TECHNIQUES IN MOLECULAR BIOLOGY – METHODS FOR PLASMID DNA ISOLATION

DNA isolation:

The application of molecular biology techniques to the analysis of complex genomes depends on the ability to prepare pure plasmid DNA. Most plasmid DNA isolation techniques come in two flavors, simple - low quality DNA preparations and more complex, time consuming, but high quality DNA preparations. For many DNA manipulations such as restriction enzyme analysis, subcloning and agarose gel electrophoresis, the simple methods are sufficient. The high quality preparations are required for most DNA sequencing, PCR manipulations, transformation and other techniques.

Most methods start with a large number of bacterial cells, which contain the plasmid of choice and centrifuging down to a pellet. The cells are then lysed by a mixture of the detergent sodium dodecylsulfate (SDS) in basic conditions or by adding a protease (lysozyme) to weaken and disrupt the host cell wall. The result of both of these methods leads to the release of compact supercoiled plasmid DNA molecules into solution. The next problem is to separate the RNA, genomic DNA and other cellular constituents from the cells. How this is accomplished depends on the method used.

The alkaline lysis preparation is the most commonly used method for isolating small amounts of plasmid DNA, often called minipreps. This method uses SDS as a weak detergent to denature the cells in the presence of NaOH, which acts to hydrolyze the cell wall and other cellular molecules. The high pH is neutralized by the addition of potassium acetate. The potassium has an additional effect on the sample. Potassium ions interact with the SDS making it the detergent insoluble. The SDS will easily precipitate and can be separated by centrifugation. In doing so the insoluble SDS traps the larger genomic DNA and removes it from the supernatant. This leaves the plasmid DNA and RNA in solution. The RNA is often removed by digestion with the addition of RNaseA. This leaves only proteins, carbohydrates and RNA nucleoside monomers in solution. A primary alcohol, such as ethanol or propanol is used to precipitate the DNA. This is accomplished by the re-ordering of the water, making the DNA aggregate and become insoluble. The result is a somewhat pure pellet of DNA that can be resuspended in a mildly buffered solution or water.

The boiling miniprep is recommended for preparing small amounts of plasmid DNA from a large number of cultures. While this method is extremely quick, the quality of DNA produced is lower than that from the alkaline lysis miniprep. In the alkaline lysis miniprep method, lysozyme is used to hydrolyze the extensive crosslinked proteins that are responsible for giving the bacterial cell wall its strength. The cells are then boiled to further denature the proteins and disrupt the cell walls. The plasmid DNA is then precipitated with alcohol.

Both of these methods will yield only a few µg of plasmid DNA. For larger quantities that are considerably higher in purity, many additional steps are required. Separating DNA based on their density by centrifuging in a cesium chloride density gradients at very high forces of gravity. The cesium chloride gradients yields high quality plasmid DNA free of most contaminants but uses ethidium bromide to identify the DNA (a potential mutagen) and requires long ultracentrifuge runs to establish the density gradient. This method is initiated by lysing the cells using the alkaline lysis method and the sample is centrifuged for 14 hours at 350,000 x g. First a CsCl gradient is made in a small tube and the DNA is added with ethidium bromide. While spinning, the DNA will migrate down the tube until it reaches the density of the CsCl that is the same as the plasmid. Thus larger DNA will be separated from the compact plasmid DNA. The plasmid band is visualized by UV light, removed with a needle and the procedure repeated. As you can see this is a very complicated and tedious method for isolating DNA that is not often used with the advent of column separations.

A more popular method now exists that take advantage of the differences in physical properties of plasmid DNA and the contaminants found in the alkaline lysis method. Nucleic acids are negatively charged and can therefore be purified away from contaminants using anion-exchange

chromatography. These are often sold as kits by several different companies and are the most used method of purifying plasmid DNA today.

These kits come in several sizes that allow the processing of a few µg of plasmid all the way up to the mg range. These are usually called mini, midi and maxi preps. One such kit which is often used is from the QIAGEN company. QIAGEN Anion-Exchange Resin yields DNA or RNA of a purity and biological activity equivalent to at least two rounds of purification in CsCl gradients, in a fraction of the time. Purified nucleic acids are ideal for sensitive downstream biological applications, such as transfection, microinjection, sequencing, and gene therapy research. QIAGEN Resin is a macroporous silica-based resin with a high density of diethylaminoethyl (DEAE) groups, which was developed exclusively for isolation of nucleic acids. Purification on QIAGEN Resin is based on the interaction between negatively charged phosphates of the nucleic acid backbone and positively charged DEAE groups on the surface of the resin (Figure 1). The salt concentration and pH conditions of the buffers used in each step control binding, wash stringency, and elution of nucleic acids. The exceptional separation properties of QIAGEN Anion-Exchange Resin arise from its high charge density. The resin consists of defined silica beads with a particle size of 100 µm, a large pore size of 2000–4000 Å, and a hydrophilic surface coating. The proprietary chemistry allows dense coupling of the DEAE groups. This provides the extraordinary broad separation range that allows selective separation of nucleic acids from other substances, such as proteins, polysaccharides, and metabolites

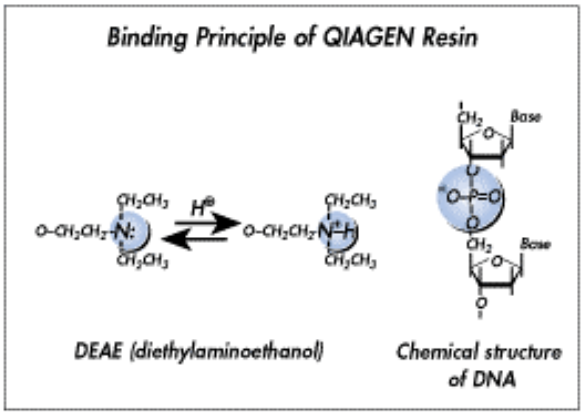

Figure 1 Chemical structure of positively charged DEAE groups of QIAGEN Resin, and negatively charged phosphate groups of the DNA backbone which interact with the resin.

A diatomaceous earth-based method is used to isolate the plasmid from cell lysate using the alkaline lysis method (Fig. 2). This is also called a siliga gel method. For this treatment the DNA pellet is resuspended in RNaseA to remove the RNA by digestion. This is necessary because the RNA will compete with DNA for binding to the diatomaceous earth. After RNaseA treatment, the DNA containing supernatant is bound to the diatomaceous earth in a chaotropic buffer often guanadine chloride or urea. The chaotropic buffer will force the silica (diatomaceous earth) to hydrophobically interact with the DNA. Purification using silica-technology is based on a simple bind-wash-elute procedure. Nucleic acids are adsorbed to the silica-gel membrane in the presence of high concentrations of chaotropic salts, which remove water from hydrated molecules in solution. Polysaccharides and proteins do not adsorb and are removed. After a wash step, pure nucleic acids are eluted under low-salt conditions in small volumes, ready for immediate use without further concentration.

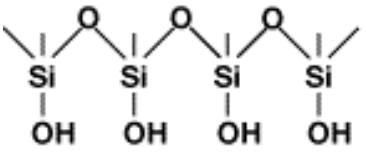

Figure 2 Structure of silica-gel materials

The diatomaceous earth is centrifuged and contaminants are left behind in the supernatant. DNA is eluted during incubation at 65°C and the removed from the particles. This is a little more difficult than the anion-exchange chromatography methods but is significantly cheaper.

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.)

Other 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?

At the end of this document is a description/recipe for each buffer and some additional background information. The information will serve as a good reference for your future use.

Cell Culture - PREPARE TWO 5 ml CULTURES: LABLE EACH TUBE WITH THE NAME OF PLASMID AND YOUR NAME.

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.

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.

12) **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.*

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*.****

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.

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.

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.*

Preparation Notes:

- 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.

25 x TEA Buffer

1M tris, 15mM EDTA, 125 mM Na acetate (pH 7.8) To 750 ml of distilled water add 121 g of Tris base, 10.2g of sodium acetate, 18.6 g of EDTA. Adjust the pH to 7.8 with glacial acetic acid and QS to 1 liter. Store at 4°C

Lysis

P1

50 mM Tris base	6.06 g
10 mM $Na_2EDTA.2H_2O$	3.72 g
HCl	*to pH 8*
H_2O	*to 1000 ml*

Dissolve the Tris and EDTA in 800 ml H_2O and adjust the pH to 8. Make the volume up to 1000 ml with H_2O.

Just prior to use, add RNAseA 50 µg/ml AND LyseBlue to the amount of P1 needed for that day.

P2

200 mM NaOH	8 g
1% SDS	10 g
H_2O	*to 1000 ml*

Dissolve NaOH in 800 ml of water, then add SDS while stirring with stir bead. Q.S to 1000ml with water.

P3

H_2O	500 ml
Potassium Acetate	294.5 g
Glacial Acetic Acid	110 ml
H_2O	*to 1000 ml*

Add the potassium acetate to 500 ml of H_2O and dissolve by agitation. Will be 3.0 M K Acetate buffer when finished. Add the glacial acetic acid slowly with gentle swirling and test the pH (it should be between 5.4 and 5.6). If not, pH using acetic acid. Make the volume up to 1000 ml with H_2O.

Using LyseBlue reagent (1000X = 43 mg/ml thymolphthalein in ethanol)

LyseBlue is a color indicator that provides visual identification of optimum buffer mixing. LyseBlue can be added to the resuspension buffer (Buffer P1) bottle before use. Alternatively, smaller amounts of LyseBlue can be added to aliquots of Buffer P1, enabling single plasmid preparations incorporating visual lysis control to be performed.

LyseBlue reagent should be added to Buffer P1 at a ratio of 1:1000 to achieve the required working concentration (e.g., 10 µl LyseBlue into 10 ml Buffer P1). LyseBlue precipitates after addition into Buffer P1. This precipitate will completely dissolve after addition of Buffer P2. Shake Buffer P1 before use to resuspend LyseBlue particles.

The plasmid preparation procedure is performed as usual. After addition of Buffer P2 to Buffer P1, the color of the suspension changes to blue. Mixing should result in a homogeneously colored suspension. If the suspension contains localized regions of colorless solution or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved. Upon addition of neutralization buffer (Buffer S3), LyseBlue turns colorless. The presence of a homogeneous solution with no traces of blue indicates that SDS from the lysis buffer has been effectively precipitated.