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

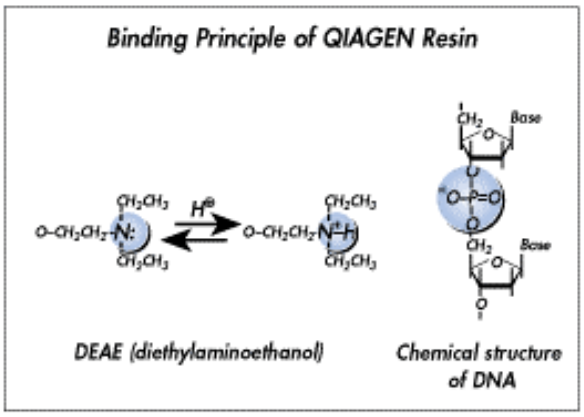

Figure 1 Chemical structure of positively charged DEAE groups of QIAGEN Resin, and negatively charged phosphate groups of the DNA backbone which interact with the resin.

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.

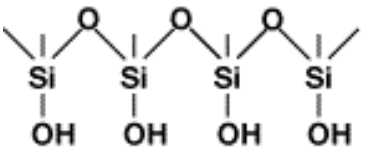

Figure 2 Structure of silica-gel materials

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.

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

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.

Lysis

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.