Biol/Chem 405 Biochemistry Lab I
MDH-GFP-His – DNA E
Experiment 3 Part 1
Methods for Plasmid DNA Isolation

Project background: This is the beginning of a multiple-week project where you will isolate DNA, express, purify and finally characterize a fusion protein called Malate Dehydrogenase- green fluorescent protein-6XHis (MGH).

For the rest of the semester, your job will be to isolate the plasmid DNA for MGH, transform your purified DNA into a bacterial cell line suitable for protein expression, purification of your fusion protein using two chromatogaphies, determination of the protein's purity and molecular weight, to identity the protein by western blot analysis and to analyze the kinetics of the enzyme.

The final write up for this lab will be a scientific manuscript written using the style guide for the Journal of Biological Chemistry. It is important that you take good notes in your lab book to make this process easier. There will be more about the MGH in next week's lab handout. Today's experiment will be isolating the clone for MGH.

It is very common for scientists to purchase or share clones. In most cases, that clone will be shipped to the researcher in one of two forms; either a very small amount (<10μl) of pure DNA or the clone may already be transformed into a bacteria cell line for DNA purification. If the purpose is to use the DNA to produce protein (expression in *E. coli*), then the biochemist will need to place the DNA into a bacterial cell line suitable for protein expression (transformation). In most cases, DNA is stored and purified in a cell line that is best for isolating DNA but not protein.

For our experiment, we have received the plasmid containing the gene for our fusion protein, MGH in the E. coli strain DH5α'. This cell strain is appropriate for DNA purification but is a poor choice for protein expression. Because our goal is to make pure protein, we will first, need to isolate and quantitate the DNA before transforming into a suitable protein expression cell line.

Required Reading: *At the Bench, pages 279 – 288*.

DNA isolation introduction:

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 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.

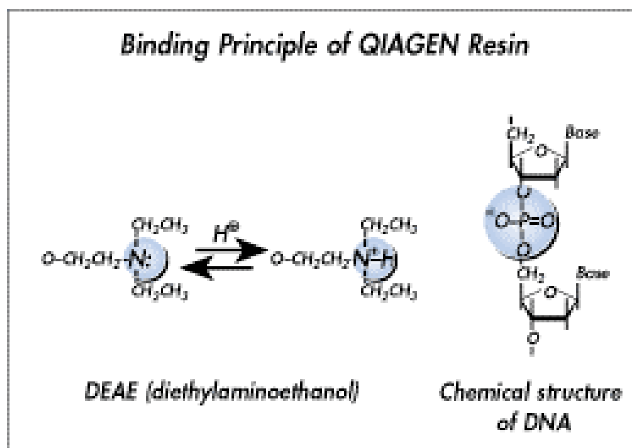

Figure 1 Chemical structure of positively charged DEAE groups of QIAGEN Resin, and negatively charged phosphate groups of the DNA backbone which interact with the resin.

Newer and more popular methods 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

Recently, a diatomaceous earth-based method was 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.

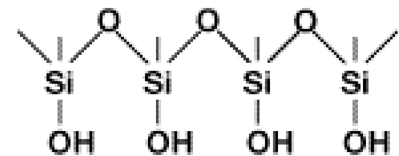

Figure 2 Structure of silica-gel materials

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.

Experiment:

The lab will be conducted as follows:

- The bacterial cultures prepared by your instructor.
- Prepare the pellet, lyse the cells and isolate the DNA as directed
- Determine the concentration and purity of the DNA spectraphotometrically
- If there is time this week we will finish by running an analytical agarose gel. Otherwise we will run the gel next week.

Labs report – All observations and calculation go in the lab book. The lab book should have an individual entry for this and each of the related experiments. In the lab book include a title, a purpose (this can be as short as a one sentence description). Include a flow chart for the methods used in the experiment and reference the handout for details. In the results include all calculations observations such as appearance of the solutions pellets and so on. Don't forget to include the picture of the DNA gel (with figure legend and an appropriately labeled gel)

- ***<u>The following points should be addressed in the paper</u>*** (not the lab book). Introduce the methods of DNA purification. 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 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?

Method

<u>Inoculation and cell preparation</u>

(The first step was conducted by your instructor – aren't you lucky?
But you are still responsible for understanding what I did.)

1) **Select a well isolated colony for inoculation.** This will ensure maintenance of the plasmid and that the plasmid is from a single cell. Inoculate into 3 ml of LB media containing 100 µg/ml ampicillin and grow for 8 – 10 hours at 37°C. This is best started in the morning the day before starting the purification. Expand the culture into 50 ml of LB media containing ampicilin and incubate overnight (10 – 12 hours) in a 150 ml Erlenmeyer flask. Grow on the orbital shaker at about 250 rpm.

2) **Transfer the 2 ml of your cells to a 2 ml microfuge tubes.** Harvest the cells by centrifugation at 3,000 x g (3500 rpm in the micro centrufuge) for 15 - 30 seconds 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.

<u>Plasmid isolation</u>

3) **Resuspend the bacterial pellet in 200 µl of buffer Cell Resuspension Buffer.**
Use a 1.0 ml (P1000) pipettor and rinse the pellet in and out of the pipet with the tip placed near or at the bottom of the container. Go slowly, do not aspirate the material into the pipet or sheer the DNA. Make sure that the bacterial pellet is completely resuspended and no clumps remain. The RNase A will degrade the RNA and it will not bind to the column.

4) **Add 250 µl of Cell Lysis Buffer and mix the sample by inversion 10 times. Incubate at room temp for 5 min.** DO NOT VORTEX
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<u>. Incubate for exactly 5 minutes</u>. 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.

5) **Add 250 µl of neutralization buffer and mix immediately but gently by inversion (5 – 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.

6) **Mix the sample by inversion 5-6 more times and centrifuge at 20,000 x g (max rpm in the microfuge) for 5 min. at room temperature. Remove the supernatant immediately (pipet). Keep the supernatant.**

DO NOT USE GLASS TUBES, the DNA will stick the silica walls of the tube. It is important that the supernatant is clear and free of cellular debris. The supernatant (cleared lysate) at this step contains the plasmid DNA.

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

7) **Equilibrate the column matrix while waiting for the centrifuge.**

Insert a spin column into one of the 2 ml microfuge wash tubes. Mix the matrix by vortexing or repeated shaking and inversion of the bottle to insure that it is completely suspended. The silica has already been equilibrated in binding buffer.

8) **Transfer the cleared lysate (supernate) from step 6 to a spin colum and add 200 µl of thoroughly suspended matrix, then pipet up and down to mix. Centrifuge for 30 seconds max rpm .**

There should be little to no time between step 6 and this step. If there is, the solution may turn cloudy and will need further centrifugation or filtration. The salt and pH of the buffers used ensure that only plasmid DNA binds to the column. RNA, cellular proteins and metabolites do not bind and are found in the eluate (flow through fraction). Keep a 10 µl sample for an analytical gel. This is the flow through sample.

9) **Remove the spin column and rinse out the tube with water. Add 400 µl of wash buffer and wash the matrix by centrifugation for 30 seconds at max RPM..**

Do not try to force out the remaining buffer. The column is then washed with a high salt buffer containing more guanadine chloride (chaotropic salt). This will wash off residual contaminants still mixed in the matrix. The low concentration of alcohol in the wash buffer eliminates nonspecific hydrophobic interactions increasing the purity of the plasmid DNA. Keep a 20 µl sample for an analytical gel. This is the wash sample.

10) **Wash the column again.**

Remove the spin column from the 2 ml tube, discard the filtrate at the bottom of the tube and replace the column in the same tube. Add 400 µl of wash buffer and wash the matrix by centrifugation for a full 2 minutes to remove any residual traces

of ethanol. Keep a 20 µl sample for an analytical gel. This is the second wash sample.

11) **Elute the DNA from the matrix.**

Remove the spin column and discard the microcentrifuge tube. Place the column in a standard 1.5 ml microfuge tube. Add 100 µl of 65°C deionized water. Promptly elute the DNA for 1 minute at top speed. The decreased concentration of salts and increased temperature allow the DNA to be re-solubilized and decrease the affinity to the hydrophobic silica matrix. Save the eluted DNA.

13) **Determine the concentration of DNA in the sample. See page 289 of At the Bench for background.**

Use the sipper for this analysis.

- Prepare one microfuge tube with 1.5 ml of TE buffer. This will be your blank.
- In a separate microfuge, add 7.5 µl (exactly) of DNA to the 1.5 ml of TE Blank the spec with TE buffer at 260 nm and 280nM.
- 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.*

14) **Analyze the sample by agarose minigel (see Pages 373 and 380 of At the Bench for background and additional details)**: These gels are useful for separating small amounts of DNA. They can be used to monitor the progress of enzyme action on DNA or the purity of the DNA preparation. Add 5 µl of DNA Sample buffer to your saved samples (10 µl of your pure DNA) and load the whole sample. In your notebook indicate the loading order. Include 5µl of the DNA ladder. Don't forget to write down the masses for the ladder.

- Assemble the gel apparatus
- Melt 0.8 g of agarose in 100 ml 1 x TEA buffer in a 250 ml flask. Use the microwave.
- Add 5µl of 10 mg/ml ethidium bromide stock solution to 100 ml of molten agarose maintained at 50 to 60°C.
- Fill the mold with agarose and immediately insert the comb. Act quickly to remove any air bubbles by touching them with a pasture pipet.
- Allow the gel to solidify at room temperature and gently remove the comb
- Remove the tape/rubber dam and fill the buffer reservoirs with 1xTEA buffer containing ethidium bromide until it just covers the gel
- In a microfuge tube, add 4 µl of tracking dye to 10 µl of sample
- Load the sample
- Run the gel at 80 volts for 1 hour. Remember DNA is negatively charge so it will "run to red" (the positive electrode)
- Visualize and take the picture.

<u>Below is a list of what each buffer is made. A good reference for your future use.</u>

Buffers

<u>Cell Resuspension Buffer</u>

50 mM Tris-Cl, pH 8.0; 10 mM EDTA 100 µg/ml Dnase free Rnase A (good for 6 months add more Rnase if needed) Store at 4°C
1.515 g Tris Base, 0.93 g EDTA add 150 ml deionized H_2O adjust pH to 8.0 and bring final volume to 250 ml with DI water

<u>Cell Lysis Buffer</u>

200 mM NaOH, 1% SDS store at room temp
4.0 g NaOH in 475 ml DI water and 25 ml of 20% SDS. Final volume of 500 ml

Neutralization Solution

3.0 mM potassium acetate, pH 5.
73.62 g K acetate in100 ml DI water. Adjust the pH to 5.5 with glacial acetic acid ~ 25 ml. Final volume of 250 ml Store at room temp

<u>25 x TEA Buffer</u>

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

<u>Equilibration Buffer (Matrix storage buffer)</u>

6 M guanidine hydrochloride.

<u>Wash buffer</u>

80% isopropanol diluted with water. Ethanol can be used but is more expensive.

Decontamination of Ethidium Bromide Solutions and Surfaces

WARNING: EtBr is toxic and mutagenic. Hypophosphorus and its solutions are\corrosive. Decontamination solution gives off a small amount of nitrogendioxide, a toxic gas, when initially mixed.

Laboratory Safety Practices and Equipment:

Prepare decontamination solution in the fume hood.
Wear two layers of gloves, lab coat and safety glasses.
Turn off electrical equipment before decontamination.

Preparation of Decontamination Solution: Prepare fresh solution on day of use. Add hypophosphorus acid to an aqueous solution of sodium nitrite:

	1 liter	2 liters	3 liters
Sodium nitrite	13.6g	27.2g	40.8g
Hypophosphorus acid	67ml	134ml	200ml

Decontamination of EtBr Waste:

Procedure has been validated for EtBr solutions of less than 0.5mg/ml in water, TBE buffer, MOPS buffer and CsCl, gradients. For solutions greater than 0.5 mg/ml, dilute to 0.5 mg/ml with water.

- Liquid waste: Add 1 part decontamination solution to 3 parts EtBr solution (25%).

- Solid waste: Ensure that all waste is submerged in the decontamination solution and in contact with it.
- Mix and hold for 20 hours.
- Neutralize with sodium bicarbonate and discard as non-hazardous aqueous waste for solutions. For solids, seal in plastic bags and discard.

References:

1.Lunn, G. and Sansone, E.B. (1987) Ethidium bromide: destruction and decontamination of solutions. Anal. Biochem 162:453.
2.Lunn, G. and Sansone, E.B. (1989) Decontamination of ethidium bromide spills. Appl Ind Hyg 4:234.