# Chapter 11

# Forensic Biochemistry: DNA Testing of Plant Material

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## Introduction

This laboratory exercise is used for a survey course in Biochemistry for undergraduates in the school of Agriculture. Originally we did this experiment with plants grown from seeds and used the CTAB isolation method, which Clint Chapple was using in his research lab. After using this exercise for several semesters we found that the research labs were using newer methods, namely the kit described in Appendix B. Also, we found that the seeds could be planted and grown in petri dishes a week earlier than the seedlings used for the DNA isolation by either method. If the instructor is not familiar with the procedures used here, the preparation may take much longer. Also, if the instructor has access to already prepared solutions when only small amounts are needed, preparation time will be less. The students will take about two and one-half hours to prepare, mark, and add 5 µL of the DNA samples to the PCR tubes so that the instructor can run the PCR. It helps if the students have had an introduction to DNA and PCR in the lecture section, but it can be covered in the laboratory section if necessary.

## List of Materials (for CTAB procedure)

*Part 1, Extraction of DNA:*

- Plants or seeds for Lansberg and Columbia Arabadopsis

Equipment:

- Microcentrifuge tube pestle, 6 per pair

- CTAB Isolation Buffer, 600 µL per pair
- CTAB Wash Buffer, 1.20 mL per pair
- TE, 600 µL
- Isopropanol – keep in freezer, 360 µL
- Chloroform:isoamyl alcohol (24:1, v/v), 600 µL
- Microfuge tubes, 1.5 ml and 0.5 ml, 7 each
- Microcentrifuge
- Pasteur pipettes and bulbs, 6
- 60°C water bath
- Adjustable pipettors, 200 and 20 µL size, 1 each

*Part 2, PCR:*

- 10X PCR buffer (500mM KCl, 100mM TrisCl pH 9.0, 0.1% Triton X-100), 30 µL
- 10X Mg++ solution (15mM $MgCl_2$), 30 µL
- 10X dNTP solution (2 mM each dATP, dCTP, dGTP and dTTP), 30 µL
- 10X Forward primer (50mM oligonucleotide), 6 µL
- 10X Reverse primer (50mM oligonucleotide), 6 µL
- Enzyme (5 U/µl or 2 U/µl Taq DNA Polymerase), 3 µL

*Part 3, Analysis of PCR product by agarose gel*

- 50x TAE (Tris-acetate EDTA)
- 242 g Tris
- 57.1 mL glacial acetic acid
- 100 mL 0.5 M EDTA
- Cold distilled water for TAE or TBE dilution
- 1X TAE for running buffer. Keep COLD! (Can also use 1X TBE buffer which will not get as hot.)
- 4% agarose in 1X TAE + 0.1% EtBr
- Gel loading dye
- 100 bp DNA ladder
- Gel apparatus and power source
- 10X TBE buffer (Tris Boric Acid Buffer).

## Student Outline

### Introduction to the two-week exercise

Forensic biochemistry is the application of standard biochemical techniques and assays to criminal investigations. In many cases, very simple techniques can be employed to determine a great deal of information about the chemical nature of a sample of material that has been collected as evidence at a crime scene. In fact, for many of these tests, it is important that the tests are simple and the results reliable. After all, in the context of a criminal investigation, the results obtained from these tests may have a dramatic impact on the life of a person accused of a crime. In this laboratory exercise, we'd like to give you the opportunity to perform some tests on "evidence" to determine who may have committed the crime in question.

Let's consider this scenario: The body of a male, the obvious object of foul play, is found in a farmer's cornfield in southern Indiana. The police cordon off the site and collect material that they think may help them identify the person who may have committed the crime.

Beside the body is found an empty can of regular (not diet) soda. No fingerprints are found on the can, but is highly unlikely that it belonged to the person killed since he was diabetic. Perhaps the can of soda belonged to the murderer. If so, it suggests that the murderer prefers regular soda to diet soda.

The cornfield in which the body was found was recently fertilized. The police deduce that it might be useful to collect soil samples near the body. Plant fertilizers commonly contain high levels of phosphate to improve plant growth. If the murderer had walked through this field, he or she may have picked up samples of this phosphate-rich soil in the treads of their shoes.

Through a number of tips and investigations, the police identify four suspects living in the Lafayette area. After obtaining the appropriate warrants, the police search the automobiles of each of the suspects. From each vehicle the police collect samples of the dirt found in the driver's side footwells. In each vehicle they are also lucky enough to find a cup from McDonalds that contains a few milliliters of residual soda.

Your task is as follows. First, analyze soil samples for fertilizer (as inorganic phosphate) taken from the shoes of each suspect and from near the body. Second, determine whether the soda in the cup recovered from the suspect's vehicle is diet or regular by analyzing it for the presence of the reducing sugars glucose and fructose that are found in the corn syrup often used to sweeten soft drinks.

*The suspects:*

- Suspect 1 looks rather suspicious. His name is Bill Power. He recently arrived in Lafayette and was hired as a bag boy at the local grocery near campus.

- Suspect 2 is a mild-mannered looking fellow. His name is Bjorn Olaf and is the president of a local smelt packing plant.

- Suspect 3 is John Smith and he sells used cars at a local dealership. He was recently fired for unscrupulously selling an automobile for its actual retail value.

- Suspect 4 is Dr. David Franklin. He is a local podiatrist and runs his hobby farm in his spare time.

In the first week of this exercise, we analyzed the soil samples found with the body and suspects by making a standard curve for phosphate, and finding out which suspect samples matched the amount of phosphate in the field. Then we tested the soda samples for reducing sugars to find out which samples were diet soda. Finally, the seeds found were grown in pots and we isolated DNA from the leaves.

In our first labs dealing with forensic biochemistry, you analyzed samples of soil and soda in an attempt to associate one specific individual with evidence found at a crime scene. The results from these experiments incriminated two of the suspects. In this lab, you will conduct further investigations in an attempt to accumulate more evidence that either incriminates or vindicates each of the suspects

*Seeds and Plants*

The cornfield in which the body was found is in southern Indiana near Evansville, while all of the suspects live in the Lafayette area. All of the suspects have denied having visited Southern Indiana in the past 6 months. Between the county road that runs alongside the field and the site where the body was found, the police identified a large patch of a common weed that grows throughout the state. The plants had completed their life cycle and were covered in dry, ripe seeds. In each of the soil samples recovered from the driver's side footwells of the suspects' cars were found seeds of the plant. This was not surprising to the police since the plant is so common, and at the time the crime occurred, this weed was setting seed throughout Indiana. Fortunately, this weed has been studied in some detail by researchers at Purdue University, and it is known that different varieties or "ecotypes" grow in different areas of the state. The "Columbia" ecotype grows in the upper half of the state, while the "Landsberg" ecotype is restricted to the southern third of Indiana. These two ecotypes show DNA differences that can be detected using the polymerase chain reaction (PCR). If it could be shown that the seeds found in one of the suspects' cars were of the "Landsberg" ecotype, this would strongly suggest that he had recently visited the southern portion of the state, and could be the murderer.

Your task is as follows: You will be provided with seedlings grown from the seeds found in the suspects' cars, as well as seedlings of authentic "Columbia" and "Landsberg" ecotypes. You will extract DNA from the seedlings using a CTAB DNA isolation procedure, and precipitate the DNA from solution using isopropanol. After drying the DNA, you will dissolve the DNA in buffer, and amplify specific regions of the DNA by PCR. Finally, you will analyze the PCR products by gel electrophoresis and compare them to determine whether the seeds found in the cars belonging to any of the suspects were from the ecotype growing in southern Indiana.

## PART 1: Extraction of plant DNA by CTAB

DNA can readily be extracted from tissues by grinding in a solution containing detergent. In many cases, other components are also added to the extraction solution to prevent the action of nucleases that would otherwise degrade the extracted DNA into nucleotides. Once extracted, DNA can readily be precipitated from solution by the addition of cold ethanol or isopropanol. The pelleted DNA is thereby separated from most of the other cellular components that remain in the supernatant.

You will find the following solutions in the supply area.

- CTAB Isolation Buffer
- CTAB Wash Buffer
- TE

*Procedure:*

1. Preheat CTAB Isolation Buffer to 60°C in a water bath.
2. Label six 1.5-mL microcentrifuge tubes as follows: Col, Ler, 1, 2, 3, and 4.
3. Obtain plant tissue samples for DNA extraction. For each sample to be extracted, 2 - 4 leaves should be sufficient.

4. Obtain leaves corresponding to the two authentic samples of the Columbia and Landsberg genotypes, and place them in the tubes labeled *Col* and *Ler*, respectively.
5. Obtain leaves corresponding to each of the four suspects and place them in the tubes labeled *1* through *4*.
6. Add 100 µL of pre-heated CTAB Isolation Buffer to each of the six tubes and grind the leaves in each of the tubes in the waterbath for 30 seconds using six microcentrifuge tube pestles. Once you have finished grinding the plant tissue, leave the pestles in the microfuge tubes.
7. Incubate the sample at 60°C for 30 minutes with gentle stirring of the sample every 5 minutes using the pestle. At the end of the 30 minutes, remove the pestles from each of the tubes and wash them carefully with soap and water.
8. Add 100 µL of chloroform:isoamyl alcohol (24:1, v/v) to each of the samples *(please be careful with chloroform; it is a strong organic solvent and is potentially hepatotoxic)*. Cap each of the microcentrifuge tubes, and mix the contents by shaking the microcentrifuge tube vigorously several times.
9. Centrifuge the samples in the microcentrifuge at maximum speed for 5 minutes.
10. Label another set of six 1.5-mL microcentrifuge tubes as follows: Col, Ler, 1, 2, 3, and 4.
11. Remove the upper, aqueous layer from each of the samples and transfer it to a similarly-labeled new microfuge tube. Although you should try and transfer as much of the upper phase as possible, it is not necessary to remove all of the upper phase, and you must be sure not to contaminate your sample with any of the lower (chloroform:isoamyl alcohol) phase.
12. Set aside the old tubes containing the lower chloroform:isoamyl alcohol phase. These samples must be disposed of appropriately because chloroform is a halogenated compound.
13. Add 60 µL of cold isopropanol to the each of the new tubes containing the upper phase. Cap the tubes and mix the samples by gently inverting the tubes several times.
14. Incubate the samples at room temperature for 30 minutes. During this time, the DNA will precipitate from solution, although it may not be visible.
15. Centrifuge the samples at room temperature in a microcentrifuge at maximum speed for 5 minutes.
16. Remove as much of the supernatant as possible using a pipette. A pellet may or may not be visible at the bottom of the tube.
17. Add 200 µL of CTAB Wash Buffer to each of the tubes. Recap the tubes and invert them several times to wash the pellet. Incubate the samples for 20 minutes at room temperature, inverting the tubes every 5 minutes.
18. Centrifuge the samples in a microcentrifuge at maximum speed for 5 minutes. Using a pipette, remove as much of the supernatant as possible.
19. Dry the pellet in a microcentrifuge by centrifuging for approximately 3 minutes *without capping the tubes*. If the pellets are not dry at the end of this treatment, repeat the centrifugation until the pellet is dry.
20. Dissolve the DNA pellet in 100 µL TE. Mix the sample carefully with a yellow pipette tip until the pellet is fully suspended.

**Part 2: Polymerase chain reaction (PCR) amplification of SSLP markers**

The DNA that you isolated in Part 1 can now be used as a template for the polymerase chain reaction. In this procedure, a small region of DNA is amplified *in vitro* using a heat-stable DNA polymerase isolated from the archaebacterium *Thermus aquaticus*. The amplified DNA can be used for a number of purposes, including cloning and sequencing.

In this lab, you will separate the amplified DNA by gel electrophoresis and visualize it by ethidium bromide staining. The primers that you used for amplification were designed to amplify a region of the plant genome that lies between two genes. Because this region of the genome does not encode a specific protein, there are fewer evolutionary constraints on its sequence and its length. In other words, this region of the genome can be more variable than those regions that encode proteins (*i.e.,* genes). These variations can be used to generate molecular markers that can be used to distinguish between species or varieties of animals, plants, or fungi. The region that you have amplified contains a simple sequence length polymorphism (SSLP) between the Columbia and Landsberg ecotypes. In this type of molecular marker, the number of nucleotides between the PCR primer sites differs. Thus, the length of the DNA amplified using the primers that flank this polymorphism will also differ. This difference can readily be visualized following agarose gel electrophoresis. (See Appendix A for a brief introduction to Polymerase Chain Reaction.)

*Procedure to prepare DNA for PCR*

The following solutions will be available in the lab for you:

- 10X PCR buffer (500mM KCl, 100mM TrisCl pH 9.0, 0.1% Triton X-100)
- 10X Mg++ solution (15mM $MgCl_2$)
- 10X dNTP solution (2 mM each dATP, dCTP, dGTP and dTTP)
- 10X Forward primer (50mM oligonucleotide)
- 10X Reverse primer (50mM oligonucleotide)
- Enzyme (2 U $\mu L^{-1}$ Taq DNA Polymerase)

1. Obtain six 0.5-mL thinwall microcentrifuge tubes. Label them with your group number, followed by Col, Ler, 1, 2, 3, and 4.
2. Add 5 µL of the DNA sample labeled *Col* to the 0.5-mL tube labeled *Col*. Repeat for each of the remaining DNA samples prepared in Part 1, placing each sample in the appropriately-labeled tube.
3. Give your samples to your TA. When the thermocycler is available, they will add the following reagents to each of the tubes.
    - 5 µL PCR buffer
    - 5 µL $Mg^{2+}$ solution
    - 5 µL dNTP solution
    - 5 µL Forward primer
    - 5 µL Reverse primer
    - 0.5 µL Enzyme mixture
    - 19.5 µL $H_2O$
4. Once these solutions have been added, your TA will put the tubes in the thermocycler.
5. The following temperature program will be used:

Step 1
94°C, 2 minutes

Step 2 (repeated 40 times)
94°C, 30 seconds
55°C, 60 seconds
72°C, 30 seconds

Step 3
72°C, 15 minutes

6. Your samples will be ready for you to analyze by electrophoresis next week.

**PART 3: Analysis of PCR-amplified SSLP markers by agarose gel electrophoresis.**

A common technique used in the analysis of nucleic acids is gel electrophoresis. Gel electrophoresis separates nucleic acids on the basis of size. Unlike proteins, nucleic acids have a uniform mass:charge ratio since each nucleotide residue is linked to the next via a phosphodiester linkage. The phosphate residues have a uniform negative charge at neutral pH. As a result, nucleic acids migrate in an electric field toward the positive electrode.

Although acrylamide gels are sometimes used to resolve DNA fragments that differ by only a few bases in length, agarose gel electrophoresis is the most commonly employed technique. To prepare an agarose gel, powdered agarose is first melted in a buffered solution (TAE) containing enough ions to conduct an electric current. The gel solution also contains ethidium bromide. Ethidium bromide is a fluorescent substance that intercalates into DNA. *Be cautious with ethidium bromide! It is a mutagen!* The melted agarose solution is then poured into a tray to solidify. Before the gel hardens, a vertically oriented piece of plastic (a "comb") is inserted into the molten gel to create slots or "wells." Once the gel has solidified, the comb is removed, and the gel is placed in an apparatus (a "gel box") with electrodes at each end. Buffer is added to the gel box until it covers the gel. DNA samples are then pipetted into the wells, and current is applied to the apparatus. During the electrophoresis process, small DNA molecules will move through the gel with fewer restrictions than large molecules because the agarose gel has a sieving effect. As a result, small DNA molecules will move further through the gel than larger DNA molecules in the same amount of time.

After electrophoresis, the relative position of the DNA molecules in the gel can be visualized on a UV-transilluminator. During the electrophoresis process, ethidium bromide molecules will have intercalated into the DNA. When placed on the UV-transilluminator, the nitrogenous bases of the DNA will absorb UV light, and pass on this energy to the ethidium bromide. The ethidium bromide in turn releases this energy in the form of a red-orange fluorescence. This allows the position of the DNA molecules in the gel to be readily identified.

The following solutions and equipment will be prepared for you:

- 50x TAE (Tris-acetate EDTA)
- cold distilled water
- molten 4% agarose in 1x TAE
- gel loading dye
- 100 bp DNA ladder

1. Obtain a gel tray, clamp apparatus, and comb. Seal the ends of the tray with the clamp apparatus and insert the comb. Label the set up with your name on a piece of tape attached lightly to the top of the comb. Place the set up on the lab bench beside the water bath containing the molten 4% agarose. *Remember, this agarose solution contains ethidium bromide; handle it with care.*
2. Carefully pour enough agarose solution into the casting tray to fill to a depth of about 8 mm. The gel should cover only about one-half the height of the comb teeth. Use a pipet tip to move large bubbles or solid debris to sides or end of tray while gel is still molten.
3. Allow the gel to solidify for at least 30 minutes. The gel will become cloudy as it solidifies. Be careful not to move or jar the casting tray while the gel is solidifying.
4. When the agarose has set, *put on a pair of gloves* and remove the clamp apparatus from the ends of the gel tray. *DO NOT touch the gel with your bare hands because it contains ethidium bromide.*
5. Obtain a gel box. Place the gel tray in the gel box so that comb is at negative (black) electrode. *(Remember: run to red!)*
6. If you have not already done so, gently remove the comb by pulling up, taking care not to rip the wells.
7. Using the 50x TAE stock solution, prepare 500 mL of cold 1x TAE (490 mL cold distilled water plus 10 mL 50x TAE).
8. Add enough 1x TAE to the gel box so that the solution just covers the entire surface of gel. Make certain that sample wells left by the comb are completely submerged.
9. Returning to your DNA samples, label another set of six 1.5-mL microcentrifuge tubes as follows: Col, Ler, 1, 2, 3, and 4. Transfer 16 µL of the contents of each of the 0.5-mL thin-walled microcentrifuge tubes containing the products of your PCR reaction to the appropriately labeled 1.5-mL microcentrifuge tube.
10. Obtain a microcentrifuge tube containing gel loading dye. This solution contains a glycerol or sucrose to increase the density of your sample so that it will sink to the bottom of the agarose gel well. The gel loading dye also contains bromphenol blue, which migrates through the gel and will allow you to monitor the progress of the electrophoresis. Add 4 µl of dye to each 1.5-mL microcentrifuge tube into which you pipetted the 16 µL of PCR product. Mix each sample well by pipetting in and out several times.
11. Obtain one tube containing a sample of the 100 bp DNA ladder.
12. Next, load the entire contents of the seven tubes (your six samples plus the DNA ladder) into separate wells in the gel, making sure that you use a fresh tip for each sample. To load a sample into the well, take the sample up in the pipet. Next, dip the pipet tip through surface of buffer, center it over the well, and <u>gently</u> depress the pipet plunger to slowly expel sample. The sample should slowly drift

toward the bottom of the well as you pipet it in. Be careful not to punch the tip of the pipet through the bottom of the gel.

13. Put the cover on the top of the gel box and connect electrical leads to a power supply. Make sure both electrodes are connected to the same channel of power supply.
14. Turn the power supply on, and set it to 200 volts. The ammeter should register over 100 mA.
15. Electrophorese for 30 min.
16. Turn off the power supply. Disconnect leads from the gel box and remove the top of the gel box.
17. *Put on a pair of gloves* and carefully remove the gel tray from the gel box. Be careful that the gel does not slip out of tray and break.
18. Take the gel to your TA. He or she will help you to view and photograph the gel. Keep the gel photo as experimental results.

*Questions*

1. A. Using the photograph of your gel taken on the transilluminator, measure the distance between the well of the lane containing the 100 bp ladder and each of the DNA marker bands. Your TA will supply you with the length (in base pairs, bp) of each of the marker bands. Plot a graph of the log of the marker length versus the distance migrated.

   B. Measure the distance migrated by the products amplified from the Columbia and Landsberg DNA. From the graph generated in Part A above, interpolate the size (in bp) of these DNA products.

   - Which of the two products is the smallest?
   - Which of the two products runs farther during gel electrophoresis?
   - On what basis does agarose gel electrophoresis separate DNA?

2. A. For each of the DNA fragments amplified in the "suspect" samples, what size of DNA products was amplified?

   B. What was the ecotype of the plants grown from the seeds recovered as evidence from each of the suspects?

   C. If you take into account the results of the previous laboratory (the sugar-in-soda and phosphate-in-soil experiments) as well as this experiment, which of the suspects appears to be guilty? Why?

3. A. How would you have interpreted your results if in one of the "suspect" lanes, you had seen both the Columbia- and Landsberg-associated DNA products? Remember, you used several plants in each of your preparations.

   B. Theoretically, could you have amplified two bands from a DNA preparation of a single plant? Remember, the plant you are working with is a diploid! For those students who have taken genetics (e.g. AGRY 320), if you describe the PCR-based amplification of DNA as a phenotype, would it be a dominant, co-dominant, or recessive phenotype? Why?

4. To amplify DNA, the thermocycler cycles through several different temperature regimes (94, 55, and 72 ºC) during each cycle. What happens during each of these cycles?

5. Finally, putting all the information together from your forensic tests, who is the murderer and what is your proof?

## Notes for the Instructor

### Preparation and TA notes for CTAB procedure

*General Notes:*

- The plants have to grow for about 3 weeks.
- Suspects 2 & 3 are Landsberg and suspects 1 & 4 are Columbia.
- It ends up that suspect #2 is guilty.
- If plants are small, be sure to use 4 or 5 plants for each sample.

*TA Notes for part 1 and 2:*

- Dispense the listed reagents into one eppendorf tube of each reagent for each pair.
- Have the students do steps 1 and 2 in Part 2, then give the TA the marked tubes. The instructor or TA will add the PCR mixture and run in the thermocycler.

*Part 1, Extraction of DNA*

1) CTAB Isolation Buffer (makes 500 ml in water) (Each pair needs 600 µL)

- *2% (w/v) cetyltrimethylammonium bromide (CTAB) -- CTAB: Sigma H 5882………10 g
- 1.4 M NaCl……………………………………………………………………40.91 g
- 0.2% (v/v) 2-mercaptoethanol………………………………………………………1 ml
- 20 mM ethylenediamine tetracetic acid (EDTA)……0.5 M EDTA, pH 8.0……………20 ml
- 100 mM Tris-HCl (pH 8.0)…………………………1.0 M Tris, pH 8.0…………….50 ml

*Note: CTAB needs gentle warming with a microwave or hot plate to go into solution. Warm CTAB and water until CTAB is in solution. Cool, and then add remaining components.

2) CTAB Wash Buffer (makes 500 ml in water)

- 76% (v/v) ethanol……………………………………200 proof………………………380 ml
- 10 mM ammonium acetate…………………………………………………………0.385 g

3) TE

- 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA

4) Isopropanol – keep in freezer

5) Chloroform:isoamyl alcohol (24:1, v/v)
- This needs to be dispensed at the last moment. It will evaporate from the eppendorf tubes fairly quickly (overnight). You can also just leave it in a glass bottle for the class to use.

*To dispense to students*: Put all solutions in 1.5-ml eppendorf tubes. Make either one tube each per pair, or recover tubes and refill. Either way, if you put more than enough for one pair in one tube, they will run out. Put 1 ml in each tube except CTAB Wash Buffer, which should be 1.3 mL.

6) Equipment:
- Microcentrifuge tube pestle, 6 per pair
- 60$^{o}$C water bath
- Microfuge tubes, 1.5-ml and 0.5-ml
- Pasteur pipettes and bulbs
- Microcentrifuge
- Adjustable pipettors, 200 and 20 µL size.

*Part 2, PCR*

1) 10X PCR buffer (500mM KCl, 100mM TrisCl pH 9.0, 0.1% Triton X-100) -- make 10 ml; *Aliquot into 1 ml portions, store frozen at –20$^{o}$C.*

| Final conc | Prep to store | Dilute to this to use (10 ml) |
|---|---|---|
| 500 mM KCl | 3 M KCl | 1.67 ml stock |
| 100 mM Tris | 1 M Tris, pH 9.0 | 1.00 ml stock |
| 0.1% Triton X-100 | 10% Triton (w/v) | 0.1 ml stock |

(Triton X-100: Available from Research Organics 3103T)
Note: see comments below on alternative prep.

2) 10X Mg++ solution (15mM $MgCl_2$)

3) 10X dNTP solution (2 mM each dATP, dCTP, dGTP and dTTP) -- make 500 µl
- As supplied, dNTPs are 100 mM. Mix 10 µl each dATP, dCTP, dGTP, dTTP with 460 µl TE (pH8.0) for 500 µl total. DNTP sets available from Fisher PR-U1240 (Promega number available from Fisher). *Aliquot for each semester and store frozen at –20$^{o}$C.*

4) Enzyme (5 U/µL or 2 U/µL Taq DNA Polymerase)

5) 10X Forward primer (50mM oligonucleotide)*

6) 10X Reverse primer (50mM oligonucleotide)*

* <u>Primers are ordered from</u>:
Integrated DNA Technologies, Inc
1710 Commercial Park
ATG-3'
Coralville, IA 52241
Phone: 800-328-2661
Fax: 319-626-8444
e-mail: orders@idtdna.com
Http://www.idtdna.com

<u>Primer sequences are</u>:
forward 5'-GTT TTG GGA AGT TTT GCT GG-3'
reverse 5'CAG TCT AAA AGC GAG ACT ATG

*NOTE:* Primers come as an amount in nanomoles converted to milligrams. For use, add TE in milliliters equal to 10 times the number of nanomoles of primer to make a concentration of 100 picomoles per microliter.

*Alternative prep:*
Instead of making the PCR buffer, I buy Taq DNA Polymerase, 5 U/μL, from Promega which comes with PCR buffer ($MgCl_2$ free) and $MgCl_2$. Make this into a "cocktail mix" and put into the students small eppendorf tubes for PCR.

"Cocktail Mix":

| | For one tube | For 5 sets of 6 tubes (30) |
|---|---|---|
| dNTP | 5 μL | 150 μL |
| PCR buffer | 5 μL | 150 μL |
| $MgCl_2$ | 5 μL | 150 μL |
| Forward primer | 1 μL | 30 μL |
| Reverse primer | 1 μL | 30 μL |
| Taq | 0.5 μL | 15 μL |
| $H_2O$ | 27.5 μL | 825 μL |

Put 45 μL in each tube containing the 5 μL sample and put in PCR thermocycler.

*Part 3, Analysis of PCR product by agarose gel (The students will do this part the second week. The TA will give the students the amplified DNA to run.)*

1) 50x TAE (Tris-acetate EDTA)

- To make 1 liter, keep at 4$^{o}$C. (242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA)
- Dilute to 1X TAE for running buffer. Keep COLD! You can also use 1X TBE buffer, which will not get as hot.
- Cold distilled water for TAE or TBE dilution

2) 4% agarose in 1X TAE + 0.1% EtBr

- The gels are very thick and get HOT and sometimes melt.
- Melt 6 g agarose in 150 ml TAE in a 500-ml erlenmeyer flask; add 15 µl EtBr. Keep warm.
- *I pour the gel and wrap in plastic wrap and keep in the refrigerator until the students are ready for them.*
- If using a microwave, be sure to use a large flask. When heated, the agarose solution will foam up quite a bit. Let sit for a few minutes until bubbles disappear before pouring. The EtBr can be put in the running buffer instead of the gel if desired.

3) Gel loading dye
- 0.04 M Tris-acetate containing 50% glyerol and 0.25% bromophenol blue, pH 8.0.

4) 100 bp DNA ladder (New England Biolabs #323-1L, 250 µg for $200 US)
- Dilute to use for standard marker: 40 µl DNA, 40 µl water, 16 µl loading dye.

5) Gel apparatus and power source

6) 10X TBE buffer (Tris Boric Acid Buffer) (to make 1 liter):
- Add to 700 ml water 1 g NaOH, 108 g Tris base (MW 121.1), 55g Boric Acid (MW 61.80), 7.4 g EDTA (MW 372.24).
- Stir to dissolve then bring to 1 liter. Dilute to 1X for use. Keep at $4^{o}$ C.

## Appendix A: A brief introduction to Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an *in vitro* method of amplifying DNA sequences. The tremendous significance of this discovery was recognized by the awarding of the 1993 Nobel Prize in Chemistry to Kary Mullis for the development of PCR.

Using PCR, specific DNA fragments are isolated and amplified to produce large quantities of the DNA fragments. In a test tube, these components -- the DNA of interest, a pair of small nucleotides (known as primers) complementary to the ends of the piece of DNA to be amplified, the nucleotide precursors of DNA, and an enzyme that copies DNA (Taq DNA polymerase) --are combined to rapidly produce many copies of that DNA. The process is so rapid because the number of copies increases exponentially. More than a billion copies of a piece of DNA can be synthesized in an afternoon.

The basic steps (see Figure 1) of PCR are:

1. The use of high temperature to denature a template DNA molecule to be copied.
2. The annealing of pairs of oligonucleotides of specific sequences (primers) chosen to be homologous to the ends of the region of the template DNA molecule to be amplified.
3. DNA polymerase begins from the primers to make a complementary copy of the template DNA molecule. This step is called the extension step. dNTPs (the precursors to synthesizing DNA) must be present in the reaction.

These three steps are repeated many times (for many cycles) to amplify the template DNA. If one copy is made of each of the strands of the template in each cycle, the number of DNA molecules produced doubles in each cycle. Because of this doubling each cycle, at the end of 20 cycles, more than one million copies of the template DNA are made.

What has made this *in vitro* DNA amplification so efficient was the discovery of heat stable DNA polymerases, such as *Taq* DNA polymerase from *Thermus aquaticus*, a eubacterium that grows in the elevated temperatures of aquatic hot springs. Although the temperature and time for each of the steps of one cycle described above will be varied according to the sequence of the primers used, a general example of the steps is:

1. Denature template, 99$^{O}$C, 1 minutes.
2. Anneal primers to template, 55$^{O}$C, 1 minutes.
3. Extension of the primers by DNA polymerase, 72$^{O}$C, 1 minutes.

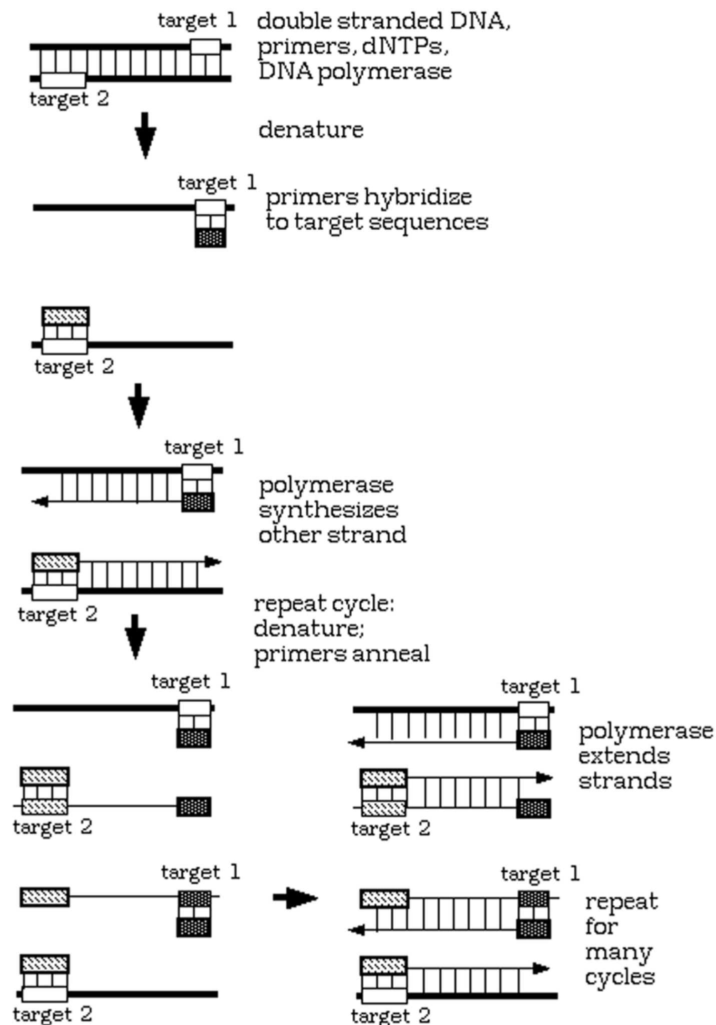


**Figure 1.** The cycles of polymerase chain reaction.

## Appendix B: REDExtract-N-Amp™Plant PCR Kit for DNA Isolation including materials list and preparation

An alternate procedure for isolating DNA from plants is to use a kit, REDExtract-N-Amp™Plant PCR Kit purchased from Sigma. This kit for 100 sample preps costs about $150 US including shipping making the cost per sample about $1.50 US. However, with this kit you don't need to add *Taq* DNA polymerase or the dNTP mixtures. The *Taq* costs about $16 for 30 samples and a set of dNTP costs about $200 but will last for quite a while. The kit is much easier to use and takes a lot less time to do the isolation.

The instructions are included in the kit, but our procedure is a little different.

*A. DNA extraction*

1. Use a spatula tip to collect seedlings grown on filter paper in petrie dishes, or use small plants from pots to put samples in 1.5 mL microfuge tubes.
2. Add 100 µL of Extraction Solution to the tube.
3. Grind the plants with a microcentrifuge pestle. Close the tube and vortex briefly. Microfuge briefly to bring all the material to the bottom of the tube.
4. Incubate at 95$^{o}$ C for 10 minutes in a water bath.
5. Add 100 µL of Dilution Solution and vortex to mix.
6. The leaf extract may be stored at 2-8$^{o}$ C.

*B. PCR amplification*

PCR reactions can be assembled at room temperatures.
The REDExtract-N-AMP PCR Reaction Mix is formulated to compensate for components in the Extraction and Dilution Solutions.

1. Add the following reagents to a thin-walled PCR microfuge tube.

| Reagent | volume |
|---|---|
| Water, PCR grade | 4 µL |
| REDExtract-N-AMP PCR reaction mix | 10 µL |
| Forward primer | 0.5 µL |
| Reverse primer | 0.5 µL |
| Leaf extract | 4 µL |
| Total volume | 20 µL |

2. Mix gently and briefly centrifuge to collect all components to the bottom of the tube.
3. Use the same cycling parameters as in the CTAB procedure.
4. The amplified DNA can be loaded directly onto an agarose gel after the PCR is completed. It is not necessary to add a separate tracking dye.

## List of Materials for REDExtract-N-Amp™Plant PCR Kit

*Part 1, Extraction of DNA*

- Plants or seeds for Lansberg and Columbia Arabadopsis
- Plants or seeds for suspects 1, 2, 3, & 4
- Extraction Solution from kit. (100 µL per pair)
- Dilution Solution from kit (100 µL per pair)

Equipment:

- Microcentrifuge tube pestle, 6 per pair
- Microfuge tubes, 1.5-mL and 0.5-mL
- Microcentrifuge
- Vortex Mixer
- 95°C water bath
- Adjustable pipettors, 200 and 20 µL size

*Part 2, PCR*

- Water, PCR grade (4 µL per pair)
- REDExtract-N-Amp™Plant PCR reaction mix (10 µL per pair)
- Forward primer (0.5 µL per pair)
- Reverse primer (0.5 µL per pair)
- PCR microfuge tube, 0.5-mL (6 per pair)
- Thermocyler

*Part 3, Analysis of PCR product by agarose gel*

- Same as for CTAB procedure.

## Preparation and TA notes for REDExtract-N-Amp™Plant PCR Kit

- Plants have to grow for about 3 weeks.
- Suspects 2 & 3 are Landsberg and suspects 1 & 4 are Columbia.
- It ends up that suspect #2 is guilty.
- If plants are small, be sure to use 4 or 5 plants for each sample. (See Appendix C.)

*Part 1, Extraction of DNA*

Each kit should have enough material for at least 100 samples. Dispense Extraction Solution and Dilution solution into microfuge tubes. You may use one for each student pair, or let the instructor control the student access to a stock tube.

Equipment:

- Microcentrifuge tube pestle, 6 per pair
- Microfuge tubes, 1.5-mL and 0.5-mL
- Microcentrifuge
- Vortex Mixer
- 95°C water bath
- Adjustable pipettors, 200 and 20 µL size.

*Part 2, PCR*

- Water, PCR grade (4 µL per pair)
- REDExtract-N-Amp™Plant PCR reaction mix (10 µL per pair)
- Forward primer* (50 mM oligonucleotide) (0.5 µL per pair)
- Reverse primer* (50 mM oligonucleotide) (0.5 µL per pair)
- PCR microfuge tube, 0.5-mL (6 per pair)
- Thermocyler

*Primers are ordered from:
Integrated DNA Technologies, Inc
1710 Commercial Park
Coralville, IA 52241
Phone: 800-328-2661
Fax: 319-626-8444
e-mail: orders@idtdna.com
Http://www.idtdna.com

Primer sequences are:
forward 5'-GTT TTG GGA AGT TTT GCT GG-3'
reverse 5'CAG TCT AAA AGC GAG ACT ATG ATG-3'

Primers come as an amount in nanomoles converted to milligrams. For use, add TE in milliliters equal to 10 times the number of nanomoles of primer to make a concentration of 100 picomoles per microliter.

*Part 3, Analysis of PCR product by agarose gel.*
(The students will do this part the second week. The TA will give the students the amplified DNA to run.)
Reagents and equipment are the same as for the CTAB isolation procedure.

## Appendix C: Bulk Planting of *Arabidopsis* Seed in Soil

*Arabidopsis* seed can be bulked up by growing plants to maturity in soil, tying plants up to dry, and collecting the seed.

1. *Arabidopsis* are relatively easy to grow. The seeds are very small so a light, fine soil mix works well. We use Scott's "Redi-Earth Plug and Seedling" mix. We plant approximately 30 seeds per 4" plastic pot. The number of seeds can easily be determined by weight given that 1000 seeds weigh approximately 20 mg. A pot of 30 *Arabidopsis* plants will yield approximately 300 mg of seed.
   For planting a large number of pots, it works well to suspend the seeds in cold (4°C or 39°F) 0.1% agar (microwave 1 g agar in approximately 100 mL $H_2O$; bring volume up to 1 liter). Weigh the appropriate number of seeds into a flask, add 10 mL of 0.1% agar for each pot to be planted, cover with Parafilm and shake. The agar is just thick enough so the seeds suspend evenly throughout it. Pipet 10 mL of seed suspension evenly onto each pre-watered pot.
   For only a few pots, seeds may be sprinkled as evenly as possible on top of well-moistened soil.
   It is helpful to cover the pots with saran wrap for the first 3 days or so, as the humidity helps speed germination.
2. *Arabidopsis* like to be kept evenly moist, though not soggy. Bottom watering is essential, as top watering too easily damages the tiny, fragile seedlings. In the lab, we grow our *Arabidopsis* at a light intensity of 100 mE $m^{-2}$ $sec^{-1}$ at 23°C (73°C), under a photoperiod of 16h light / 8h dark. Basically, this is pretty close to normal room conditions on a bright windowsill.

3. *Arabidopsis* bolt (produce flowers) at approximately 3 - 4 weeks of age. Continue to water the plants until they are “flowered out” (no buds remain on the main inflorescence). Placing a stake (12" shish-ke-bob skewers work perfectly) in the center of the pot and tying the plants up with twine greatly speeds seed-harvesting when the plants have dried.
4. Discontinue watering the plants and allow them to dry thoroughly in a location where they will not be disturbed. Seeds shed easily as they dry and may be lost if plants are in a high-traffic area or a breeze.
5. When plants are thoroughly dry, harvest the seed. Carefully remove the stake, cut plants at soil level, invert over a piece of white paper and squeeze plants such that the seeds drop onto the paper. Seeds can be cleaned by collecting them in the center of the paper and gently blowing the chaff away.
6. Germination greatly improves if seeds are placed in a loosely covered container for approximately two weeks to dry further. The freezer is the best location for long-term storage (longer than one year), as long as seed are not allowed to become wet or dessicated by a frost-free cycle.

## Appendix D: Growing seedlings in petri dishes

1. Cut filter paper to fit petri dish. Add enough water thoroughly moisten the filter paper and leave some water standing.
2. Prepare a microfuge tube for each petri dish with seeds that will fit on the tip of a small spatula. Tap the tube with your finger or a spatula as you move the tube over the petri dish so the seeds fall in a scatter pattern onto the filter paper. Try to scatter them well.
3. Put the cover on the petri dish and seal with parafilm.
4. Sit in the sun until seeds germinate and grow as much as you want. Several days will be enough for the *Col*, but it will take about a week to grow the *Ler*. In both pots and petri dishes, the *Col* will grow bigger faster.

## Appendix E: Seedlings in Petrie Dish and Grinding

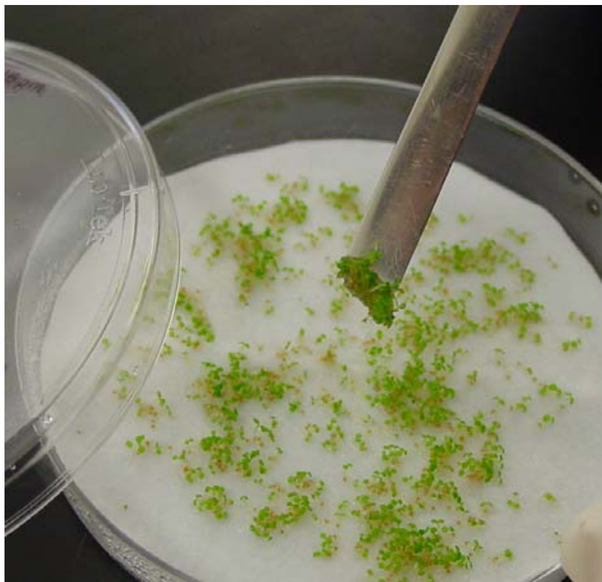

**Figure 2.** Seedlings grown in petrie dish

**Figure 3**. Grinding seedlings with the microfuge tube pestle.