

## ISOLATION OF GENOMIC DNA

Isolation of genomic DNA from plants is more difficult than from animal cells. Plant tissues, with thick cell walls and high levels of phenolic compounds, have proven more challenging to recover high molecular weight DNA from than either bacteria or mammalian tissues. The thick cell walls must be broken effectively to release the DNA. However, the mechanical breakage method cannot be so vigorous that it leads to shearing of the released DNA. Similarly, once the cells are broken, partitioning of extracted phenolic materials and chlorophyll away from the DNA must occur without damage to the high molecular weight DNA. The method described below, which you will follow, is one of several (1,2) that have proven effective. One way to minimize both of these problems is to use young etiolated (grown in the dark) seedlings. The walls are less developed, and the cells have lower phenolics and chlorophyll.

Genomic DNA is isolated for various purposes including construction of genomic libraries, PCR amplification of specific sequences and to search for the presence of specific genes or sequences in the genome by Southern blot analysis. Your objective will be to isolate clean, high molecular weight DNA from tobacco leaf tissue. Once the genomic DNA has been isolated you will quantify the amount of DNA recovered. Small samples of the DNA will be digested and the fragments generated will be examined by agarose gel electrophoresis. You will also use your prepared DNA to PCR amplify a selected gene sequence during the second week.

### Extraction of Genomic DNA from Plant Tissues

#### WEEK #1

- 1) Weigh 0.5 g of tobacco leaf tissue. Place into a mortar with a “pinch” of fine grinding sand and grind the tissue to a fine paste - the finer the better!
- 2) Immediately add 2.0 ml of hot (65°C) CTAB buffer and grind the tissue again.
- 3) Transfer each sample of homogenate to a 15 ml polypropylene tube and incubate the tubes at 65°C for 20 minutes. At 5 min intervals gently mix the extraction mixture by several inversions. (Vigorous mixing will shear extracted DNA.)

#### CTAB buffer

2 % CTAB (cetyltrimethyl ammonium bromide)  
50 mM Tris-HCl, pH 8.0  
20 mM EDTA (ethylenediamine tetraacetic acid)  
1.4 M NaCl

NOTE: ALL STEPS INVOLVING CHLOROFORM ARE DONE IN THE FUME HOOD

- 4) Add an equal volume of chloroform:isoamyl alcohol (24:1). Mix for about 5 min by repeated inversion. This step will result in the denaturation of protein and extraction of

any chlorophyll into the organic phase.

- 5) Centrifuge at 10,000g for 5 min at RT . Transfer the upper aqueous phase to a clean 15 ml tube using a pipette (A wide mouthed pipette combined with slow uptake and expulsion will minimize shearing of the high molecular weight DNA).
- 6) Repeat the chloroform extraction once. Transfer the aqueous phase to a clean 15 ml tube.
- 7) To the aqueous phase add 0.4 volume of 5 M ammonium acetate, (mix gently) and 2 volumes of isopropanol (again mix gently). This procedure should cause the precipitation of the DNA without precipitating protein. After 15 min on ice the DNA is recovered by centrifugation at 12000g for 10 min. Wash the resulting pellet in 70% ethanol (500  $\mu$ l) to remove the isopropanol. Remove ethanol and allow tube to drain and air dry.
- 8) Redissolve pellet in 100  $\mu$ l of TE buffer and transfer to a microfuge tube.

# Genomic DNA Part II

## Quantitative Analysis, Digestion and Selective Amplification

Before proceeding to enzyme digestion and PCR amplification you will need to have a good estimate of the amount of DNA in your sample. The quality of DNA can be measured by a variety of techniques but will ultimately depend on what the intended use of the DNA will be. The purity of the DNA preparation, un-complexed with polyphenolic material and free of carbohydrates, is critical to enzymic manipulation or PCR amplification of the DNA.

WEEK #2

### Quantitative Analysis of DNA

**Spectrophotometric method** The amount of DNA will be estimated by absorbance at 260 nm. Past experience has shown that you should recover approximately 100 ug of genomic DNA from the 2.5 g of starting tissue, however an accurate value is required. Assume your DNA has been dissolved in 100 µl of water. Calculate in advance of coming to lab what dilution you will need to make to obtain an accurate estimate of the DNA concentration of this DNA solution ie. calculate the dilution needed to get an absorbance (260 nm) of approximately 0.1.

Verify with the demonstrator how you will prepare 700 µl of the appropriate dilution before actually making it. You will be shown how to run a scan of your sample with a Nanodrop spectrophotometer to measure the absorbance at 230, 260 and 280 nm. This measurement may be made later in the lab period.

### EcoRI Digestion of Genomic DNA

- 1) From the concentration you determine in step 3), calculate the volume that will contain 5 ug of genomic DNA and carefully transfer this volume into a clean and labelled microcentrifuge tube. This DNA will be digested with the restriction enzyme EcoRI. Label (name, tube1/tube2) and prepare digests as follows in the order given:

Sterile water	35-N µl
DNA (your sample)	N µl
10x EcoRI Digestion buffer	4 µl
EcoRI enzyme (10 units/ul)	1 µl
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	40 ul

- 2) Mix the components carefully and incubate the digests at 37°C for 1 hour.

Prepare a full sized agarose (1.6 g) gel in TAE (0.04 M Tris-acetate, 0.001 M EDTA) electrophoresis buffer (200 ml) containing 0.5 ug/ml ethidium bromide. Follow the same procedure you used previously to prepare the mini-gel for the analysis of your plasmid

preparation and allow it to gel while you wait for the digests.

- 3) When the digestions are completed add 5  $\mu$ l of agarose gel loading buffer ( contains 0.25% bromophenol blue and 0.25% xylene cyanol as tracking dyes and 40% (w/v) sucrose to make the solution dense to facilitate loading). Stir briefly to ensure complete mixing and centrifuge for 10 seconds to drive all the liquid to the bottom of the tube. Carefully load the complete digest into the wells in a recorded manner.
- 4) Connect the electrophoresis apparatus to the power supply in the correct orientation and adjust the voltage to a constant output of 30 volts. This will be allowed to run overnight to separate the DNA fragments in terms of size.
- 5) The following morning the power will be turned off and the gel examined on a UV light box. A photograph may be taken to record the results but you are encouraged to examine the gel yourself sometime the next day.

### Polymerase Chain Reaction

The PCR technique allows a unique piece of DNA to be amplified through a cyclic reaction involving a thermostable DNA polymerase, two primer molecules and the four deoxynucleotides. If template DNA (dsDNA sequence containing the regions complimentary to the two primers) is present in the reaction mixture then a product of defined size should be produced. If the DNA which is present does not contain the appropriate sites for primer annealing within  $\sim$ 3000bp of each other no products should be produced. The presence of an appropriate sized product can be demonstrated by separating the reaction products on an agarose gel (along with standard DNA sample markers of a known size) and visualizing the products with ethidium bromide. (For small products,  $<$ 300bp, a polyacrylamide gel is normally used)

In this test you are attempting to verify the presence of the neomycin phosphotransferase II (NPT II) gene and of the  $\beta$ -glucuronidase (GUS) gene in your tobacco genomic DNA preparation. The NPT II gene produces a product which is able to phosphorylate kanamycin and thereby detoxify it. The GUS gene is a representative target gene in pBI121 that should be present in successfully transformed tobacco plants. The sequence of the gene and the location of the two primers you will be using are shown in Figure 1. Primers are typically 10-25 nt long with longer primers providing a high degree of selectivity.

### **Procedure**

1 From the concentration you determine, calculate the volume you will need to contain 100 ng of DNA. You may have to dilute your DNA in order to have a workable volume (a volume less than 1  $\mu$ l cannot be measured accurately). A good target concentration would be 10 ng/ $\mu$ l allowing a 10  $\mu$ l volume to be used.

2) Label the top of two 0.2 ml microcentrifuge tube with your name and add your genomic DNA sample (100 ng in 10  $\mu$ l) to each. Label one tube **N** (for NPT II) and label the second tube **G** (for GUS). Place on ice.

3) To minimize pipetting error and simplify the addition process a master mix containing all necessary buffers and reagents has been prepared and its relative composition is shown below. Add 40  $\mu$ l of the appropriate master mix to your tube, (always use a new pipet tip to sample from the master mix to avoid contaminating the master mix). **Important:** Keep your reaction tubes on ice!

<b>Master mix</b>	<b>Composition per 50 <math>\mu</math>l Reaction</b>
10x PCR buffer	5.0 $\mu$ l
25 mM dNTP	0.4 $\mu$ l
25 mM MgCl <sub>2</sub>	0.75 $\mu$ l
primer 1 (20 pmoles/ $\mu$ l)	1.0 $\mu$ l
primer 2 (20 pmoles/ $\mu$ l)	1.0 $\mu$ l
Taq polymerase (5 units/ $\mu$ l)	0.5 $\mu$ l
sterile H <sub>2</sub> O	30.6 $\mu$ l

4) Master Mix **N** contains primers for the NPT II gene while Master Mix **G** contains primers specific for the GUS gene.

5) Place tubes in thermocycler. The cycle you will run is:

95°C	3.0 minutes	
95°C	0.5 minutes	
58°C	0.5 minutes	40 cycles
72°C	1.0 minutes	
Hold at 4°C		

This reaction will take about approximately 3 hours to run to completion. Your samples will be analysed on a 1% agarose minigel with ethidium bromide. A copy of the photo of this gel will be supplied for you to determine the result of your PCR reaction.

## References

### DNA extraction

1. S.O. Rogers and A.J. Bendich, 1988. Extraction of DNA from plant tissues. Plant Molecular Biology Manual A6: 1-10.
2. M.G. Murray and W.F. Thompson, 1980. Rapid isolation of high molecular weight plant

DNA. Nucleic Acid Research 8: 4321-4325.

PCR

The Polymerase Chain Reaction. Ed K.B. Mullis, F. Ferre, R.A. Gibbs. 1994 Birkhauser Boston

## **Discussion**

Consider in looking at the electrophoresis results that the original chromosomes were greater than 100,000,000 bp long. The more streaking of the DNA which occurs on the gel the greater is the degree of degradation.

Calculate the recovery ( $\mu\text{g}/\text{leaf tissue}$ ) and relative purity (260/280 ratio). Discuss these values and the purity criteria you use in making your judgement. Discuss the isolation procedure and your results in terms of the goal of obtaining clean, high molecular weight DNA and whether or not you feel the DNA sample you have prepared would be suitable for creating a genomic library.

Also answer the following questions as part of your discussion of the experiment.

Do your PCR results confirm that your tissue is transformed?

What would be the effect of having the annealing temperature (the low temperature in the cycle) lower than the optimum? Discuss.

What are the appropriate PCR control reactions that should be run to ensure that the presence of a band on a gel is indicative of the expected product?

Using the attached sequence information (Figure 1b) for NPT II provide the sequences for primer #1 and primer #2 assuming perfect complementation.

Primer 1 is **complementary** to the lower strand of the NPT II gene from bp#11 to bp#31.  
Primer 2 is **complementary** to the upper (coding) strand of the NPT II gene from bp#766 to bp#786.

### Sequence of Neomycin Phosphotransferase II

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1   agaactcgtc aagaaggcga tagaaggcga tgcgctgcga atcgggagcg
51  gcgataccgt aaagcacgag gaagcgggtca gccattcgc cgccaagctc
101 ttcagcaata tcacgggtag ccaacgctat gtcctgatag cgggccgcca
151 caccagccg gccacagtcg atgaatccag aaaagcggcc atttccacc
201 atgatattcg gcaagcaggc atcgccatgg gtcacgacga gatcctcgcc
251 gtcgggcatg cgcgcttga gcctggcgaa cagtccggct ggcgagagcc
301 cctgatgctc ttcgtccaga tcctcctgat cgacaagacc ggcttccatc
351 cgagtacgtg ctcgctcgat gcgatgtttc gcttggtggt cgaatgggca
401 ggtagccgga tcaagcgtat gcagccgccg cattgcatca gccatgatgg
451 atactttctc ggcaggagca aggtgagatg acaggagatc ctgccccggc
501 acttcgcca atagcagcca gtcccttccc gcttcagtga caacgtcgag
551 cacagctgcg caaggaacgc ccgtcgtggc cagccacgat agccgcgctg
601 cctcgtcctg cagttcattc agggcaccgg acaggtcggt cttgacaaaa
651 agaaccgggc gccctgctgc tgacagccgg aacacggcgg catcagagca
701 gccgattgtc tgttgtgccc agtcatagcc gaatagcctc tccaccaag
751 cggccggaga acctgcgctgc aatccatctt gttcaatcat
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