplies and Equipment:

- Transformation plates from Lab 8
- LB broth (5 mL aliquots)
- Inoculating loops
- Bunsen burners
- Kanamycin Stock (1000x)
- IPTG Stock (1 M)
- Sterile culture tubes (w/ wire racks)
- 37 °C shaking incubator
- Micropipettors w/ gel loading tips
- Microcentrifuges (RT)
- SDS-PAGE apparatus w/ 15 well combs
- Power supply (200 V)
- Temed
- APS (100 mg/mL)
- Resolving gel premix
- Stacking gel premix
- Scintillation vials
- Pasteur pipets
- 95% EtOH
- Filter paper
- Running buffer (10x)
- Graduated cylinders (100 and 1000 mL)
- 2x Reducing sample buffer
- 95 °C water bath (or heat block)
- Coomassie stain and destain
- Orbital shaker
- White light box

Procedure:

Use the procedure as outlined in Lab 9 making the appropriate changes as required.

\textit{If you wish this lab can be split across two lab periods with the following modifications.}

1. \textit{Once the gels have been cast, they can be wrapped in paper towel and stored at 4 °C in a plastic container with a small volume of running buffer until the next lab period.}

2. \textit{The cell pellets can also be stored at 4 °C prior to the addition of the 2x RSB (once the 2x RSB has been added you must run the gel the same day). Alternatively, the cultures can be left to induce overnight but should be pelleted and stored at 4 °C the following morning.}