TECHNIQUES IN MOLECULAR BIOLOGY – LABORATORY - PLASMID PURIFICATION

In this lab you will prepare plasmid from your pQE60 wgMDH culture. One preparation will be using an alkaline lysis method and the other a spin-column format using materials from the company, Qiagen.

A key to success in protocols like this is to know *where the desired component of the reaction is located at all times:* the **supernatant** or the **pellet**. (In this case, plasmid DNA is the desired component.)

Important notes on this plasmid mini-prep technique

– Once cells have been lysed, mixing should be done thoroughly *but gently*, to avoid breaking plasmid and bacterial chromosomal DNA. **Do not vortex** after cell resuspension, but mix by inversion.
– After the protein precipitation step, the supernatant should be transferred as soon as possible since the pellet tends to soften quickly.
– Make sure that a centrifuge is available for spinning immediately after adding isopropanol.

Experiment: The lab will be conducted as follows:

- Students will initiate two 5 ml cultures as described below the DAY BEFORE this laboratory.
- Each student will conduce an alkaline lysis DNA prep and a Qiagen Mini Spin column DNA preparation

The following points should be considered after the experiment is finished. What are the pros and cons of the methods of purification? What method did you use? What is the final yield (in milligrams) of your DNA? Was it pure, how can you tell that your final prep was pure? What might possible contaminants be and what steps might be used to avoid contamination? What is the difference between the two methods in terms of time, yield, cost and purity? When might you use one method vs the other method to purify DNA? What is in each of the buffers and what is the role of EACH of these components? Did your results with the spectrophotometer reflect the agarose gel analysis?

I. Cell Culture - PREPARE TWO 5 ml CULTURES: LABLE EACH TUBE

1) **Select a single well isolated colony** from your freshly transformed cell culture. Alternatively a streak from a glycerol stock can be used if re-streaked before using. Use a toothpick or pipette tip to transform the colony into a 5 ml LB media with the appropriate antibiotic in loosely capped tube. Leave tip in the liquid.
2) **Culture overnight WITH SHAKING** at an angle at 37°C. This is best done after noon but before 5pm.
3) **Centrifuge the Cells.** Harvest the cells by centrifugation at 3,000 x g for 5 minutes at 4°C. Remove all traces of supernatant by inverting the centrifuge tube until all media has been drained. The pellet can then be frozen at –20°C for later use.

Bacterial Culture Information:

- From a freshly grown bacterial colony, select an isolated colony and inoculate 5.0 ml of LB media with the appropriate antibiotic in a loosely capped (use the plastic tube caps or cotton plug. *See the bacterial culture handout for antibiotic and media preparation information*.)
- Incubate the culture in a rolling mixer for 12 – 14 hours. Much longer and the cells will be too dense to work well and cells may have begun to lyse and die. Less time will result in poor or no DNA yield.

BEFORE STARTING:

- Add RNaseA and LyseBlue to P1 (see buffer instructions)
- Check Buffer P2 for SDS precipitation due to low storage temperatures (in winter, the room's temp will cause some ppt). If necessary, dissolve the SDS by warming to 37°C.
- Pre-Chill Buffer P3 at 4°C.

METHOD ONE - Qiagen QIAprep Mini prep DNA Purification Method c/n 27014 *Plasmid isolation Procedure (taken directly from the Qiagen Handbook with additional notation for clarity)*

1) **Resuspend the bacterial pellet in 250 µl of Buffer P1.**

Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet

If LyseBlue reagent has been added to Buffer P1, vigorously shate the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain

2) **Add 250 µl of Buffer P2 and mix the sample by inversion 4-6 times. Incubate at room temp for 5 min.**

Mix Gently by inverting the tube. If Lyseblue has been added to Buffer P1 the cell suspension will trun blue after addition of Buffer P2. Mixing should result in a homogenously colored suspension. If suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the soltuion until a homogeneously colored suspension is achieved.

Vortexing will result in shearing of the genomic DNA, leaving free chromosomal fragments to contaminate the plasmid DNA. This will result in a co-purification of both DNA types and will ruin any further experiments. The lysed cells be very thick, almost like snot. The solution should become slightly more clear if the cells have lysed. This solution contains NaOH and will alkaline lyse the cells.

Incubate for exactly 5 minutes. Longer incubations will lead to inappropriate degradation of genomic DNA. SDS solubilizes the phospholipid and protein components. NaOH denatures the DNA as well as proteins. Optimal lysis time allows maximal release of plasmid DNA from the cells without release of genomic DNA. Long exposure to alkaline conditions may cause the plasmid DNA to become irreversibly denatured. This DNA will run faster on a gel and is resistant to restriction enzyme digestion.

3) **Add 350 µl of Buffer N3 and mix immediatelyand thoroughly by inverting the tube 4-6 times..**

After addition of this buffer the solution becomes cloudy and very viscous. The lysate is neutralized by the addition of acidic potassium acetate in the neutralization buffer. The high salt (K^+) concentration causes the SDS to become insoluble and precipitate. Plasmid DNA is too small to be trapped in the precipitate while genomic DNA and protein is. Any remaining SDS in solution will inhibit DNA binding to the column later so make certain that the solution is well mixed.

To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. >5 ml) may require inverting up to 10 times. The solution should be come cloudy.

If LyseBlue has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively preciptiated.

4) **Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.**

A compact white pellet will form

Remove and save 10 µl of the supernatant for an analytical gel. Save as cell lysis.

5) **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**

6) **Centrifuge for 30-60 s. Discard the flow-through .**

7) **Recommended: Wash the QIAprep spin column by adding 0.5 ml BUffer PB and centrifuging for 30-60 s. Discard the flow through.**

This step is necessary to remove trace nuclease activity when using endA$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5 alpha do not require this additional wash step

8) **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 s.**

9) **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10) **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min..**

Important: SAVE THIS ELUTION - this contains your plasmid. RECORD THE VOLUME!!!!

Method two - Aklkaline Lysis DNA Mini Prep - For semi-pure purification of plasmid DNA.

****This protocol is for Mini (up to 20 µg) preparations of high-copy plasmid DNA from cultures of *E. coli*.****

Alkyline Lysis Protocol (5 ml culture preparation)

1. **Resuspend pellet. Add 200 µl Buffer P1. Resuspend and transfer to a microcentrifuge tube**.
   - Ensure that RNaseA has been added to the buffer.
   - No clumps should be visible
   - If using Lyse Blue, ensure all particles are completely dissolved by vortexing buffer P1 PRIOR to use.
   - The bacteria should be resuspended completely by vortexing or pipetting up and down.
2. **Lyse cells. Add 200 µl Buffer P2 and mix by inverting the tube 4-6 times.**
   - Incubate at room temp for 5 min.
   - DO NOT VORTEX or vigorously invert– will shear genomic DNA causing contaminant in your elution.
   - DO NOT allow lysis reaction to go longer than 5 min.
   - Solution should turn blue. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.
3. **Precipitate protein and SDS with KOAc**. **Add 300 µl Buffer P3. Mix <u>immediately</u> and thoroughly by inverting 4-6 times.**
   - Precipitation is enhanced by using chilled Buffer P3. After addition of Buffer P3, a fluffy white precipitate containing genomic DNA, proteins, cell debris, and KDS 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. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated
4. **Clarify Lysate:** Centrifuge at 10 min at 13,000 rmp (~17,900 x g) in a table-top centrifuge.
   - Remember to leave the hinge of the tube on the outside edge of the centrifuge
   - A tight white pellet should form on the outside/side bottom of the tube. This is the cell debris, SDS-potassium ions and trapped genomic DNA. Plasmid DNA is in solution (supernate) of this centrifugation.
5. **Precipitate Plasmid DNA: Precipitation of DNA with isopropanol should be carried out at room temperature in order to minimize salt precipitation.**
   - Transfer supernatant to fresh-labeled tube. Supernate should be close to 700 µl. Discard pellet.
   - Add 0.7 volume (490 µl) of room temperature isopropanol and mix by inversion.
   - Centrifuge at max speed in microfuge for 20 min at room temp. Plasmid DNA will ppt.
   - Remove supernatant solution and use kimwipe to wick off remaining solution.
   - Carefully add 1 ml of ice cold 70% ethanol and resuspend the pellet by a short vortex.
   - Centrifuge to ppt the plasmid DNA at max rpm (~17,900 x g) for 30 min at 4°C.
   - The pellet will be difficult to see (some describe it as glass) and carefully remove ethanol. The 70% ethanol serves to remove precipitated salt, as well as to replace isopropanol with the more volatile ethanol, making the DNA easier to redissolve. You may need to tip the tube upside down on Kimwipes to remove residual ethanol.
   - Marking the outside of the tube before centrifugation allows the pellet to be easily located. *Watch the tube hinge placement for identification of the pellet.*

For BOTH Purification Methods- Determine the concentration of DNA in the sample.

- Determine the absorbance of your sample at 260 and 280 nm.
- The concentration of DNA will be 10 times the absorbance at 260 nm. The units will be mg/ml.
- The actual purity of the sample can be determined by taking the ratio of absorbance at 260 to 280 nm*. If the ratio is greater than 1.8 the absorption is due to nucleic acids. A high quality prep should have a ratio of 2.0 to 1.8. If the ratio is below 1.6 there may be proteins or other organic contaminates and the DNA can be extracted by chloroform phenol extraction for a final clean up.*
- ***SAVE EACH SAMPLE in the -20°C freezer.***

General Preparation Notes and FAQ:

- To ensure high yields of pure DNA, use no more than 3 ml LB culture for high-copy number plasmids (e.g., pUC, pBluescript®). For low-copy-number plasmids (e.g., pBR322), use no more than 10 ml LB culture and refer to the recommendations on page 13. We do not recommend the use of rich media such as TB or 2xYT for culture. When low-copy-number plasmids containing the ColE1 replication origin are prepared, the yield can be improved by amplification in the presence of chloramphenicol (34 mg/ml). They should then be treated as high-copy-number plasmids.
- Add the provided RNase A solution to Buffer P1 before use (spin down RNase A briefly before use). Buffer P1 should then be stored at 2–8°C and is stable for 6 months.
- Check Buffer P2 for SDS precipitation due to low storage temperatures. If necessary, dissolve the SDS by warming to 37°C.
- After use, the bottle containing Buffer P2 should be closed immediately to avoid any reaction between the NaOH and CO_2 in the air. If the buffer is left open for any length of time, it should be prepared fresh from stock solutions.
- Chill Buffer P3 at 4°C.
- Optional: To confirm purification or to identify a problem, samples may be taken at specific steps for analysis on an agarose gel. Appropriate samples and volumes are indicated in the protocol below.