Experiment 13

Large-Scale Purification of Plasmids pRIT4501 and pRIT4501 by Qiagen Ion Exchange Column Chromatography

In Experiment 6 you purified your chimeric plasmids by the classic CsCl density gradient centrifugation method. This procedure is used in many laboratories, but it is now replaced by pre-packaged kits based on ion exchange chromatography. One such kit is produced by Qiagen. The density gradient method produces larger amounts of DNA, probably at higher purity, but the kit method is very fast and convenient. The reason for doing both isolations in this course is that they each take advantage of different aspects of DNA structure and chemistry.

The alkaline lysis method that you used in experiment 11 produces a small amount of a crude lysate that you run on an analytical gel to determine desired recombinant products. In contrast, the procedure in Experiment 12 produces a large amount of crude lysate that is then purified by density gradient centrifugation. Similarly, the Qiagen HiSpeed Plasmid Purification procedure produces a large amount of crude lysate that is purified by ion exchange column chromatography. The lysis technique that you will employ here is the same alkaline lysis method that you used in Experiment 11, although the details of the reagents are different. There is the same lysis and DNA denaturation by alkali and SDS, followed by subsequent reannealment with Potassium Acetate.

Purification occurs when the lysate is passed through an anion exchange column. The column is a resin made of silica beads coated with diethylaminoethanol (DEAE). DEAE can be either neutral or negatively charged, depending on the salt concentration and the pH. At low salt concentration and pH, the DEAE is positively charged, so it attracts and binds the negatively charged phosphates of the phosphodiester backbone of DNA. Of course RNA and proteins are negatively charged and also bind. Their overall negative charge, however, is lower than that of DNA. After the lysate is bound in the column, the column is washed with QC buffer. This is a medium salt and neutral pH buffer that makes the DEAE somewhat neutral so that the protein and RNA impurities are washed from the column while the DNA remains bound. The wash step is followed by the elution buffer, QF. QF is a high salt high pH buffer that finally releases the DNA and allows it to pass through the column.
Qiagen Ion Exchange Column Chromatography

Materials
- QIAfilter cartridge
- QIAfilter cartridge cap
- HiSpeed Midi Tip
- QIAprecipitator Midi Module
- 20 ml syringe
- 5 ml syringe
- isopropanol
- 70% ethanol

Qiagen Reagents:
- P1, P2, P3, QBT, QC, QF, and TE

Two Days Before
- Streak cultures containing pRIT4501 and pRIT4502 on MacConkey plates containing the appropriate selective antibiotic for isolated colonies.

The Day Before
- Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. Incubate for approximately 8 hours at 37°C with vigorous shaking (~300 rpm). Dilute this starter culture 10/500 to 1/1000 in 50 ml LB + antibiotic, and incubate overnight.

Lysis Procedure
- Harvest your culture as follows:
  - Transfer each culture to a 250 ml polypropylene bottle, balance, and centrifuge at 7,000 rpm for 10 minutes. Decant the supernatant.
Resuspend the pellet in 15 ml LB broth and transfer to a 50 ml polypropylene Oakridge centrifuge. Centrifuge the cells at 10,000 rpm in the large floor model centrifuge. Decant the pellet.

_This is a convenient breaking point if you wish to stop the protocol and continue later, freeze the cell pellet at −20°C. We will proceed directly to the next step._

2. Resuspend the pellet in 6 ml of Buffer P1. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

3. Add 6 ml of Buffer P2 and mix gently but thoroughly by inverting 4–6 times. Incubate at room temperature for 5 min.

_Donot vortex, as this will result in shearing of chromosomal DNA._

The lysate should appear viscous.

_Do not allow the lysis reaction to proceed for more than 5 min._

After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO2 in the air.

During the incubation prepare the QIAfilter Cartridge:
Screw the cap onto the outlet nozzle of the QIAfilter Midi and place the QIAfilter cartridge into a 25 ml disposable conical centrifuge tube

4. Add 6 ml of chilled Buffer P3 to the lysate, and mix immediately but gently by inverting 4–6 times. Proceed directly to step 5.

_Do not incubate the lysate on ice._

Precipitation is enhanced by using chilled Buffer P3.

After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and SDS becomes visible. The buffers must be mixed completely. If the mixture appears still viscous and brownish, more mixing is required to completely neutralize the solution.

It is important to transfer the lysate into the QIAfilter Cartridge
Chromatography Procedure

1. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10 min.

   Do not insert the plunger!

   This 10 min incubation at room temperature is essential for optimal performance of the QIAfilter Cartridge. Do not agitate the QIAfilter Cartridge during this time. A precipitate containing proteins, genomic DNA, and detergent will float and form a layer on top of the solution. This ensures convenient filtration without clogging.

   If, after the 10 min incubation, the precipitate has not floated to the top of the solution, carefully run a sterile pipet tip around the walls of the cartridge to dislodge it.

2. Equilibrate a HiSpeed Midi Tip by applying 4 ml of Buffer QBT and allow the column to empty by gravity flow. Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the HiSpeed Midi Tip to drain completely. HiSpeed Midi Tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

3. Remove the cap from the QIAfilter outlet nozzle. Gently insert the plunger into the QIAfilter Midi Cartridge and filter the cell lysate into the previously equilibrated HiSpeed Midi Tip. Filter until all of the lysate has passed through the QIAfilter Cartridge, but do not apply extreme force.

   Approximately 15 ml of lysate is generally recovered after filtration.

4. Allow the cleared lysate to enter the resin by gravity flow.

5. Wash the HiSpeed Midi Tip with 20 of Buffer QC. Allow Buffer QC to move through the HiSpeed Tip bygravity flow.
6. Elute DNA with 5 ml of Buffer QF. Collect the eluate in a tube with a minimum capacity of 10 ml.

   *If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.*

7. Precipitate DNA by adding 3.5 ml of room-temperature isopropanol to the eluted DNA. Mix and incubate at room temperature for 5 min.

   *All solutions should be at room temperature in order to minimize salt precipitation.*

8. During the incubation remove the plunger from a 20 ml syringe and attach the QIAprecipitator onto the outlet nozzle.

   *Do not use excessive force, bending, or twisting to attach the QIAprecipitator!*

9. Place the QIAprecipitator over a waste bottle, transfer the eluate/isopropanol mixture into the 20 ml syringe, and insert the plunger. Filter the eluate/isopropanol mixture through the QIAprecipitator using constant pressure.

10. Remove the QIA precipitator from the 20 ml syringe and pull out the plunger. Re-attach the QIA precipitator and add 2 ml of 70% ethanol to the syringe. Wash the DNA by inserting the plunger and forcing the ethanol through the QIA precipitator using constant pressure.

   *Important: Always remove the QIAprecipitator from the syringe before pulling up the plunger!*

11. Remove the QIA precipitator from the 20 ml syringe and pull out the plunger. Attach the QIA precipitator to the 20 ml syringe again, insert the plunger, and dry the membrane by pressing air through the QIA precipitator quickly and forcefully. Repeat this step several times.

12. Dry the outlet nozzle of the QIA precipitator with absorbent paper to prevent ethanol carryover.
13. Remove the plunger from a new 5 ml syringe and attach the QIA precipitator onto the outlet nozzle.

*Do not use excessive force, bending, or twisting to attach the QIA precipitator!*

Hold the outlet of the QIA precipitator over a microfuge tube. Add 1 ml of Buffer TE to the 5 ml syringe. Insert the plunger and elute the DNA into the collection tube using constant pressure. Be sure that the QIA precipitator is held over the collection tube when Buffer TE is poured into the syringe, as eluate can drip through the QIA precipitator before the syringe barrel is inserted. Be careful, as residual elution buffer in the QIA precipitator tends to foam when expelled.

14. Remove the QIA precipitator from the 5 ml syringe, pull out the plunger and reattach the QIA precipitator to the 5 ml syringe.

Transfer the eluate to the 5 ml syringe and elute for a second time into the same microfuge tube. This re-elution step ensures that the maximum amount of DNA in the QIA precipitator is solubilized and recovered.
Reagents for the Qiagen HiSpeed Plasmid Purification are provided in the kit

<table>
<thead>
<tr>
<th>Buffer P1 (Resuspension Buffer)</th>
<th>Buffer QF (Elution Buffer)</th>
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<tbody>
<tr>
<td>50 mM Tris-Cl, pH 8.0</td>
<td>1.25 M NaCl</td>
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<tr>
<td>10 mM EDTA</td>
<td>50 mM Tris-Cl pH 8.5</td>
</tr>
<tr>
<td>100 µg/ml RNase A</td>
<td>15% isopropanol (v/v)</td>
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<tr>
<td><em>store at 2–8°C after addition of RNase A</em></td>
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<tr>
<th>Buffer P2 (Lysis Buffer)</th>
<th>Buffer TE</th>
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<tbody>
<tr>
<td>200 mM NaOH</td>
<td>10 mM Tris-Cl pH 8.0</td>
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<tr>
<td>1% SDS (w/v)</td>
<td>1 mM EDTA</td>
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<tr>
<th>Buffer P3 (Neutralization Buffer)</th>
<th>Buffer QC (Wash Buffer)</th>
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<tbody>
<tr>
<td>3.0 M potassium acetate pH 5.5</td>
<td>1.0 M NaCl</td>
</tr>
<tr>
<td><em>can be stored either at room temp or 2–8°C</em></td>
<td>50 mM MOPS pH 7.0</td>
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<td></td>
<td>15% isopropanol (v/v)</td>
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<thead>
<tr>
<th>Buffer QBT (Equilibration Buffer)</th>
<th>Buffer QF (Elution Buffer)</th>
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<tbody>
<tr>
<td>750 mM NaCl</td>
<td>1.25 M NaCl</td>
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<tr>
<td>50 mM MOPS pH 7.0</td>
<td>50 mM Tris-Cl pH 8.5</td>
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<tr>
<td>15% isopropanol (v/v)</td>
<td>15% isopropanol (v/v)</td>
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<tr>
<td>0.15% Triton® X-100 (v/v)</td>
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<tr>
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<th>Buffer TE</th>
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<tbody>
<tr>
<td>1.0 M NaCl</td>
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