Plasmid Purification, Restriction Digest, and Lithium Acetate Transformation

adapted from BI 125 Lab Manual

Over the next three weeks of lab you will continue your research on the anticancer ruthenium complex KP1019. Specifically, you will use the budding yeast *Saccharomyces cerevisiae* as a model organism to examine the effects of this drug on expression of the ribonucleotide reductase (RNR) subunit Rnr3. As you will read in next week’s Journal Club article, DNA damage elevates the expression of RNR. This enzyme complex is involved in nucleotide synthesis and may play a role in providing the building blocks needed to replace damaged DNA. In order to verify that KP1019 causes genotoxic stress (i.e.- DNA damage), you will examine Rnr3 expression in wild-type yeast through use of a reporter construct, a type of DNA technology wherein scientists fuse the regulatory sequence of a gene of interest to a gene that is easy to measure. In our case, the regulatory sequence of *S. cerevisiae RNR3* is fused to the *lacZ* gene from *E. coli*. The *lacZ* gene encodes the enzyme β-galactosidase, which is easily measured as it is capable of catalyzing the conversion of a colorless substrate into a colored product. The basic timeline for this series of experiments is as follows:

Week 1 – purify RNR3-lacZ plasmid from *E. coli*, transform into yeast, digest with restriction enzymes
Week 2 – agarose gel of restriction digest, treat transformed yeast with KP1019, and freeze yeast samples
Week 3 – β-galactosidase assay and data analysis

To fully understand this series of experiments, let’s begin with a little background…

**Introduction to DNA and Plasmids**

In 1928 Frederick Griffiths first demonstrated transformation of bacteria in his studies of pneumococcus. Subsequently, in 1944, Oswald Avery, Colin MacLeod, and Maclyn McCarty determined that the transforming principle was DNA. This result, however, was not widely accepted for a number of reasons. Nevertheless, the Hershey-Chase experiment of 1952 convinced the scientific community that DNA was the genetic material, and the discovery of the structure of DNA in 1953 by James Watson and Francis Crick ushered in the era of modern molecular biology, which gave birth to the field of recombinant DNA technology.

One reason that the results of Avery and his coworkers were not readily accepted was that many researchers were unable to reproduce transformation for certain genes. In particular, many scientists unsuccessfully tried to transform bacterial strains that were sensitive to antibiotics into strains that were resistant. It is possible that this lack of
success was due to the fact that many genes for antibiotic resistance are typically found not on the chromosome of a bacterium, but on plasmids, which are small, circular, autonomously replicating, extrachromosomal DNA molecules. These plasmids have become very important in the techniques of genetic engineering.

The key breakthrough in recombinant DNA technology that allowed the cloning of genes was the discovery of restriction endonucleases and their characteristics by Werner Arber and Hamilton Smith in the late 1960's and early 1970's. These enzymes make sequence-specific cuts in DNA molecules. For example, the restriction endonuclease EcoRI recognizes the following DNA sequence…

\[ -----NNNGAATTCCNNN----- \\
-----NNNCTTAAGNNN----- \]

…and cuts between T and C, making a staggered cut that leaves behind cohesive (or “sticky”) ends, as shown below. The ends are called sticky or cohesive because under conditions that favor base pairing, the two ends may stick to one another via hydrogen bonding between the complementary bases.

\[ -----NNNGAATT \quad CNNN----- \\
-----NNNC \quad TTAAGNNN----- \]

Restriction endonucleases allow both the creation and identification of recombinant DNA molecules. Both the gene of interest and a suitable plasmid, in this case a plasmid containing an antibiotic resistance gene, are cut with a restriction enzyme to produce cohesive ends on the two molecules. The plasmid and the DNA fragment containing the gene are mixed together \textit{in vitro} under conditions that favor base-pairing of the cohesive ends. The gene fragment is then covalently inserted into the plasmid by the action of DNA ligase to produce a recombinant plasmid. This plasmid is then used to transform a bacterial cell, which is an inefficient process. Thus, after transformation, there will be a large number of unwanted untransformed bacteria and only a few transformed cells. The transformed bacteria can be distinguished from nontransformed bacteria by culturing them in the presence of an antibiotic; only the bacteria that contain the plasmid will grow, since these are the bacteria that possess antibiotic resistance. These bacteria will then multiply and produce a limitless supply of the gene of interest.

Often in the production of a recombinant plasmid, the cohesive ends of the plasmid will stick to one another and be covalently linked by DNA ligase, without the inclusion of foreign DNA. Such self ligation is favored because the two cohesive ends are present in the same molecule. These non-recombinant plasmids will also transform bacteria for antibiotic resistance, but will not contain the gene of interest. Restriction endonucleases can be used in conjunction with agarose gel electrophoresis to distinguish between recombinant and non-recombinant plasmids. The recombinant and non-recombinant plasmids will give different patterns because of the presence or absence of the foreign gene.
When a particular DNA molecule is digested with restriction endonucleases, it will give a unique set of fragments, called its restriction map. These DNA fragments can be separated on the basis of their size by electrophoresis on an agarose gel. In the gel, the smaller pieces move more easily through the gel matrix than do the larger fragments; the distance migrated by a fragment is inversely proportional to the logarithm of its molecular weight in nucleotide pairs. The actual molecular weight of the fragments produced can be obtained by comparing the distances migrated with the distance traveled by DNA molecules of known molecular weight. The standard means of visualizing the location of the DNA fragments in the gel is to add ethidium bromide, a chemical dye that binds to DNA and fluoresces orange under ultraviolet light.

**Instructions for Today’s Experiment**

You will be given a culture of *E. coli* that contains the RNR3-lacZ plasmid. In addition to encoding RNR3-lacZ, this plasmid carries a selectable marker that allows identification of bacteria that carry the plasmid. Specifically, bacteria transformed with this plasmid are resistant to the antibiotic ampicillin. Since this plasmid is also designed for transformation into yeast, it carries a second selectable marker, the yeast HIS3 gene. Thus, yeast cells carrying this plasmid are able to make their own histidine, which allows for selection of transformed yeast on media lacking histidine.

To purify this plasmid from *E. coli*, thus preparing it for transformation into yeast, you will use the QIAprep Spin Miniprep plasmid preparation kit from QIAGEN. Wear gloves while completing this protocol. Although you must write-up a pre-lab describing this plasmid preparation protocol, you do not need to include it in your final lab report. Instead, when writing your lab report, simply state that the plasmid was purified using the QIAprep Spin Miniprep plasmid preparation kit.

1. Pellet 3 ml of bacterial culture (the entire culture) by centrifugation of 1.5 ml of culture for 1 minute at top speed in two microfuge tubes in a microcentrifuge. Use a pipetman to remove the supernatant without disturbing the cell pellet.

2. Completely resuspend the two cell pellets by vortexing well in a total of 250 μl of Buffer P1 (125 μl per tube). **Combine these two suspensions in one of the microfuge tubes.**

   *Resuspension solution: 50mM Glucose, 10mM EDTA, and 25 mM Tris-HCL pH 8.0. The Tris buffers the cells while EDTA binds divalent cations in the lipid bilayer weakening the cell envelope.*

3. Add 250 μl of Buffer P2 and **mix by inverting** the tube 4-6 times to mix (DO NOT VORTEX). Observe suspension for clearing to insure lysis is complete.
This cell lysis solution consists of SDS-NaOH (Sodium dodecyl sulfate-sodium hydroxide). This alkaline mixture lyses the bacterial cells. The detergent SDS dissolves the lipid components of the cell envelope, as well as cellular proteins. The sodium hydroxide denatures the chromosomal and plasmid DNA into single strands. The intact circles of plasmid DNA remain intertwined. The NaOH/SDS solution is made by adding 1 ml of 10% SDS and 1 ml of 2 N NaOH to 8 ml of distilled water.

4. Add 350 μl of Buffer N3 and mix by inverting the tube 4-6 times (DO NOT VORTEX).

The neutralization solution consists of sodium acetate/acetic acid. The acetic acid returns the pH to neutral, allowing DNA strands to renature. The large disrupted chromosomal strands cannot rehybridize perfectly, but instead collapse into a partially hybridized tangle. At the same time, the sodium acetate precipitates the SDS from the cell suspension along with the proteins and lipids with which it has associated. The renaturing chromosomal DNA is trapped in the SDS/lipid/protein precipitate. Only smaller plasmid DNA and RNA molecules escape the precipitate and remain in solution.

5. Centrifuge the bacterial lysate at 13,000 rpm in a microcentrifuge for 10 minutes at room temperature. Be sure to balance the microfuge!

A white pellet will form.

6. Transfer the supernatant from step 5, approximately 850 μl, by pipetting onto a QIAprep Spin Column in a collection tube.

The QIAprep DNA Purification System uses a silica-based membrane to which the DNA sticks in the presence of high salt.

7. Centrifuge the QIAprep Spin Column in a collection tube for 1 minute at room temperature. A cap is not needed. Following centrifugation, remove the QIAprep Spin Column from the tube and discard the flow through from the Collection Tube.

8. Wash the column by adding 750 μl of Buffer PE to the QIAprep Spin Column.

The column wash solution contains ethanol and removes RNA and protein from the column.

9. Centrifuge at 13,000 rpm in a microcentrifuge for 1 minute at room temperature. Remove the QIAprep Spin Column from the tube and discard the flow through from the Collection Tube.

10. Return the Spin Column to the Collection Tube and centrifuge at 13,000 rpm in a microcentrifuge for an additional 1 minute at room temperature to remove residual wash buffer.
11. Remove the cap from a new 1.5 ml microcentrifuge tube by twisting and tearing it off, and transfer the Spin Column to this tube. (If the top is not removed it will likely be sheared off in the microcentrifuge).

12. Elute the plasmid DNA by adding 50µl of EB to the Spin Column. Wait 1 minute to allow the EB to remove the DNA from the filter at the bottom of the column. Centrifuge at 13,000 rpm for 1 minute at room temperature in a micro-centrifuge.

   *The DNA does not remain bound to the column in the absence of a high salt concentration.*

13. Label the microfuge tube "RNR3-lacZ" along with your initials and store the tube on ice until you need this purified plasmid DNA for today’s other experiments. At the end of lab, your instructor will store your DNA in the freezer for potential use later in the semester.

**Week 1 – Yeast Transformation - Continues on next page.**
**Lithium Acetate Transformation**

*NOTE: A description of this transformation procedure should be included in BOTH your pre-lab write-up and your final lab report.*

1. Pipette 1ml of wild-type culture into a microfuge tube, one labeled with your initials.

2. Pellet the cells by spinning in a microfuge for one minute at 13,000rpm. Be sure to balance the microfuge, perhaps by coordinating with another group.

3. Use a pipetman to remove the media without disturbing the cell pellet.

4. Resuspend the cell pellet in 500μl of sterile water, pipetting up and down to evenly distribute the cells in the water.

5. Pellet the cells by spinning in a microfuge for one minute at 13,000rpm. Be sure to balance the microfuge, perhaps by coordinating with another group.

6. Use a pipetman to remove the water without disturbing the cell pellet.

7. Resuspend the cell pellet in 200μl of 100mM lithium acetate, pipetting up and down to evenly distribute the cells in the solution.

8. Pellet the cells by spinning in a microfuge for one minute at 13,000rpm. Be sure to balance the microfuge, perhaps by coordinating with another group.

9. Use a pipetman to remove the lithium acetate solution without disturbing the cell pellet.

10. Repeat steps 7, 8, and 9.

11. Add the following ingredients on top of the cell pellet in the order listed:
   - 240 μL PEG3350 (50% w/v)
   - 36μL 1.0 M LiAc
   - 25μL carrier DNA (2mg/mL)
   - 5μL purified plasmid DNA
   - 45μL sterile water

12. Vortex the tube vigorously until the cell pellet has been completely resuspended (about 1 minute).

13. Heat shock in a water bath at 42°C for 40 minutes.

14. During this 40 minute incubation, set up your restriction digest.

15. After the 40 minute incubation, pellet the cells by spinning in a microfuge for one minute at 13,000rpm. Be sure to balance the microfuge, perhaps by coordinating with another group.

16. Use a pipetman to remove the lithium acetate solution without disturbing the cell pellet.
17. Resuspend the pellet in 1ml of sterile water by gently pipetting up and down (It is extremely important to be gentle at this step, as the cells are very delicate after the exposure to harsh chemicals and heat!).

18. Pellet the cells by spinning in a microfuge for one minute at 13,000rpm. Be sure to balance the microfuge, perhaps by coordinating with another group.

19. Remove approximately 800μL of water from your sample, then resuspend the cells in the remaining liquid and plate it on an SDC-his plate as follows:
   a. Wipe off your work space with 70% ethanol and turn on your Bunsen burner.
   b. Label your SDC-his plate with your initials, the date, and wt+RNR3-lacZ.
   c. Turn the plates right/lid-side up, then use a pipetman to transfer your sample of transformed yeast onto the media in your appropriately labeled plate.
   d. Sterilize the cell spreader that is currently resting in a beaker containing 70% ethanol by slowly pulling the wet spreader through the flame of your Bunsen burner, leaving it in the flame just long enough to catch the alcohol on fire. While holding the spreader away from the Bunsen burner, watch while the ethanol burns off, sterilizing the spreader.
   e. Use your free hand to lift the lid off your Petri dish. While holding the lid with one hand (DO NOT PLACE THE LID ON YOUR LAB BENCH) and the spreader in the other, touch the spreader to a portion of the plate where there are no cells. As long as you don’t hear any sizzling (which you shouldn’t!), it’s safe to gently spread the cell suspension evenly across the surface of the agar.
   f. After you are finished spreading your first sample, close your Petri dish and let it rest right-side up for a few minutes while the liquid is absorbed.
   g. Once all the liquid has been absorbed into the solid media, flip the Petri dish upside down and place it in the 30°C incubator.

20. In 2-3 days, your instructor will (1) verify that there are plenty of colonies on the plate and (2) place your plate in the refrigerator for storage.

Week 1 - Restriction Analysis of Purified plasmid - Continues on next page.
Week 1 Continued - Restriction Analysis of Purified Plasmid

Wear latex gloves when assembling the reaction mixtures below. **Pulse all tubes in microcentrifuge before pipetting.** You will perform the following reactions.

N  = plasmid DNA, plus the restriction enzyme Ncol  
H  = plasmid DNA, plus the restriction enzyme HindIII  
N+H  = plasmid DNA, plus both the restriction enzymes Ncol and HindIII

1. Since restriction enzymes are expensive, and because they are used in extremely small quantities (quantities too small to pipette with a p20), these enzymes have been aliquoted for you. Thus you should obtain the, N, H, and N+H tubes from your instructor. Add your initials to the tube so you will know it is yours. Then you will add the rest of the reagents to these tubes, using the matrix below as a checklist. **Use a fresh tip for each reagent.** Additional detailed directions that follow this table.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Plasmid</th>
<th>Buffer (10X)</th>
<th>Enzyme(s)</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5 ul</td>
<td>2 ul</td>
<td>2 ul</td>
<td>11 ul</td>
</tr>
<tr>
<td>H</td>
<td>5 ul</td>
<td>2 ul</td>
<td>2 ul</td>
<td>11 ul</td>
</tr>
<tr>
<td>N+H</td>
<td>5 ul</td>
<td>2 ul</td>
<td>2 ul</td>
<td>11 ul</td>
</tr>
</tbody>
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2. Add 5 ul of miniprep **PLASMID DNA** to each of your tubes. **STORE THE REMAINDER OF YOUR PLASMID DNA AT -20°C FOR POTENTIAL USE LATER IN THE TERM.**

3. Use a **fresh tip** to add 2 ul of restriction buffer (RB) to each reaction tube.

4. Use a **fresh tip** to add proper volumes of distilled water to each of the tubes.

5. Close tube tops. Pool and mix reagents by pulsing in a microfuge or sharply tapping the tube bottom on the lab bench.

6. Place reaction tubes in 37°C heat block, and incubate for 30-60 minutes. **Do not overincubate. During longer incubations, DNases in miniprep may degrade plasmid DNA.**

7. Store the digests at -20°C until the following week for analysis by agarose gel electrophoresis.

8. Take time for responsible cleanup by properly disposing of culture tubes and micropipettor tips that have come in contact with *E. coli*, wiping down your lab bench with 70% ethanol, and washing your hands before leaving the laboratory.