DNA: Isolation, Quantitation and Physical Characterization

Strawberry DNA: Isolation and Characterization

Chromosomal DNA isolation is the foundation of a wide variety of biochemical investigations of DNA. Thus there are many different protocols for the isolation of chromosomal DNA, even chromosomal DNA from the same organism. For our purposes, we will choose a species which is amenable to high yields of chromosomal DNA through a relatively safe and quick process.

The common cultivated or garden strawberry (*Fragaria* × *ananassa*) is a hybrid organism which is octoploid. With eight copies of each of its seven chromosomes (n=7) in each cell, this strawberry is a potent source of DNA. The soft nature of the fruit makes the disruption of the cells relatively easy. With this easy homogenization, not much effort is required to bring the DNA out of aqueous solution. Large DNA molecules are not very soluble in ethanol, and we can isolate strawberry DNA from strawberry homogenate with a simple ethanol "precipitation."

Materials

Lysis Buffer (0.15 M NaCl, 1 % SDS)
TE buffer, pH 8 (10 mM Tris-Cl and 1 mM EDTA, pH 8; see appendix of **DNA Science** for preparation procedure)
Ethanol, 95%, -20 °C
Semi-micro blender, chilled
Vacuum filtration apparatus with Whatman #1 filter paper
2 50 mL conical tubes
15 mL conical tube

Preparing Strawberry DNA

1. Chill the semi-micro blender by placing it in the freezer for 10 minutes.
2. Remove the leaves and hull of one strawberry and cut into smaller pieces.
3. Tare a weigh boat and add strawberry pieces until you have 3 to 5 grams of strawberry flesh.
4. Record the starting mass of strawberry.
5. Add the strawberry to a semi-micro blender along with 10 mL of lysis buffer.
6. Blend the strawberry and lysis buffer for 60 seconds.
7. Allow the pulverized strawberry to rest in lysis buffer for 2 minutes.
8. Vacuum filter the homogenate over Whatman #1 filter paper.
9. Discard the filter paper and fibrous strawberry remnants.
10. Add 30 mL of ice cold ethanol to a 50 mL conical tube.
11. Add the filtrate from the strawberry homogenate to the conical tube of cold ethanol and allow it to sit for five minutes. During this time the DNA will condense (precipitate) and rise to the surface.
12. Transfer the DNA mass (catching it with a spatula works well) to a second 50 mL conical tube and wash with 30 mL of cold ethanol. The purpose of this rinse is to remove red pigment from the fruit. Allow the DNA to sit in this ethanol for five minutes.
13. Remove the DNA to a clean 15 mL conical tube, being sure to allow excess ethanol to drain from the ball of DNA. Dissolve the DNA in 10 mL of TE buffer (this may take a while).

ULTRAVIOLET EVALUATION OF DNA PURITY

A pure solution of double-stranded DNA at 50 μg/ml has an optical density of 1.0 at 260 nm and an OD_{260}/OD_{280} ratio of 1.8. Contamination with protein will give a ratio significantly less than 1.8, and contamination with RNA gives a ratio greater than 1.8. For pure RNA, the ratio equals 2.0.

Dilute the strawberry DNA/TE buffer solution in TE buffer and measure OD_{260} and OD_{280} with a UV/Vis spectrophotometer. You will need to guess at the first dilution and work from there to find the proper dilution. You are looking for an OD_{260} in the range of 0.2-0.8 in order to be able to make a reasonable measurement.

From the concentration of your purified strawberry DNA, calculate the total mass amount of DNA you purified. Compare this number to the mass of strawberry used for the purification. What percentage of the strawberry is DNA?

ESTIMATION OF MOLES %GC OF DNA

The UV absorbance of DNA is due to the aromatic organic bases (purines and pyrimidines). Solutions of 5' deoxyribonucleotides at pH 8 have the following A_{280}/A_{260} ratios: 0.14 (dAMP), 0.73 (dTMP), 0.67 (dGMP), and 0.99 (dCMP). Assuming that these values can be used for the absorption of a nucleotide residue in the context of a polynucleotide chain, one can calculate the A_{280}/A_{260} values for DNA having various base compositions. For example, note the following:

Mole %				
A	**T**	**G**	**C**	**A_{280}/A_{260} (pH 8)**
30	30	20	20	0.592
25	25	25	25	0.632
20	20	30	30	0.672

Note that the A_{280}/A_{260} ratio increases by 0.04 for every increment of 10% in moles %.GC. If you assume that your DNA solution is at pH 8 and that your protein and RNA contaminations are negligible, estimate the moles % GC content of your DNA preparation. If protein were present in your DNA sample, would the calculated moles % GC value be too high or too low? Explain.

SPECTRA OF THE dNMPs

The deoxynucleotide monophosphates (dNMPs) are easily isolated and are easily dissolved in water. You can use the spectrophotometer to measure the absorbance spectra of the free nucleotides to see the contribution of each nitrogenous base to the spectrum of a long, mixed composition DNA molecule.

Using the analytical balance, prepared ~1 mg/ml stock solutions of each of the nucleotide monophosphates in distilled water. Be sure to record the actual masses of each dNMP used, and,

using the molecular weights given below, calculate the concentration of each dNMP in units of μmoles/L (μM).

dNMP	Molecular Weight (g/mol)
2'-deoxyadenosine 5'-monophosphate, sodium salt	331.2
thymidine 5'-monophosphate, disodium salt, hydrate	366.2
2'-deoxyguanosine 5'-monophosphate, disodium salt, hydrate	347.2
2'-deoxycytidine 5'-monophosphate, sodium salt	351.2

Using your stock solutions, prepare 1 ml total volume dilutions of each dNMP at a concentration of 100 μM. Use the Model 14 to measure the absorbance spectra of the dNMPs in self-masking 1 mL semi-micro cuvettes from 200 to 350 nm (the ultraviolet range). The Model 14 is not currently connected to the network, physical media will be useful for moving data files.

Prepare a single plot of absorbance as a function of wavelength for all four dNMPs. Use your data, and the Beer-Lambert Law (A=ε*l*c) to calculate the molar extinction coefficients of each dNMP at 260 nm (*hint: the pathlength of the cuvette is 1 cm, and the typical units of the extinction coefficient,* ε, *are* $M^{-1}cm^{-1}$). Which nucleotide makes the greatest contribution to a DNA molecule's absorbance at 260 nm on a per molar basis? Which the least? Make a qualitative description as to why the DNA spectrum is so smooth and peaks at 260 nm when the spectra of the contributing monomers are so distinct.

The extinction coefficients of the dNMPs have been measured (Cavaluzzi and Borer, 2004). Those values are 15.06 (A), 8.56 (T), 12.18 (G) and 7.10 (C). All extinction coefficients are in units of $mM^{-1}cm^{-1}$. Calculate the percent error of your measurements. *Hint:*

$$PercentError = \frac{(MeasuredValue - AcceptedValue)}{AcceptedValue} * 100\%$$

REFERENCES

Cavaluzzi, M. J., and Borer, P. N. 2004. Revised UV extinction coefficients for nucleoside-5'-monophosphates and unpaired DNA and RNA. *Nucleic Acids Res*. 32, e13.

PLASMID DNA: ISOLATION AND CHARACTERIZATION

PLASMID MINIPREP

In this laboratory, you will isolate plasmid DNA from transformed *E. coli* cells using a "mini-prep" procedure. The plasmid mini-prep is a common laboratory procedure for the isolation of small amounts of DNA. *E. coli* cells taken from an ampicillin-resistant colony are grown to stationary phase in suspension culture. The cells are harvested and lysed, and then the cellular debris and chromosomal DNA is separated from the plasmid DNA. This procedure yields 1-3 micrograms (μg) of intact plasmid DNA.

There are several protocols for the mini-prep and each varies in the method used to lysis the cells. You will be performing an alkaline lysis protocol in which the cells are disrupted by NaOH and a detergent (*hint: see the figure on page 48 in* DNA Science *for an explanation of each reagent's role*). The other common protocol involves the lysis of cell by elevated temperature and is known as the boiling lysis protocol.

MATERIALS

~5 ml *E. coli* suspension (from overnight stationary phase culture)
Glucose/tris/EDTA (GTE, ice cold)
Potassium acetate (KOAc, ice cold)
SDS/NaOH (room temp)
Isopropanol (room temp)
95% ethanol (room temp)
Hair dryer
Tris/EDTA (TE, room temp)
Crushed ice
Microcentrifuge
*(hint: see **DNA Science**, p. 95, for the complete protocol and the solution preparation guide)*

ALKALINE LYSIS PLASMID MINI-PREP PROTOCOL

(Perform the following procedure in duplicate)

1. Using a 1000 μl micropipette, add 1.5 ml of *E. coli* suspension to a microcentrifuge tube. Pellet the cells by centrifugation at full speed in a microcentrifuge. Drain the growth media being careful not to disturb the pellet. To the same tube add another 1.5 ml of *E. coli* suspension and pellet the cells. Repeat this once more so that you have the cells from ~4.5 ml of dense culture in your microcentrifuge tube.
2. Resuspend the pellet in 100 μl of ice-cold Glucose/Tris/EDTA (GTE).
3. Allow the GTE-cell suspension to stand for 5 minutes at room temperature.
4. Add 200 μl of room temperature SDS/NaOH to the tube. Close the cap and mix the solutions rapidly by inverting the tube several times.
5. Let the tube stand on ice for 5 minutes. The contents of the tube will become relatively clear. This is the alkaline lysis portion of the mini-prep.
6. Add 150 μl of ice-cold potassium acetate (KOAc) to the tube. Close the cap and mix the solutions rapidly by inverting the tube several times.

7. Let the tube stand on ice for 5 minutes. White flocculent will form; this is precipitated protein. High molecular weight DNA also becomes trapped in the SDS/protein layer.
8. Centrifuge the tubes at high speed in a microcentrifuge for 5 minutes. The white material will pellet on the side of the tube.
9. Use a micropipette to remove the supernatant from the tube and transfer to a clean microcentrifuge tube. Be careful not to disturb or remove any of the pellet.
10. Add 400 µl of isopropanol to the supernatant. Close the cap, and mix vigorously by rapidly inverting tubes. Let stand at room temperature for *only* 2 minutes. (It is important to do this entire step quickly. Isopropanol precipitates the nucleic acids in solution quite rapidly; however, after some time it will also begin to precipitate any proteins that are remaining in the solution.)
11. Centrifuge for 5 minutes. This pellets the nucleic acids which will appear as a small translucent pellet (*hint: since the pellet is subtle, or invisible, it is helpful to place the microcentrifuge tube into the rotor in the same orientation each time. This will allow you to know where the pellet is, even if you can't see it.*).
12. Being careful not to disturb the pellet, pour off the supernatant, invert the tube over a paper towel and tap gently on the bench to drain.
13. Add 200 µl of 95% ethanol and cap. Flick the tube several times to wash the pellets (*hint: DNA is not soluble in ethanol; this will not resuspend the pellets. If you are ready to stop, you can safely freeze the sample at this point.*).
14. Spin the tube for 2 minutes.
15. Being careful not to disturb the pellet, pour off the ethanol, invert the tube over a paper towel and tap gently on the bench to drain.
16. Direct a stream of hot air from hair dryer into the open ends of the tubes for about 3 minutes. *(caution: Do not blow DNA chip out of tube or bake the DNA. The hot air evaporates remaining ethanol. It is important that all ethanol is evaporated before going on to the next step. If there is still ethanol evaporating you can usually detect an alcohol odor when you sniff the opening of the tube.)*
17. Add 50 µl of Tris/EDTA (TE) to the tube and resuspend the pellet by smashing with a pipet tip and pipeting in and out vigorously. Check carefully to see that all DNA is solubilized and that no solid DNA is stuck in tip or on side of tube (*hint: If you are ready to stop, you can safely freeze the sample at this point.*).

PURIFICATION OF PLASMID DNA BY SOLVENT EXTRACTION AND QUANTITATION

Plasmid DNA can be further purified to remove protein contaminates by solvent extraction. The following protocol will use phenol to remove contaminating proteins. Phenol must be of high quality so as not to damage DNA. Phenol will burn the skin and should be used with gloves under the hood.

With your plasmid DNA samples, reserve one as it is and use the other for phenol/chloroform extraction. These samples will then be assayed for purity using the UV spectrophotometer. The primary measure of purity will be the OD_{260}/OD_{280} ratio used early. Pure DNA will have OD_{260}/OD_{280} ratio of 1.8. Protein contamination will lower this ratio; RNA contamination will raise it. Compare the mini-prep sample to the phenol/chloroform extracted sample. Did the phenol/chloroform extraction eliminate protein?

MATERIALS

Chloroform (mixture of chloroform and isoamyl alcohol [24:1 (v/v)])
Ethanol (95%) at -20° C
Phenol
TE buffer (pH 8)

SOLVENT EXTRACTION PROTOCOL

1. Mix 50 µl of the mini-prep of plasmid DNA sample in a microcentrifuge tube with 50 µl of TE buffer (pH 8) to obtain a workable volume.
2. To this mixture add 100 µl of phenol. This organic solvent serves to denature and extract protein. (Phenol is in the freezer in a brown bottle. Scrape some phenol crystals into a small beaker and warm gently to liquefy prior to use)
3. Mix the contents by inverting the tube gently several times until an emulsion forms. This avoids the breakage of DNA that occurs by shear forces generated in vortexing and violent stirring.
4. Centrifuge in the micro centrifuge for 20 seconds at top speed.
5. With a micropipettor, transfer the upper aqueous phase to a clean microcentrifuge tube.
6. Reextract the remaining lower organic (phenol) phase and interphase in the original tube by adding 100 µl of TE buffer. Mix by inversion. Centrifuge as in Step 4. Collect the aqueous phase and combine with the first aqueous phase collected.
7. Extract the combined aqueous phases by adding about 100 µl of phenol and 100 µl of chloroform. The chloroform also serves to extract and denature protein. Mix by inversion, Centrifuge as in Step 4, and transfer the upper phase to a clean microcentrifuge tube.
8. Extract the upper aqueous phase from Step 7 with 200 µl of chloroform only. Mix, centrifuge, and collect the upper phase that contains purified DNA, which should be stored at 4 ^{o}C.

CONCENTRATION OF PLASMID DNA

MATERIALS

Ammonium acetate (10 M)
Ethanol (95%) at -20 ^{o}C
TE buffer (pH 8)

CONCENTRATION PROTOCOL

1. Adjust the concentration of the DNA solution by adding ammonium acetate to make a final solution of 2 M ammonium acetate. This is done by estimating the volume of the DNA sample, which is in TE buffer (pH 8). This is necessary to allow precipitation of DNA by ethanol.
2. Add 2 volumes of ice-cold 95% ethanol. Mix and put on ice for 30-60 minutes. (If the DNA is smaller than 1 kb, or it is present at a concentration of less than 100 ng/ml, the solution should be stored at -70°C for about 4 hours. For DNA less than 0.2 kb, the addition of 10 mM $MgCl_2$ improves recovery.)
3. Centrifuge at 4 ^{o}C for 10 minutes in the microcentrifuge at top speed.

4. Discard the supernatant. Invert the tubes on a layer of absorbent paper to allow drainage of ethanol. Solvent traces can be removed in a vacuum desiccator or vacuum centrifuge.
5. Dissolve the pellet in 50 µl of TE buffer (pH 8). Rinse the tube walls with buffer to ensure dissolution of DNA. Heating to 37 ºC for at least 5 minutes may help get DNA in solution.
6. Store the DNA solution at 4 ºC. This phenol/chloroform extracted mini-prep DNA will be compared in the following section to mini-prep DNA that has not been further purified.

QUANTITATION PROTOCOL

Remembering that a pure solution of double-stranded DNA at 50 µg/ml has an optical density of 1.0 at 260 nm and an OD_{260}/OD_{280} ratio of 1.8, collect the UV spectrum of both plasmid DNA samples. A reasonable starting point might be diluting your plasmid preparations 1:100 in TE buffer. If this solution is too concentrated, you may have to dilute further, if too dilute, you may have to make a more concentrated sample. Calculate the concentration of DNA in each preparation. Calculate the purity of each preparation. Did the phenol/chloroform extraction increase the purity of your sample? How much, if any, DNA was lost during the phenol/chloroform extraction?

REFERENCES

Lunn, E. G., and Sandstone, F. 1987. Ethidium bromide: Destruction and decontamination of solutions. *Anal Biochem*. 162, 453-8.

Sambrook, I., Fritsch, E. F., and Maniatis, T. 1989. **Molecular Cloning: A Laboratory Manual, 2nd Ed.** Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

THE STRUCTURE AND STABILITY OF THE DNA DOUBLE HELIX

Read the paper Watson JD and Crick FHC. 1953. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. *Nature*. **171:** 737-8. This is the landmark paper suggesting the double helix structure for DNA. From your reading of this paper, answer the following questions:

1. The X-ray data showed that the double helix has a constant diameter. How would Watson and Crick reconcile this finding with the fact that purines and pyrimidines have different sizes?
2. How were they helped in this view by Chargaff's work?
3. Why did Watson and Crick conclude that the strands are antiparallel?
4. How did they calculate the number of bases per turn of the helix?
5. What evidence suggested a double helix?

Read the introductory article to a series of papers on DNA strand separation entitled "Hydrophobic Forces in the DNA Molecule" in Freifelder D. 1978. **The DNA Molecule: Structure and Properties, Original Papers, Analyses and Problems**. W.H. Freeman Co. San Fransisco, California, pages 265-72. From this paper, remembering the molecular forces, answer the four questions given at the end of that article on pages 271-2.

After reading "Hydrophobic Forces in the DNA Molecule", look at figures 1 & 2 (thermal dentaurations of DNA) in the following paper (Marmar J and Doty P. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol*. **5**: 109-18, page 275 in Freifelder's book) as you will be designing similar experiments using commercially purified DNA. These figures are excellent illustrations of the hyperchromic effect visible on DNA strand separation with increasing temperature.

THERMAL AND CHEMICAL STABILITY OF DNA

The DNA double helix can be melted by increasing the temperature of a DNA solution. This strategy is used during PCR to produce strand separation in preparation for priming. The process of strand separation is easily observed in the spectrophotometer at 260 nm. Double strand DNA, with bases tightly packed, absorbs less UV light than the same molecules in the single strand form; this is known as the hypochromic effect. As a DNA double helix melts, UV absorbance increases and the increase in absorbance is the hyperchromic effect.

MATERIALS

Oligonucleotide GGACGTCC, stock soln at ~ 2500 μg/ml
TE buffer

PROTOCOL

Using the information presented in "Hydrophobic Forces in the DNA Molecule", design two protocols to investigate the melting of the DNA double helix. First convince yourself that the oligonucleotide GGACGTCC will, upon heating, form duplex DNA. Ok, believe it? The melting temperature of this duplex is 23.9 ^{o}C. You will have the opportunity to perform, at most, ten individual thermal denaturations, five experiments each week (be sure you start these late in the day as other experiments depend on the Model 14 for data collection). The thermal denaturation cuvettes require 3000 μL in total sample volume. A 100 fold dilution of the stock solution should produce a reasonable absorbance at 260 nm. What absorbance will you expect in your cuvettes at low temperature? The basic design then is to melt the duplex in dilute buffer, and compare that melting curve to similar curves collected in slightly different solution conditions. Likely choices for your experiment are the addition a cosolvent such as guanidine hydrochloride (GdnHCl) *(hint: the solubility limit of GdnHCl is about 6 M)* or urea (*hint: the solubility limit of urea is 10.49 M)* to your DNA solution, a change of pH (including buffer), the addition of a salt *(hint: the solubility limit of NaCl is about 4.8 M, but the exciting effects happen at much lower concentrations, see the concentrations in Marmar & Doty)* or the addition of a less polar cosolvent such as methanol, ethanol or propanol. For any of these variables you choose to examine, thermal denaturations in different salt concentrations are illustrated in the figures of the Marmar & Doty article which will

serve as a template for your data. The key point in each of these curves is the midpoint of the transition, in the neighborhood of 24 ºC for the duplex in question. The higher the temperature at the midpoint, the more stable the DNA duplex (note that Marmar & Doty are using a purified DNA molecule from a microorganism. The transitions you see there are very sharp, that is they are complete over just 10 ºC. This is a good way to qualitatively demonstrate the effects of the variable on the duplex DNA structure. In some cases, the change will stabilize the double helix; in others the double helix will be destabilized. Is the double helix more or less stable (or unaffected) by the variables you have chosen? What feature of your data suggest the answer to the previous question? Provide a physical interpretation of the action of the molecule you chose on the double helix. Does your experiment have any physiological relevance to humans or other organisms?