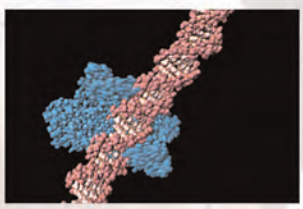
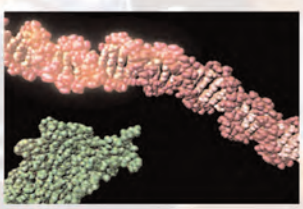
Detecting Genetically Modified Foods by PCR

DNA KITS
Learning Center



DOLAN
DNA
LEARNING
CENTER

CAROLINA



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IMPORTANT INFORMATION

Storage: Upon receipt of the kit, store 35S primer/loading dye mix, tubulin primer/loading dye mix, Tris/EDTA (TE) Buffer with RNase A, and DNA marker pBR322/*Bst*NI in the freezer (approximately –20°C). All other materials may be stored at room temperature (approximately 25°C).

Use and Lab Safety: The materials supplied are for use with the method described in this kit only. Use of this kit presumes and requires prior knowledge of basic methods of gel electrophoresis and staining of DNA. Individuals should use this kit only in accordance with prudent laboratory safety precautions and under the supervision of a person familiar with such precautions. Use of this kit by unsupervised or improperly supervised individuals could result in injury.

Limited License: Polymerase chain reaction (PCR) is protected by patents owned by Hoffman-La Roche, Inc. The purchase price of this product includes a limited, non-transferable license under U.S. Patents 4,683,202; 4,683,195; and 4,965,188 or their foreign counterparts, owned by Hoffmann-La Roche Inc. and F. Hoffmann-La Roche Ltd. (Roche), to use only this amount of the product to practice the Polymerase Chain Reaction (PCR) and related processes described in said patents solely for the research, educational, and training activities of the purchaser when this product is used either manually or in conjunction with an authorized thermal cycler. No right to perform or offer commercial services of any kind using PCR, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is hereby granted by implication or estoppel. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at The Perkin-Elmer Corporation, 850 Lincoln Center Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

Printed Material: The Student Lab Instructions, pages 5–18, as well as the *Carolina*BLU™ staining protocol on page 24, may be photocopied as needed for use by your students.

REAGENTS, SUPPLIES, AND EQUIPMENT CHECKLIST

Included in the kit:

DNA extraction and amplification (all kits):

- ☐ 16 wild-type soybean seeds
- ☐ 16 Roundup Ready® soybean seeds
- ☐ 20 mL Edwards' buffer
- ☐ 10 mL 100% isopropanol
- ☐ 3 mL Tris/EDTA (TE) buffer with RNase A
- ☐ 500 µL 35S primer/loading dye mix
- ☐ 500 µL Tubulin primer/loading dye mix
- ☐ 25 *Ready-to-Go™ PCR Beads
- ☐ 5 mL mineral oil
- ☐ 1 130-µL tube pBR322/*Bst*NI markers (0.075 µg/µL)
- ☐ 15 pellet pestles
- ☐ 25 microcentrifuge tubes
- ☐ Instructor's manual with reproducible Student Lab Instructions
- ☐ GM Food CD-ROM

***Electrophoresis with ethidium bromide staining (Kits 21-1368 and 21-1369):*

- ☐ 8 g agarose
- ☐ 150 mL 20× TBE
- ☐ 250 mL ethidium bromide, 1 µg/mL
- ☐ 4 latex gloves
- ☐ 6 staining trays

***Electrophoresis with Carolina*BLU™ *staining (Kits 21-1370 and 21-1371)*:

- ☐ 8 g agarose
- ☐ 150 mL 20× TBE
- ☐ 7 mL *Carolina*BLU™ Gel and Buffer Stain
- ☐ 250 mL *Carolina*BLU™ Final Stain
- ☐ 4 latex gloves
- ☐ 6 staining trays

Needed but not supplied:

- ☐ Planting pot or flat
- ☐ Potting soil
- ☐ Soy or corn food products
- ☐ Micropipets and tips (1 µL to 1000 µL)
- ☐ Microcentrifuge tube racks
- ☐ Microcentrifuge for 1.5-mL tubes
- ☐ Thermal cycler
- ☐ Water bath or heating block
- ☐ Electrophoresis chambers
- ☐ Electrophoresis power supplies
- ☐ UV transilluminator (ethidium bromide staining)
- ☐ White light box (*Carolina*BLU™ staining, optional)
- ☐ Camera or photo-documentary system (optional)
- ☐ Permanent markers
- ☐ Containers with cracked or crushed ice
- ☐ Vortexer (optional)
- ☐ Roundup® herbicide (optional)

*Ready-to-Go™ PCR Beads incorporate *Taq* polymerase, dNTPs, and $MgCl_2$. Each bead is supplied in an individual 0.5–mL tube or a 0.2–mL tube.

**Electrophoresis reagents must be purchased separately for Kits 21-1366 and 21-1367.

Detecting Genetically Modified Foods by PCR

CONTENTS

STUDENT LAB INSTRUCTIONS

INTRODUCTION

During the "green revolution" of the 1950s through 1970s, high-yielding strains of wheat, corn, and rice, coupled with extensive use of chemical fertilizers, irrigation, mechanized harvesters, pesticides, and herbicides, greatly increased world food supply. Results were especially dramatic in underdeveloped countries. Now, genetic engineering is fueling a "second green revolution." Genes that encode herbicide resistance, insect resistance, drought tolerance, frost tolerance, and other traits have been added to many plants of commercial importance. In 2003, 167 million acres of farmland worldwide were planted in genetically modified (GM) crops—equal to one fourth of total land under cultivation. The most widely planted GM crops are soybeans, corn, cotton, canola, and papaya.

Two important transgenes (transferred genes) have been widely introduced into crop plants. The *Bt* gene, from *Bacillus thuringiensis*, produces a toxin that protects against caterpillars, reducing applications of insecticides and increasing yields. The glyphosate-resistance gene protects food plants against the broad-spectrum herbicide Roundup®, which efficiently kills invasive weeds in the field. The major advantages of the "Roundup Ready®" system include better weed control, reduction of crop injury, higher yield, and lower environmental impact than traditional weed-control systems. Notably, fields treated with Roundup® require less tilling; this preserves soil fertility by lessening soil run-off and oxidation.

Most Americans would probably be surprised to learn that more than 60% of fresh vegetables and processed foods sold in supermarkets today are genetically modified by gene transfer. In 2004, approximately 85% of soy and 45% of corn grown in the U.S. were grown from Roundup Ready® seed.

This laboratory uses a rapid method to isolate DNA from plant tissue and food products. Then, polymerase chain reaction (PCR) is used to assay for evidence of the 35S promoter that drives expression of the glyphosate-resistance gene and many other plant transgenes. Herbicide resistance correlates with an insertion allele, the 35S promoter, that is readily identified by electrophoresis on an agarose mini-gel. Amplification of tubulin, a protein found in all plants, provides evidence of amplifiable DNA in the preparation, while tissue samples from wild-type and Roundup Ready® soy plants are negative and positive controls for the 35S promoter. Since soy and corn are ingredients in many processed foods, it is not difficult to detect the 35S promoter in a variety of food products.

Castle, L.A., Siehl, D.L., Gorton, R., Patten, P.A., Chen, Y.H., Bertain, S., Cho, H.J., Wong, N.D., Liu, D., Lassner, M.W. (2004). Discovery and Directed Evolution of a Glyphosate Tolerance Gene. *Science* 304: 1151–1154.

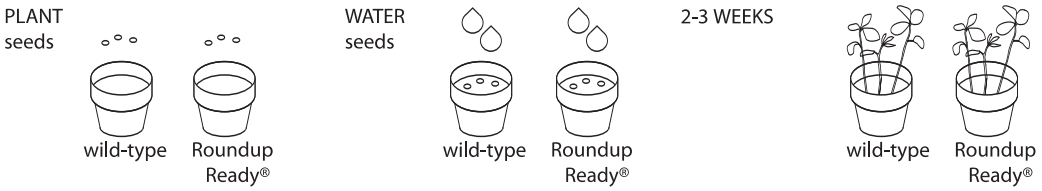
Edwards, K., Johnstone, C. and Thompson, C. (1991). A Simple and Rapid Method for the Preparation of Plant Genomic DNA for PCR Analysis. *Nucleic Acids Res.*19: 1349.

Stalker, D.M., McBride, K.E., Maiyj, L.D. (1988). Herbicide Resistance in Transgenic Plants Expressing a Bacterial Detoxification Gene. *Science* 242: 419–423.

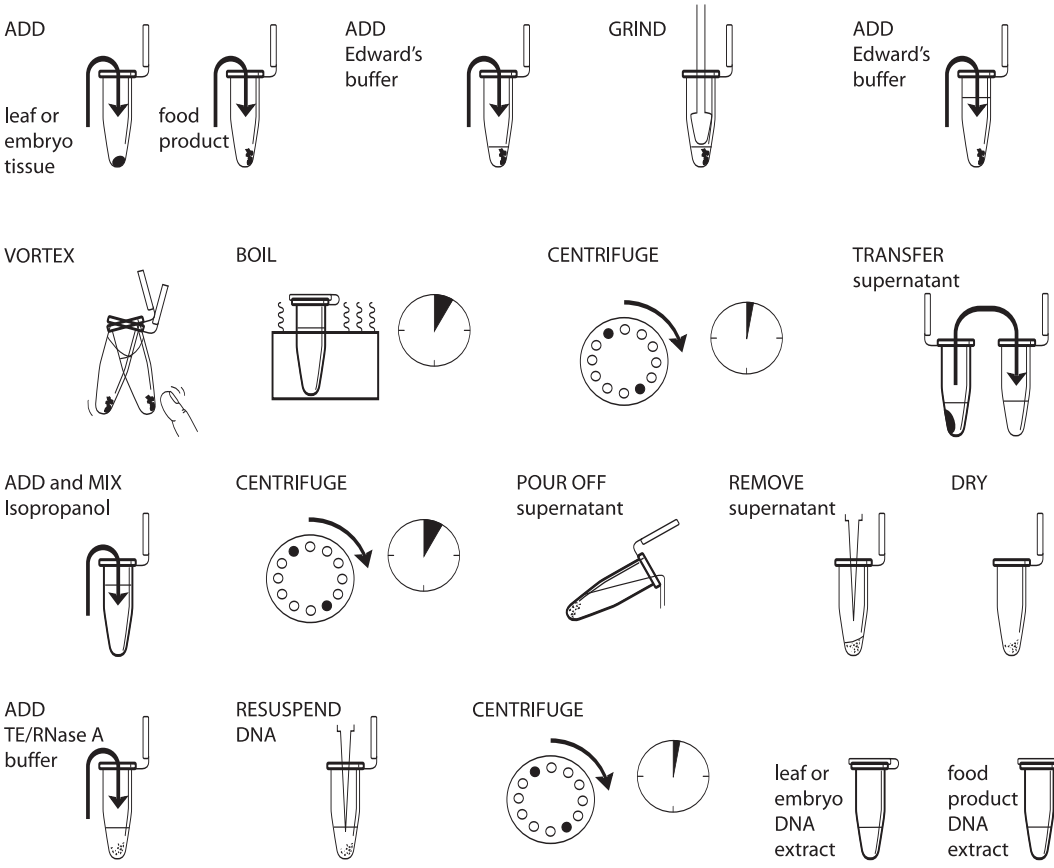
Vollenhofer, S., Burg, K., Schmidt, J., Kroath, H. (1999). Genetically Modified Organisms in Food Screening and Specific Detection by Polymerase Chain Reaction. *J. Agric. Food Chem.* 47: 5038–5043.

LAB FLOW

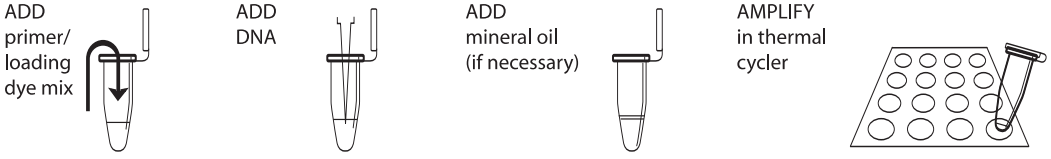
I. PLANT SOYBEAN SEEDS



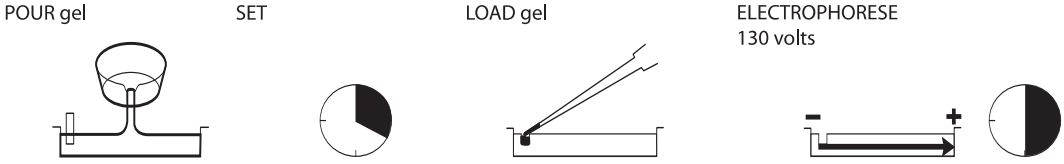
II. ISOLATE DNA FROM SOY AND FOOD PRODUCTS



III. AMPLIFY DNA BY PCR



IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS



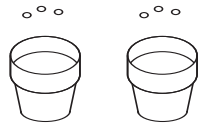
METHODS

I. PLANT SOYBEAN SEEDS

To extract DNA from leaf tissue, you must plant the soybean seeds 2–3 weeks prior to DNA isolation and PCR.

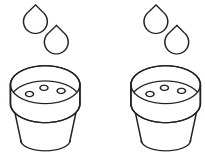
Reagents	Supplies and Equipment
Wild-type and Roundup Ready® soybean seeds	Planting pot or flat Potting soil

For best results, use a potting soil formulated specifically for soybeans.



1. Fill the planting pots or flat evenly with potting soil, but do not pack the soil tightly.
2. Label half of the pots "Roundup Ready®," and half of the pots "wild-type."
3. Plant only three of the appropriate seeds per pot, or one per flat cell, to allow optimal growth and easy observation.
4. Use your finger to make a 0.5-inch depression. Add a seed, cover with soil, and lightly tamp.

Germination requires a humid environment.



5. Water the plants from above to prevent the soil from drying out. Drain off excess water, and do not allow the pot or flat to sit in water.
6. Grow the plants close to a sunny window at room temperature or slightly warmer. A growth light may be used.
7. Harvest plant tissue for PCR as soon as the first true leaves become visible. These will follow the cotyledons, or seed leaves. This should be about 2 weeks after planting, depending on light and temperature conditions.

The first true leaves may be visible 2 weeks after planting, depending on light and temperature conditions.

8. Allow the plants to continue to grow if you plan to test later for Roundup® sensitivity/resistance (optional).

II. ISOLATE DNA FROM SOYBEAN AND FOOD PRODUCTS

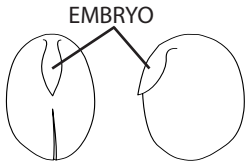
If extracting DNA from seed embryos, soak the wild-type and Roundup Ready® soybean seeds in separate containers of distilled water for a minimum of 30 minutes. This will soften the seeds, making the embryos easier to remove.

Reagents (at each student station)	**Supplies and Equipment**
Soy or corn food products	Plastic pestles
Wild-type or Roundup Ready® soybean tissue	Permanent marker
Edwards' buffer, 2.2 mL	1.5-mL microcentrifuge tubes
Isopropanol, 1 mL	Micropipet and tips (100–1000 µL)
Tris/EDTA (TE) buffer with RNase A, 300 µL	Microcentrifuge tube racks
	Microcentrifuge
	Vortexer (optional)
	Water bath or heating block (95–100°C)
	Container with cracked or crushed ice

Your instructor will assign you either wild-type or Roundup Ready® soybean control.

1. Prepare tissue from wild-type or Roundup Ready® soybeans.
   a. From soy leaves: Cut two pieces of tissue approximately 1/4 inch in diameter. Place the leaf tissue in a clean 1.5-mL tube, and label with soybean type and your group number.
   b. From seed embryo: The embryo is a small (3 mm) flap of tissue located beneath the hilum, the light- or dark-colored scar marking where the seed was attached to the pod. Gently remove the seed coat by rubbing the seed between your fingers. Remove the embryo flap with a scalpel or razor blade, and place it in a 1.5-mL tube. Label the tube with the soybean type and your group number.

The large end of a 1000-µL pipet tip will punch disks of this size.



SOYBEAN SEED
with seed coat removed

2. Prepare soy or corn food product. Crush a small amount of dry product on a clean piece of paper or in a clean plastic bag to produce a coarse powder. Add the crushed food product to a clean 1.5-mL tube to a level about halfway to the 0.1 mL mark. Label the tube with the food type and your group number.
3. Add 100 µL of Edwards' buffer to each tube containing the plant or food material.

Detergent in the Edwards' buffer dissolves lipids of the cell membrane. The soy tissue sample should color the buffer green. Not all of the dry food will liquefy.

4. Twist a clean plastic pestle against the inner surface of the 1.5-mL tube to *forcefully* grind the plant tissue or food product for 1 minute.
5. Add 900 µL of Edwards' buffer to each tube containing the ground sample. Grind briefly to remove tissue from the pestle.
6. Vortex the tubes for 5 seconds, by hand or machine.
7. Boil the samples for 5 minutes in a water bath or heating block.

This step denatures proteins, including DNA-digesting enzymes.

This step pellets insoluble material at the bottom of the tube.

8. Place the tubes in a balanced configuration in a microcentrifuge, and spin for 2 minutes to pellet cell and food debris.
9. Transfer 350 µL of each supernatant to a fresh tube. Maintain labels for each plant, food type, and group number. Be careful not to disturb the pelleted debris when transferring the supernatant. Discard old tubes containing the precipitates.

This step precipitates nucleic acids, including DNA.

10. Add 400 µL of isopropanol to each tube of supernatant.
11. Mix by inverting the tubes several times, and leave at room temperature for 3 minutes.

The nucleic acid pellet may appear as a tiny teardrop-shaped smear or particles on the tube side. Don't be concerned if you can't see a pellet. A large or greenish pellet is cellular debris carried over from the first centrifugation.

12. Place the tubes in a balanced configuration in a microcentrifuge, and spin for 5 minutes. Align tubes in the rotor with the cap hinges pointing outward. Nucleic acids will collect on the tube side under the hinge during centrifugation.
13. Carefully pour off and discard the supernatant from each tube. Then completely remove the remaining liquid with a medium pipet set at 100 µL.

You may dry the pellets quickly with a hair dryer! To prevent blowing the pellet away, direct the air across the tube mouth, not into the tube.

14. Air dry the pellets by letting the tubes sit with caps open for 10 minutes. The remaining isopropanol will evaporate.
15. Add 100 µL of TE/RNase A buffer to each tube. Dissolve the nucleic acid pellet by pipetting in and out. Take care to wash down the side of the tube underneath the hinge, where the pellet formed during centrifugation.
16. Incubate TE/RNase A solution at room temperature for 5 minutes.
17. Microcentrifuge the tubes for 1 minute to pellet any material that did not go into solution.

You will use 2.5 µL of the DNA extract for the PCR reactions in Part III. The crude DNA extract contains nucleases that will eventually fragment the DNA at room temperature. Keeping the sample cold limits this activity.

18. DNA may be used immediately or stored at –20°C until you are ready to continue with Part III. Keep the DNA on ice during use.

III. AMPLIFY DNA BY PCR

Reagents (at each student station)

*Food product DNA (from Part II)
*Wild-type or Roundup Ready® soybean DNA (from Part II)
*35S primer/loading dye mix, 50 µL
*Tubulin primer/loading dye mix, 50 µL
Ready-to-Go™ PCR Beads
Mineral oil, 5 mL (depending on thermal cycler)

*Store on ice

Supplies and Equipment

Permanent marker
Micropipet and tips (1–20 µL)
Microcentrifuge tube rack
Container with cracked or crushed ice
Thermal cycler

Carry on with either wild-type or Roundup Ready® soybean control, as assigned by your instructor.

1. Set up 35S promoter reactions:
   a. Obtain 2 PCR tubes containing Ready-To-Go™ PCR Beads. Label with your group number.
   b. Label one tube "35S FP" (food product). Label another tube either "35S WT" (wild-type soy plant) or "35S RR" (Roundup Ready® soy plant). Remember, the WT and RR tubes are positive and negative controls. Each group will only do one control and will share data with other groups.

The primer/loading dye mix will turn purple as the Ready-to-Go™ PCR Bead dissolves.

   c. Use a micropipet with a fresh tip to add 22.5 µL of the 35S primer/loading dye mix to each tube.
   d. Use a micropipet with a fresh tip to add 2.5 µL of food product DNA (from Part II) to the reaction tube marked "35S FP."
   e. Use a micropipet with a fresh tip to add 2.5 µL of wild-type or Roundup Ready® soybean DNA (from Part II) to the appropriate reaction tube marked "35S WT" or "35S RR."

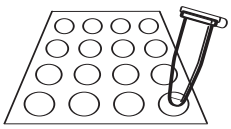
The tubulin gene is found in all plants and, so, is a positive control for the presence of amplifiable DNA.

2. Set up tubulin reactions:
   a. Obtain 2 PCR tubes containing Ready-to-Go™ PCR Beads. Label with your group number.
   b. Label one tube "T FP" (food product). Label another tube either "T WT" (wild-type) or "T RR" (Roundup Ready®). Remember, the WT and RR tubes are positive and negative controls. Each group will only do one control and will share data with other groups.

If the reagents become splattered on the wall of the tube, pool them by pulsing in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.

   c. Use a micropipet with a fresh tip to add 22.5 µL of the tubulin primer/loading dye mix to each tube.
   d. Use a micropipet with a fresh tip to add 2.5 µL of the food product DNA (from Part II) to the reaction tube marked "T FP."
   e. Use a micropipet with a fresh tip to add 2.5 µL of wild-type or Roundup Ready® soybean DNA (from Part II) to the appropriate reaction tube marked "T WT" or "T RR."

The mineral oil prevents the PCR mix from evaporating and condensing on the tube cap during cycling. Most modern thermal cyclers have heated lids that prevent condensing and DO NOT require the addition of mineral oil.



3. If necessary, add one drop of mineral oil to the top of the reactants in each PCR tube. Be careful not to touch the dropper tip to the tube or reactants, or subsequent reactions will be contaminated with DNA from your preparation. If your thermal cycler has a heated lid, you do not need to use mineral oil.
4. Store samples on ice until you are ready to begin thermal cycling.
5. Program the thermal cycler for 32 cycles of the following profile. The program may be linked to a 4°C hold program after the 32 cycles are completed.

Denaturing step:	94°C	30 seconds
Annealing step:	60°C	30 seconds
Extending step:	72°C	30 seconds

6. After cycling, store the amplified DNA at –20°C until you are ready to continue with Part IV.

IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

Reagents (at each student station)

*Food product PCR product (from Part III)
*Wild-type *or* Roundup Ready® soybean PCR product (from Part III)
2% agarose in 1× TBE, 50 mL
1× TBE, 300 mL

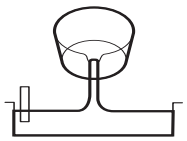
Shared Reagents

*pBR322/*Bst*NI marker, 43 µL (per two teams)
Ethidium bromide (1 µg/mL), 250 mL
or
*Carolina*BLU™ Gel and Buffer Stain, 7 mL
*Carolina*BLU™ Final Stain, 250 mL

*Store on ice

Supplies and Equipment

Micropipet and tips (1–100 µL)
1.5-mL microcentrifuge tube rack
Gel electrophoresis chamber
Power supply
Staining trays
Latex gloves
UV transilluminator (for use with ethidium bromide)
White light transilluminator (for use with *Carolina*BLU™)
Digital or instant camera (optional)
Water bath (60°C)
Container with cracked or crushed ice



Avoid pouring an overly thick gel, which is more difficult to visualize. The gel will become cloudy as it solidifies.

1. Seal the ends of the gel-casting tray with masking tape, and insert a well-forming comb.
2. Prepare enough gels for 10 wells per two lab teams. This may require two gels or one "double-combed" gel per two teams.
3. Pour 2% agarose solution to a depth that covers about 1/3 the height of the open teeth of the combs.

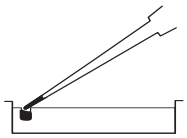
4. Allow the gel to solidify completely. This takes approximately 20 minutes.

Do not add more buffer than necessary. Too much buffer above the gel channels electrical current over the gel, increasing running time.

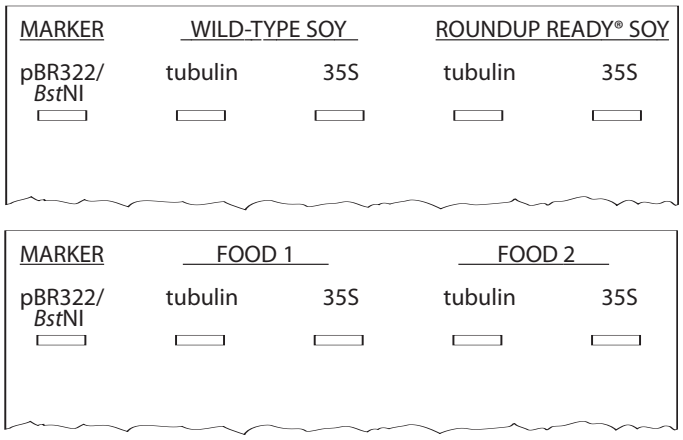
5. Place the gel into the electrophoresis chamber and add enough 1× TBE buffer to cover the surface of the gel.

6. Carefully remove the combs and add additional 1× TBE buffer to just cover and fill in wells, creating a smooth buffer surface.

Pipetting 20 µL of the 25-µL reaction takes into account undermeasurement, condensation, and spattering that typically reduce the volume of PCR product available to load. Expel any air from the tip before loading. Be careful not to push the tip of the pipet through the bottom of the sample well.

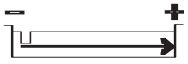


7. Use a micropipet with a fresh tip to add 20 µL of each of the sample/loading dye mixtures into different wells of a 2% agarose gel, according to the following scheme. (If you used mineral oil during PCR, pierce your pipet tip through the layer of mineral oil to withdraw the PCR products, and leave the mineral oil behind in the original tube.)



100-bp ladder may also be used as a marker.

8. Load 20 µL of the molecular weight marker (pBR322/*Bst*NI) into one well.



9. Run the gels at 130 V for approximately 30 minutes. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.

10. Stain the gel in ethidium bromide or *Carolina*BLU™:

Destaining the gel for 5–10 minutes in tap water leeches unbound ethidium bromide from the gel, decreasing background and increasing contrast of the stained DNA.

   a. For ethidium bromide, stain 10–15 minutes. Decant stain back into storage container for reuse, and rinse gel in tap water. Use gloves when handling ethidium bromide solution and stained gel, or anything that has ethidium bromide on it. Ethidium bromide is a known mutagen and care should be taken when using and disposing of it.

   b. For *Carolina*BLU™ staining, follow the directions supplied by your instructor.

Transillumination, where the light source is below the gel, increases brightness and contrast.

11. View the gel using transillumination, and photograph it with a digital or instant camera.

BIOINFORMATICS

For a better understanding of the experiment, do the following bioinformatics exercises before you analyze your results.

Biological information is encoded in the nucleotide sequence of DNA. Bioinformatics is the field that identifies biological information in DNA using computer-based tools. Some bioinformatics algorithms aid the identification of genes, promoters, and other functional elements of DNA. Other algorithms help determine the evolutionary relationships between DNA sequences.

Because of the large number of tools and DNA sequences available on the Internet, experiments done "in silico" (in silicon, or on the computer) now complement experiments done "in vitro" (in glass, or test tube). This movement between biochemistry and computation is a key feature of modern biological research.

In Part I, you will use the Basic Local Alignment Search Tool (BLAST) to identify sequences in biological databases and to make predictions about the outcome of your experiments. In Part II, you will discover some of the genes and functions that are transferred into GM plants.

NOTE: The links in these bioinformatics exercises were correct at the time of printing. However, links and labels within the NCBI Internet site change occasionally. When this occurs, you can find updated exercises at http://bioinformatics.dnalc.org.

I. USE BLAST TO FIND DNA SEQUENCES IN DATABASES (ELECTRONIC PCR)

The following primer sets were used in the experiment:

5'-CCGACAGTGGTCCCAAAGATGGAC-3'	(Forward Primer)
5'-ATATAGAGGAAGGGTCTTGCGAAGG-3'	(Reverse Primer)
5'-GGGATCCACTTCATGCTTTCGTCC-3'	(Forward Primer)
5'-GGGAACCACATCACCACGGTACAT-3'	(Reverse Primer)

1. Initiate a BLAST search.
   a. Open the Internet site of the National Center for Biotechnology Information (NCBI) www.ncbi.nlm.nih.gov/.
   b. Click on *BLAST* in the top speed bar.
   c. Click on the link to *nucleotide BLAST* under the heading *Basic BLAST*.
   d. Copy the first set of primers and paste them into the *Search* window. These are the query sequences.
   e. Omit any non-nucleotide characters from the window, because they will not be recognized by the BLAST algorithm.
   f. Under *Choose Search Set*, select the *Nucleotide collection(nr/nt)* database from the drop-down menu.

g. Under *Program Selection*, optimize for somewhat similar sequences by selecting *blastn*.

h. Click on *BLAST!* and the query sequences are sent to a server at the National Center for Biotechnology Information in Bethesda, Maryland. There, the BLAST algorithm will attempt to match the primer sequences to the millions of DNA sequences stored in its database. While searching, a page showing the status of your search will be displayed until your results are available. This may take only a few seconds, or more than a minute if a lot of other searches are queued at the server.

2. The results of the BLAST search are displayed in three ways as you scroll down the page:

   a. First, a graphical overview illustrates how significant matches, or hits, align with the query sequence. Matches to the forward primer are in blue, while matches to the reverse primer are in green.

   b. This is followed by a list of significant alignments, or hits, with links to *Accession* information.

   c. Next is a detailed view of each primer sequence (*query*) aligned to the nucleotide sequence of the search hit (*subject*). Notice that a match to the forward primer (nucleotides 1–24), and a match to the reverse primer (nucleotides 25–49) are within the same *Accession*.

3. What is the predicted length of the product that the primer set would amplify in a PCR reaction (*in vitro*)?

   a. In the list of significant alignments, notice the scores in the *E-value* column on the right. The *Expectation* or *E-value* is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the *E-value* the higher the probability that the hit is related to the query. For example, 6e-4 denotes 6 x 10-4 or 0.0006. Shorter queries, such as primers, produce higher *E-values*.

   b. Note any significant alignment that has an *E-value* less than 0.1.

   c. Scroll down to the *Alignments* section to see exactly where the two primers have landed in a subject sequence.

   d. The lowest and highest nucleotide positions in the subject sequence indicate the borders of the amplified sequence. Subtracting one from the other gives the difference between the two coordinates.

   e. However, the actual length of the fragment *includes* both ends, so add 1 nucleotide to the result to determine the exact length of the PCR product amplified by the two primers.

4. What DNA sequence does this primer set amplify? Is this the primer set to detect a GM product or the control primer set?

   a. In the list of *significant alignments*, select one hit from among those

with the lowest *E-values*.

b. Click on the the *Accession* link at the left to open the sequence datasheet for this hit.

c. The datasheet has three parts:

- The top part contains basic information about the sequence, including its basepair length, database accession number, source, and references. Typically, this basic information provides clues to the identity of the subject sequence.
- The bottom section lists the nucleotide sequence.
- The middle section contains annotations of gene and regulatory features, with their beginning and ending nucleotide positions (xx ... xx). If your datasheet does not list any features, go back and select another hit from the list of *significant alignments*.

d. Identify the feature(s) contained between the nucleotide positions identified by the primers, as determined in 3.d. above.

II. USE BLAST TO IDENTIFY TRANSGENES DRIVEN BY THE 35S PROMOTER

1. The BLAST search with the primers, in Part I above, identified numerous cloning vectors that incorporate a 35S promoter. To limit hits from cloning vectors and to find out more about transgenes driven by the promoter, do another BLAST search with the 162-basepair product amplified by our primers.
2. Return to the sequence at the bottom of the appropriate *Accession* datasheet used in Part I.3. above. Highlight all the nucleotides between the beginning of the forward primer and end of reverse primer.
3. Paste this sequence into a text document. Then, trim any extra nucleotides from the ends, and delete all non-nucleotide characters and spaces. This is the sequence of the 35S amplicon (amplified product).
4. Copy the amplicon and paste it into the BLAST search window. To narrow the search, use the *Options* menu to change *All organisms* to *Viridiplantae*, the major group of green plants.
5. What do you notice about the *E-values* obtained by this search? Why is this so?
6. Follow *Accession* links to learn about genes that follow and, therefore, are expressed by the 35S promoter. For quick information about a feature, copy the name of a gene or coding sequence (CDS), and do a *Google* search. Refine a search by adding the word "gene" to any abbreviation.

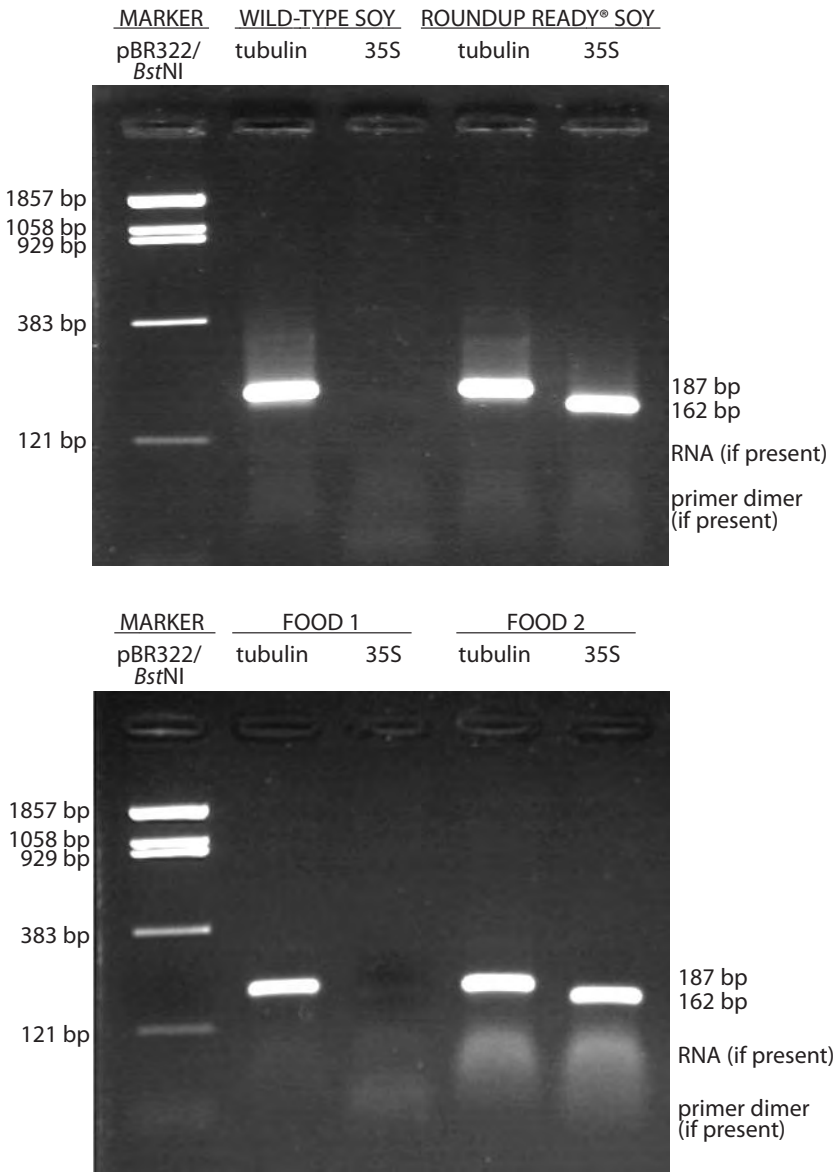
Repeat Parts I and II to predict the size of the PCR product and to learn about the gene or feature amplified by the second primer set.

RESULTS AND DISCUSSION

1. Describe the purpose of each of the following steps or reagents used in DNA isolation (Part II):
   a. grinding with pestle.
   b. Edwards' buffer.
   c. boiling.
   d. tris-EDTA (TE) buffer with RNase A.
2. What is the purpose of performing each of the following PCR reactions:
   a. tubulin?
   b. wild-type soybean?
   c. Roundup Ready® soybean?
3. Which sample(s) in the gels pictured on the next page show the following banding patterns? Explain what each pattern means. (Foods 1 and 2 shown are two examples of expected results. Remember that your own samples can yield any of the combinations for tubulin and 35S in the table below.)

187 bp (tubulin)	162 bp (35S)	Samples showing this pattern and explanation
present	present	
present	absent	
absent	absent	
absent	absent	

4. Observe the photograph of the stained gel containing your sample and those of other students. Orient the photograph with wells at the top. Interpret each lane of the gel.
   a. Scan across the photograph of your gel and others as well to get an impression of what you see in each lane. You should notice that virtually all experiment lanes contain one or two prominent bands.
   b. Now locate the lane containing the pBR322/*Bst*NI marker on the left-hand side of the gel. Working from the well, locate the bands corresponding to each restriction fragment: 1857 bp, 1058 bp, 929 bp, 383 bp, and 121 bp (may be faint). Alternatively, locate the lane containing the 100-bp ladder, with the fastest migrating band equal to 100 bp and each successive band 100 bp larger (100, 200, 300, 400, etc.).
   c. The amplification products of the 35S promoter (162 bp) and of the tubulin gene (187 bp) should align between the 121-bp and 383-bp fragments of the pBR322/*Bst*NI marker (or between the 100-bp and 200-bp markers of the 100-bp ladder).



d. It is common to see one or two diffuse (fuzzy) bands of RNA and/or primer dimer at the bottom of the gel. RNA may be found at approximately the position of the 121-bp fragment of the pBR322/*Bst*NI marker (or the 100-bp marker of the 100-bp ladder). RNA is the largest component of nucleic acid isolated from plant tissue, so some RNA may escape digestion by the RNase incorporated into the TE buffer in Part II of the experiment. Primer dimer is an artifact of the PCR reaction that results from two primers overlapping one another and amplifying themselves. Primer dimer is approximately 50 bp, and should be in a position ahead of the 121-bp marker fragment.

e. Additional faint bands, at other positions on the gel, occur when the primers bind to chromosomal loci other than 35S or tubulin, giving rise to "nonspecific" amplification products.

INFORMATION FOR INSTRUCTOR

CONCEPTS AND METHODS

This laboratory can help students understand several important concepts of modern biology:

- The relationship between genotype and phenotype.
- Forensic identification of genes.
- Methods for producing transgenic crops.
- The movement between *in vitro* experimentation and *in silico* computation.

The laboratory uses several methods for modern biological research:

- DNA extraction and purification.
- Polymerase chain reaction (PCR).
- Gel electrophoresis.
- Bioinformatics.

INSTRUCTOR PLANNING AND PREPARATION

The following table will help you to plan and integrate the four parts of the experiment.

Part		Day	Time	Activity
I.	Plant Soybean Seeds	2–3 weeks before lab	15–30 min.	Plant and care for soybean seeds.
II.	Isolate DNA	1	30 min. 30–60 min.	**Pre-lab:** Set up student stations. Isolate soy DNA.
III.	Amplify DNA by PCR	2	30–60 min. 15–30 min. 70+ min.	**Pre-lab:** Set up student stations. Set up PCR reactions. **Post-lab:** Amplify DNA in thermal cycler.
IV.	Analyze PCR Products by Gel Electrophoresis	3	30 min.	Prepare agarose gel solution and cast gels.
		4	30 min 30+ min. 20+ min. 20 min. to overnight 20 min.	Load DNA samples into gels. Electrophorese samples. **Post-lab:** Stain gels. **Post-lab:** De-stain gels. **Post-lab:** Photograph gels.

I. PLANT SOYBEAN SEEDS

Tissue samples from wild-type and Roundup Ready® soybean plants are used as negative and positive controls of the experiment. Soybean seeds must be planted 2–3 weeks before the date anticipated for DNA extraction and amplification by PCR. Plant tissue may be harvested for DNA isolation at any point after plantlets emerge from the soil. Two 1/4-inch diameter leaf disks are required for each experiment. Several wild-type and Roundup Ready® plants can be set aside for treatment with Roundup® to test for herbicide sensitivity/resistance.

The following Carolina products are suggested for growing soybean seedlings:

Standard Poly-Tray without Holes (54 x 27 x 6 cm tray)	item number 66-5666
Poly-Flats (6-cm deep cells that can be separated into individual pots):	
8-Cell Tray	item number 66-5668
24-Cell Tray	item number 66-5669
36-Cell Tray	item number 66-5670
Redi-Earth Soil (8-lb Bag)	item number 15-9701

Alternatively, DNA can be extracted from the seed embryo, without planting; however, this method is less reproducible than getting DNA from leaf tissue. In this case, seeds must be soaked in water for at least 30 minutes prior to DNA isolation and PCR. Be sure to soak wild-type and Roundup Ready® seeds separately.

II. ISOLATE DNA FROM SOYBEAN AND FOOD PRODUCTS

Divide the class into six lab teams, and assign each team a number at the outset of the experiment. This will make it easier to mark and identify the several types of small test tubes used in the experiment.

Have students bring in six different foods they want to test for transgenes. Fresh or dry food products work well with the DNA extraction protocol outlined below. Food products should contain either soy or corn as an ingredient. Products that have been tested successfully using this procedure include corn and tortilla chips, artificial bacon bits, corn muffin mix, granola and energy bars, protein powder, and pet food.

If extracting DNA from seed embryos, soak the wild-type and Roundup Ready® soybean seeds in separate containers of distilled water for a minimum of 30 minutes. This will soften the seeds, making the embryos easier to remove.

Each lab team will set up four of six kinds of reactions. Each team will test a food product of their choice. Half of the teams will set up a positive (+) and half a negative (–) control, according to the scheme below:

Test Item	35S Primers	Tubulin Primers	Teams
Soy or corn food product	✓	✓	All
Wild-type soybean tissue	✓	✓	– control team
Roundup Ready® soybean tissue	✓	✓	+ control team

The cell walls of living plant tissue and the granular structure of dried foods typically are broken up by grinding with a mortar and pestle. This can be accomplished directly in a 1.5-mL tube with the plastic pestle provided in the kit.

Set up a 95°C heating block, or one boiling water bath per 12 samples. A boiling water bath can be made in one of two ways:

- Place tubes in a floating test tube rack within a beaker of water atop a hot plate. Regulate temperature to maintain a low boil.
- Fill a beaker with water and cover tightly with a double layer of aluminum foil. Use a pencil to punch holes to hold the tubes, and maintain at low boil with a hot plate.

Watch out for lids opening as the tubes heat.

Pre-lab Setup for DNA Isolation (per student team)

Soy or corn food product
Wild-type or Roundup Ready® soybean tissue (leaf or embryo)
Edwards' buffer, 2.2 mL
Isopropanol, 1 mL
Tris/EDTA (TE) buffer with RNase A, 300 µL (thaw and store on ice)
1.5-mL microcentrifuge tubes
Permanent marker
Pellet pestles
Micropipet and tips (100–1000 µL)
Microcentrifuge tube rack
Container with cracked or crushed ice

Shared Items

Microcentrifuge
Water bath or heating block (95–100°C)
Vortexer (optional)

III. AMPLIFY DNA BY PCR

Two PCR reactions are performed for each plant or food sample. One primer set amplifies the 35S promoter from cauliflower mosaic virus. The presence of a 35S product is diagnostic for the presence of a transgene, since the 35S promoter is used to drive expression of the glyphosate (Roundup®) resistance gene or *Bt* gene in edible crops. A second primer set amplifies a fragment of a tubulin gene and controls for the presence of plant template DNA. Since the tubulin gene is found in all plant genomes, the presence of a tubulin product indicates amplifiable DNA in the sample isolated. The following table interprets the possible combinations of results for 35S and tubulin amplifications from soybeans and food products.

Plant or Plant Product	35S Promoter	Tubulin	Interpretation
Wild-type soybean	Negative	Positive	Transgene absent
Roundup Ready® soybean	Positive	Positive	Transgene present
Product A	Negative	Positive	Transgene absent
Product B	Positive	Positive	Transgene present
Product C	Negative	Negative	DNA template absent

Each Ready-to-Go™ PCR Bead contains reagents so that when brought to a final volume of 25 µL, the reaction contains 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 mM of each dNTP.

The lyophilized *Taq* DNA polymerase in the Ready-to-Go™ PCR Bead becomes active immediately upon addition of the primer/loading dye mix. In the absence of thermal cycling, "nonspecific priming" at room temperature allows the polymerase to begin generating erroneous products, which can show up as extra bands in gel analysis. *Therefore, work quickly. Be sure the thermal cycler is set and have all experimenters set up their PCR reactions as a coordinated effort. Add primer/loading dye mix to all reaction tubes, then add each student template, and begin thermal cycling as quickly as possible. Hold reactions on ice until all are ready to load into the thermal cycler.*

Each primer/loading dye mix incorporates the appropriate primer pair (0.26 picomoles/µL of each primer), 13.9% sucrose, and 0.0082% cresol red. The inclusion of loading dye components, sucrose and cresol red, allows the amplified product to be directly loaded into an agarose gel for electrophoresis. The primer/loading dye mix may collect in the tube cap during shipping; pool the reagent by spinning the tube briefly in a microcentrifuge or by tapping the tube end on the desktop.

PCR amplification from crude cell extracts is biochemically demanding, and requires the precision of automated thermal cycling. However, amplification of the 35S and tubulin loci is not complicated by the presence of repeated units. Therefore, the recommended amplification times and temperatures will work adequately for all types of thermal cyclers.

Pre-lab Setup for DNA Amplification (per student team)

Food Product DNA, from Part II (store on ice)
Wild-type or Roundup Ready® soybean DNA, from Part II (store on ice)
50 µL 35S primer/loading dye mix (thaw and store on ice)
50 µL tubulin primer/loading dye mix (thaw and store on ice)
4 Ready-to-Go™ PCR Beads (in PCR tubes)
Permanent marker
Micropipet and tips (1–20 µL)
Microcentrifuge tube rack
Container with cracked or crushed ice

Shared Items

Mineral oil, 5 mL (depending on thermal cycler)
Thermal cycler

IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

Prepare a 1× concentration of TBE by adding 75 mL of 20× concentrated stock into 1425 mL of deionized or distilled water. Mix thoroughly.

Prepare a 2% agarose solution by adding 2 g of agarose to 100 mL of 1× TBE in a 500-mL flask or beaker. Heat the flask or beaker in a boiling water bath (approximately 15 minutes) or in a microwave oven (approximately 4 minutes) until the agarose is *completely* dissolved. You should no longer see agarose particles floating in solution when the beaker is swirled. Allow the agarose to cool to approximately 60°C, and hold at this temperature in a hot water bath. Cover beaker or flask with aluminum foil, and skim any polymerized "skin" off the top of the solution before pouring.

The cresol red and sucrose in the primer mix function as loading dye, so that amplified samples can be loaded directly into an agarose gel. This is a nice time saver. However, since it has relatively little sugar and cresol red, this loading dye is more difficult to use than typical loading dyes. So, encourage students to load carefully.

Plasmid pBR322 digested with the restriction endonuclease *Bst*NI is an inexpensive marker and produces fragments that are useful as size markers in this experiment. The size of the DNA fragments in the marker are 1857 bp, 1058 bp, 929 bp, 383 bp, and 121 bp. Use 20 µL of a 0.075 µg/µL stock solution of this DNA ladder per gel. Other markers or a 100-bp ladder may be substituted.

View and photograph gels as soon as possible after appropriate staining/destaining. Over time, at room temperature, the small-sized PCR products will diffuse through the gel and lose sharpness.

Pre-lab Setup for Gel Analysis (per student lab team)

Wild-type soy 35S and tubulin PCR products from Part III (store on ice)
Roundup Ready® soy 35S and tubulin PCR products from Part III (store on ice)
Food product 35S and tubulin PCR products from Part III (store on ice)
pBR322/*Bst*NI markers, 43 µL per two teams (thaw and store on ice)
2% agarose in 1× TBE (hold at 60°C), 50 mL per gel
1× TBE buffer, 300 mL per gel
Micropipet and tips (1–100 µL)
Microcentrifuge tube rack
Electrophoresis chamber and power supply
Latex gloves
Staining tray
Container with cracked or crushed ice

Shared Items

Water bath for agarose solution (60°C)
Transilluminator with digital or instant camera (optional)
Ethidium bromide (1 µg/mL), 250 mL
or
*Carolina*BLU™ Gel and Buffer Stain, 7 mL
*Carolina*BLU™ Final Stain, 250 mL

*Carolina*BLU™ STAINING

POST-STAINING

1. Cover the electrophoresed gel with the *Carolina*BLU™ Final Stain and let sit for 20–30 minutes. Agitate gently (optional).
2. After staining, pour the stain back into the bottle for future use. (The stain can be used 6–8 times.)
3. Cover the gel with deionized or distilled water to destain. Chloride ions in tap water can partially remove the stain from the DNA bands and will cause the staining to fade.
4. Change the water 3 or 4 times over the course of 30–40 minutes. Agitate the gel occasionally.
5. Bands that are not immediately present will become more apparent with time and will reach their maximum visibility if the gel is left to destain overnight in just enough water to cover the gel. Gels left overnight in a large volume of water may destain too much.

PRE-STAINING

*Carolina*BLU™ can also be used to stain the DNA while it is being electrophoresed. Pre-staining will allow students to visualize their results prior to the end of the gel run. However, post-staining is still required for optimum viewing.

To pre-stain the gel during electrophoresis, add *Carolina*BLU™ Gel and Buffer Stain in the amounts indicated in the table below. Note that the amount of stain added is dependent upon the voltage used for electrophoresis. *Do not use more stain than recommended. This may precipitate the DNA in the wells and create artifact bands.*

Gels containing *Carolina*BLU™ may be prepared one day ahead of the lab day, if necessary. However, gels stored longer tend to fade and lose their ability to stain DNA bands during electrophoresis.

Use the table below to add the appropriate volume of *Carolina*BLU™ stain to the agarose gel:

Voltage	Agarose Volume	Stain Volume
<50 Volts	30 mL	40 µL (1 drop)
	200 mL	240 µL (6 drops)
	400 mL	520 µL (13 drops)
>50 Volts	50 mL	80 µL (2 drops)
	300 mL	480 µL (12 drops)
	400 mL	640 µL (16 drops)

Use the table below to add the appropriate volume of *Carolina*BLU™ stain to 1× TBE buffer:

Voltage	Agarose Volume	Stain Volume
<50 Volts	500 mL	480 µL (12 drops)
	3000 mL	3 mL (72 drops)
>50 Volts	500 mL	960 µL (24 drops)
	2600 mL	5 mL (125 drops)

BIOINFORMATICS

Have students do the bioinformatics exercises before starting the experiment—or analyzing results. This should improve conceptual understanding of the purpose of the tubulin control and the relationship between the 35S promoter and transgenes. It should also improve practical understanding of the results of gel electrophoresis.

ANSWERS TO BIOINFORMATICS QUESTIONS

I.3. What is the predicted length of the product that the primer set would amplify in a PCR reaction (in vitro)? **The first primer set amplifies a 162-bp product. The second primer set amplifies a 187-bp product.**

I.4. What DNA sequence does this primer set amplify? Is this the primer set to detect a GM product or the control primer set? **The first primer set amplifies the 35S promoter of cauliflower mosaic virus (cmV), and detects a GM product. The second primer set amplifies tubulin, the positive control for plant DNA.**

II.5. What do you notice about the *E-values* obtained by this search? Why is this so? **The *E-values* are much smaller (having many more decimal places), because the query sequence is longer.**

ANSWERS TO DISCUSSION QUESTIONS

1. Describe the purpose of each of the following steps or reagents used in DNA isolation (Part II):
   a. **Grinding with the pestle pulverizes dry food and breaks cell walls of soybean or other fresh tissue.**
   b. **The detergent component of Edwards' buffer, sodium dodecyl sulfate, dissolves lipids that compose the cell membrane.**
   c. **Boiling denatures proteins, including DNases that would damage the DNA template needed for PCR.**
   d. **Tris-EDTA (TE) buffer provides conditions for stable storage of DNA. Tris provides a constant pH of 8.0, while EDTA binds cations (positive ions) that are required for DNase activity. RNase A digests RNA, which composes the vast majority of nucleic acids isolated from living cells.**
2. What is the purpose of performing each of the following PCR reactions:
   a. tubulin? **Positive control for the presence of amplifiable plant DNA in the sample.**
   b. wild-type soybean? **Negative control for absence of the 35S promoter.**
   c. Roundup Ready® soybean? **Positive control for the presence of the 35S promoter.**
3. Which sample(s) in the gels pictured show the following banding patterns? Explain what each pattern means.

187 bp (tubulin)	162 bp (35S)	Sample showing this pattern and explanation
present	present	**Roundup Ready® soybean and Food 2: transgene present**
present	absent	**wild-type soybean and Food 1: transgene absent**
absent	absent	**Not shown: no template DNA**
absent	absent	**Not shown: no PCR reaction**

CD-ROM CONTENTS

The valuable companion CD-ROM is for exclusive use of purchasers of this DNA Learning Center Kit. To accommodate home or computer lab use by students, all materials may also be reached at the companion Internet site http://bioinformatics.dnalc.org/gmo.

- **Protocol:** a unique online lab notebook with the complete experiment, as well as printable PDF files.
- **Resources:** 13 animations on key techniques of molecular genetics and genomic biology, from the award-winning Internet site, *DNA Interactive*.

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