

Protocols for Detection of Begomovirus

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22 February 2007

Skills. You should be able to do the following:

1. Isolate DNA from plant samples.
 2. Perform a polymerase chain reaction assay.
 3. Analyze the PCR results by electrophoresis on an agarose gels.
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The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a powerful technique that has widespread application in molecular biology. This technique is used to amplify a specific nucleic acid fragment that lies between two regions of known nucleotide sequence, and often from an extremely small amount of target nucleic acid in biologically complex samples. Amplified fragments can then be further characterized by size fractionation on agarose gels, restriction enzyme digestion, hybridization with probes or by DNA sequencing. The specificity of the PCR is based on the use of oligonucleotide primers that are complementary to the regions flanking the fragment to be amplified. Because of the sensitivity and specificity of PCR techniques, these procedures will likely have widespread application to the detection of plant pathogens, and can be used to answer precise questions about identification of pathogens, populations and variability.

History. The polymerase chain reaction (PCR) was first proposed by Ghobind Khorana et al. in 1971, but several elements required for PCR, particularly thermostable DNA polymerase, had not yet been discovered at that time. They did not further develop their idea. In 1983, Kary Mullis independently developed PCR. By 1983, scientists had a better understanding of how DNA synthesis worked, but they did not yet have thermal stable enzymes. Mullis used *Escherichia coli* polymerase I to polymerase chains of DNA. However, enzyme had to be added for cycle of the chain reaction, so the technique was very clumsy. In 1988, Saiki and colleagues had found that by using thermal stable DNA polymerase from *Thermus aquaticus*, an archaea species that lives at high temperatures (50-85°C), they no longer needed to add additional polymerase for every cycle of the polymerase chain reaction. Since 1988, PCR has become one of the most important tools for molecular biologists.

The benefits of PCR over ELISA include:

1. PCR can detect lower pathogen amounts.
2. PCR assay development is faster and cheaper than ELISA because no antibodies need to be produced.
3. Because there are no antibodies used, antibody cross reaction is not a problem.
4. Very small amounts of sample are needed for PCR, and sample tissues can be fresh, dried, or frozen, and even partially degraded.

PCR-based assays are most advanced for the detection of important viral pathogens of humans. PCR methods are also used to detect and characterize plant viruses, viroids and phytoplasmas.

Because of the sensitivity of PCR, viral infections which cannot be detected by serological methods can be readily detected with PCR methods. And PCR is not dependent on the presence of expressed proteins (antigens) but only on the presence of the target nucleic acid sequence. As more information becomes available on the molecular genetics of other pathogens, it is expected that PCR will gain importance in diagnosis.

There are many components required for a PCR, including:

Thermal stable polymerase. Many varieties of thermal stable polymerase are now commercially available. They vary in characteristics such as efficiency and accuracy. For routine PCR, the *Taq* enzyme from *Thermus aquaticus*, is commonly used.

Primers. A pair of synthetic oligonucleotides are required for the polymerase to initiate DNA synthesis. The oligonucleotides are commonly called “oligos” or “primers.” Primers are design to anneal to DNA sequences bordering the region to be amplified. Many factors are important in designing primers (see below).

dNTPs. Standard PCRs contain equal amounts of the four nucleotides that make up chains of DNA: dATP, dCTP, dGTP, and dTTP. The DNA polymerase (*Taq*) uses these nucleotides to synthesize the new chains of DNA during PCR.

Divalent Cations. Many DNA polymerases require divalent cations for activity and the concentration of the divalent cations affects the efficacy of the PCR. Usually Mg^{2+} is used in PCRs and, since the dNTPs and primers also bind Mg^{2+} the molar concentration of the Mg^{2+} must exceed that of the dNTPs and primers so that some of the divalent cation is available to the DNA polymerase. Usually a concentration of 1.5 mM is used in PCR, but this concentration may be altered if the PCR is not successful.

Buffer. All enzymes have optimal pH values at which they are most efficient. PCRs are usually buffered with 10 mM TRIS-HCl to a pH of 8.3-8.8 at room temperature. When the PCR is incubated at 72°C, the temperature at which the *Taq* DNA polymerase replicates the chains of DNA, the pH of the buffer drops to approximately 7.2.

Template DNA. The template DNA can be either double stranded or single stranded. It can also be either DNA isolated from an organism such as a plant or bacterium, or it can be cDNA prepared from RNA from a virus or an expressed gene. PCR can amplify many copies of DNA from a single chain of DNA, but typically several thousand copies of DNA are added to a reaction mixture.

The Polymerase Chain Reaction

PCR consists of three steps that are generally repeated 30 times. After each cycle of three steps, the amount of synthesized DNA in the reaction doubles until the reaction components are used up. A thermal cycler machine is used to heat and cool the PCRs during these repeating steps.

Step 1. Denaturation

The first step is to heat the DNA to 94°C, which denatures, or melts, the double stranded DNA (dsDNA) into single strands. Generally a denaturation step lasts for 30 to 60 seconds.

Step 2. Annealing


The second step is to cool the reaction so that the primers (synthetic oligonucleotides) bind to the template DNA. The annealing (binding) temperature used depends on how cool the reaction must be to allow the primers to anneal (bind). This temperature is called the “melting” temperature of the primers and researchers have used different calculations to determine this temperature. It is important that the melting temperature be similar for both primers in the PCR (see primer design section). Generally an annealing temperature of 45-60°C is used.

Step 3. Extension.

The third step is for the thermal stable DNA polymerase (*Taq*) to extend the DNA chain from the primer by adding the dNTPs to the new, growing DNA chain. The extension time depends both on the speed of the polymerase and the length of the DNA being amplified. With *Taq* polymerase, generally 1 minute per 1000 nucleotides (1 kilobase) is used. Generally the extension step occurs at 72°C, but sometimes a lower temperature is used.

A typical PCR profile or program to amplify a 1500 nucleotide (nt) DNA fragment on a thermal cycler:

94°C for 30 seconds
55°C for 45 seconds
72°C for 90 seconds



the three steps are repeated 30 times

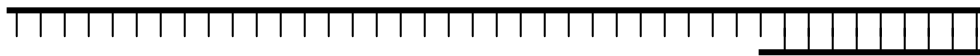
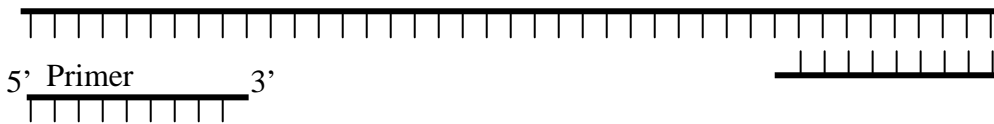
Each time the cycle is repeated, the amount of synthesized DNA doubles until one of the reaction components, such as the primers or dNTPs, is used up. Therefore after:

1 cycle	2 copies
2 cycles	4 copies
3 cycles	8 copies
4 cycles	16 copies
10 cycles	1024 copies
20 cycles	over 1,000,000 copies!



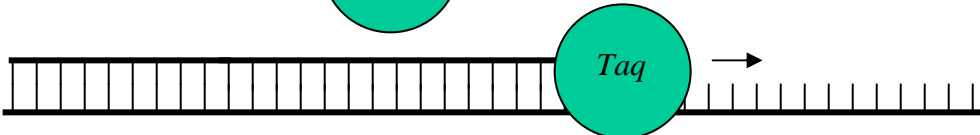
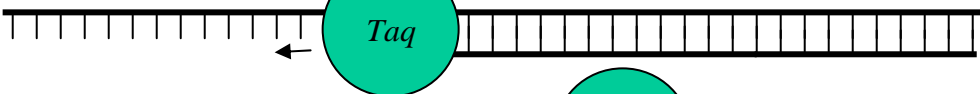
Step 1.
Denaturation

30-60 sec.
94°C



Step 2.
Annealing

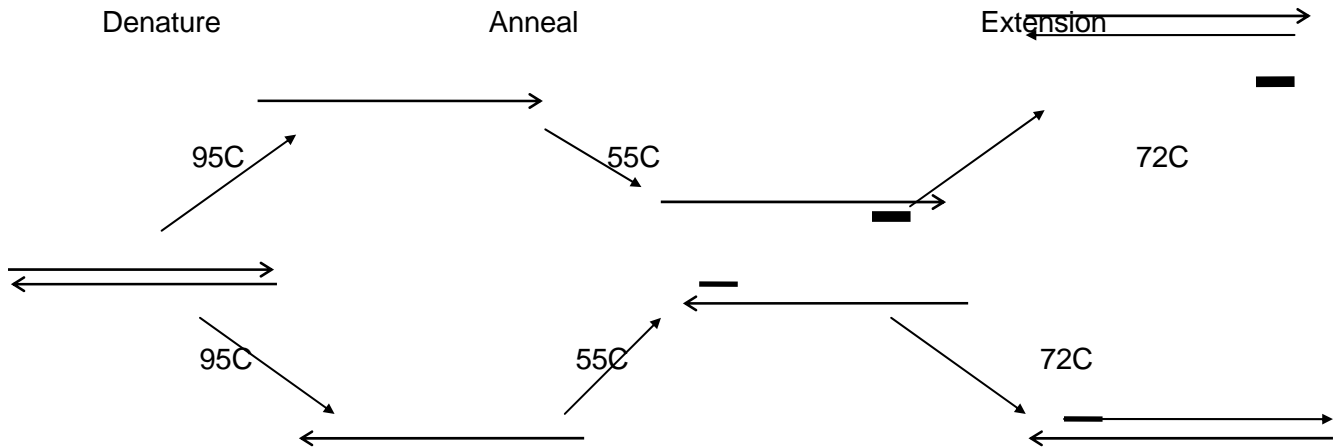
30-60 sec
40-60°C





Step 3.
Extension

60 sec/1000 nt
60-72°C

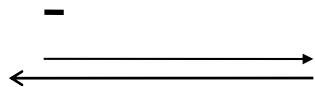
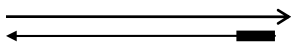
Return to
step 1.



primer 1 
 primer 2 

Schematic diagram of the polymerase chain reaction. Step-cycle temperature profiles for PCR cycling. 1 = denaturation step (95 C); 2 = annealing step (55 C); 3 = extension step (72 C).

Shown above is a diagram of the first cycle of a PCR. Complete the second cycle in the space below.



Primer Design

Length. The main goal of primer design is specificity. The primers should anneal only to the sequence that one is trying to amplify and not to multiple sites. In general, the longer the primer, the more specific it is. However, DNA in many organisms contains repetitive sequences and if a primer is accidentally designed to anneal to a repetitive sequence, it will not be specific to a particular region of DNA. Generally, primers that are 18-24 nucleotides (nt) long are long enough to anneal specifically to a particular stretch of DNA.

Template DNA. Regions of template DNA that are repetitive or that anneal itself after the denaturation step are difficult to amplify by PCR.

Self-complementary primers. Primers should not be self-complementary or they will anneal to themselves and not to the template DNA. One primer in a primer pair should also not complement the other primer.

Example of self-complementary primers:

```
5' ATTCCGGTCGACCGGAAT 3'
   |||||
3' TAAGGCCAGCTGGCCAAT 5'
```

Example of non-self-complementary primers:

```
5' ATTCCGGTCATTCCGATC 3'
   || | |
3' CTAGCCTTACTGGCCTTA 5'
```

3' primer end. The 5' end of a primer does not have to match the template sequence, but the 3' end is crucial for specificity. If possible, the 3' end should be either a G or a C. However, the primer sequence should not end with a CG or GC because these sequences are self-complementary and the primers may bind to each other and not to the template sequence.

Example:

```
5' ATTCCTATTACCAAACG 3'
      ||
3' GCAAACCATTATCCTTA 5'
```

G+C content. DNA consists of four nucleotides, G, A, C, and T. The A and T nucleotides anneal to each other with two hydrogen bonds and the G and C nucleotides anneal to each other with three hydrogen bonds. Therefore, G-C bonds are stronger than A-T bonds and the percent G or C in a primer will affect how well it anneals to the template DNA. Primers should contain 40-60% G or C.

Annealing (melting) temperature. The melting (denaturing) temperature of primers needs to be higher than the annealing temperature so that the primers anneal to the template DNA before the extension step begins. Ideally, the melting temperature of the primers in a PCR

primer pair are within 3°C of each other and should never be above 85°C. There are two simple equations that can be used to estimate the melting temperature of primers:

The Wallace equation (predicts T_m in high salt conditions):

$$T_m = 2(A+T) + 4(G+C) \quad T_m = \text{melting temperature in } ^\circ\text{C}$$

The Bolton McCarthy equation (predicts T_m in conditions up to 0.4M KCl):

$$T_m = 81.5^\circ\text{C} + 16.6 (\log_{10}[K^+]) + .41(\%[G+C]) - (675/n)$$

Where n = number of nucleotides in the primer and K^+ = potassium concentration in the buffer.

Computer programs. There are many computer programs available to assist with primer design. These programs save time and money because primers are designed more quickly and because people are less likely to make mistakes in primer design that require them to order multiple primers for a single purpose.

Primer Design Questions:

1. Write the complementary sequence below this DNA sequence:

5' AGACGGCGCGTGACCGTTAATCACATCAG 3'

2. Why would the following sequences make poor PCR primers?

5' ATCGCGAATCGCGCGAT 3'

5' ATAATCTTATATGTCTAA 3'

5' AGCGGGCT 3'

3. Design two primers from this sequence that would allow you to amplify a DNA fragment between 200 – 300 nucleotides long:

1 GACGGTAGCCGACCTGATAATCAACGCCATCCCTGACGATATACAAGTAA

51 AGGCGACCGGTATCGTAGTTACCGTCATCTTCACCCACAGCTTGACGGA

101 AACGTAGTTACGGCGCAGCGGATCCACCCGCAGGGTAACCGTCAGGCTGC

151 CGCCATACACGCCTGCCGGGCTGAGCGGCAGTAATTTACGGGCGGATTCCG

201 CCCAGCCCGCCGGAGAGCGTCTGGCTATTATTTTCGATCAGACTATGGCT

251 TTTCTCCGACGCGCTGTGCGCAAACGAAACCTGGTCAATCGTCCCAACCG

301 CCGTAGCCGCGGCCGATGACGACGTGGCTCCTGAACCCGAACTTTTCGGCG

351 GCAATGGCCGCATGATTTGCCGTTGTAGCCGCCAGCGCCAGCGCAACCAG

401 CCACGCCGGTCTGAACACCGTGTTTTTCTGCATAGGTCAGTCCCTTTTTTC

451 CAGTTTTTTATCATCCGTGCAGTCCTATCAGATGACAATGACAAAACGAA

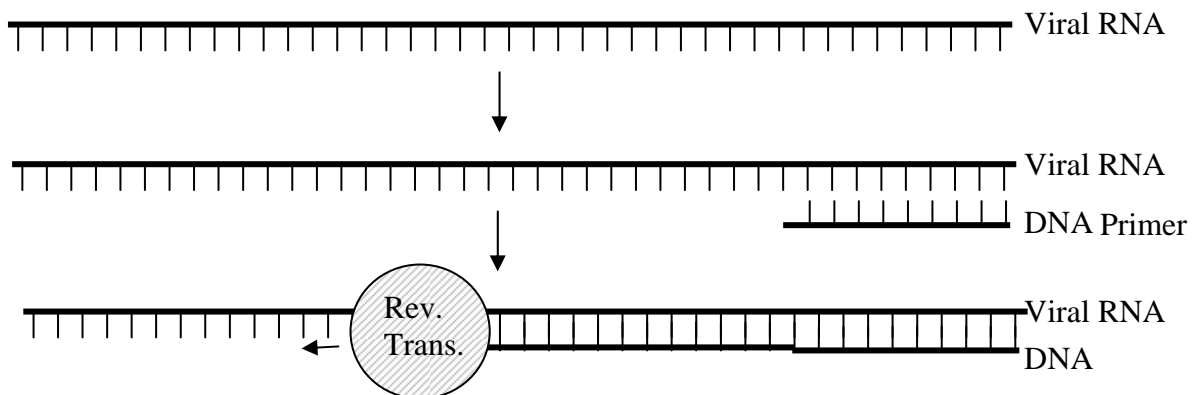
501 TACATCATTACCACACTAATACTTCCATCAATTAACAATAGTTTCTCGAC

Reverse Transcriptase PCR (RT-PCR)

The nucleic acid of most viruses is RNA, not DNA, and the Taq polymerase can only amplify DNA fragments. Therefore before PCR can be used to amplify viral RNA, the RNA must be reverse transcribed to DNA. This step is called “reverse” because usually DNA is transcribed to RNA, not the reverse of RNA being transcribed to DNA.

An enzyme called **reverse transcriptase (RT)** is used to make a DNA copy of viral RNA prior to PCR. First, a DNA oligo (primer) is annealed to the viral RNA, then the RT enzyme transcribes the RNA sequence to a complementary DNA sequence. These DNA copies are then used as the template for PCR.

Generally, the easiest way to perform RT-PCR to amplify nucleic acid sequences from viruses is to purify viral RNA using a commercial reagent such as Trizol (Life Technologies, Inc.) and RT-PCR beads (Amersham Pharmacia Biotech), which contain all reagents necessary for both the RT and the PCR steps.



Draw the two rounds of PCR using the reverse transcribed viral DNA template

PCR trouble shooting

What to do if the PCR fails?

- 1. Try adjusting the Mg²⁺ concentration.** The divalent cation Mg²⁺ is required for the DNA polymerase to function. If the concentration is not correct, the PCR will fail. Prepare four PCRs with a Mg²⁺ concentration of 1.5 mM, 2.5 mM, 3.5 mM, and 4.5 mM to determine the optimal Mg²⁺ concentration.
- 2. Try adjusting the annealing temperature.** The annealing temperature may be too high or too low for the primers to efficiently anneal to the template DNA. Many thermal cyclers have a gradient feature that allows multiple annealing temperatures to be tried at one time. If the PCR fails, prepare multiple reactions and try annealing temperatures between 45°C and 60°C.
- 3. Try a new DNA extraction method.** Many chemicals can inhibit the PCR, including proteinases, EDTA, phenol, and many substances present in plant and soil samples. Therefore, it is important to use appropriate DNA extraction protocols for a particular sample. If the PCR is repeatedly failing, different DNA extraction protocols should be tested.
- 4. Try adding DMSO.** The chemical DMSO can aid some PCR reactions. If the PCR is failing or a small amount of product is present, add DMSO to a final concentration of 2% (1 μ l in a 50 μ l PCR reaction).
- 5. If all else fails - try new primers.** If primers are designed correctly and other optimization methods have been tried, but the PCR still fails, it may be due to the primers annealing to other sections of the template DNA or to secondary structure in the template DNA inhibiting the primer annealing and polymerase extension. One solution is to re-design primers to anneal to a different section of the template DNA.

How to Avoid Contamination

PCR is a very sensitive technique and contamination is a common problem. It is critical to run a negative control with all PCRs to determine that contamination has not occurred. To reduce the chance of contaminating PCRs:

Set up all reactions in a laminar flow hood. The hood may be equipped with a UV light that can be turned on to decontaminate the pipets and hood surfaces (CAUTION – the hood opening must be covered with an opaque cloth when the UV light is on because exposure to UV light can cause blindness!)

Use filter-tips on pipettes. If these are not available, clean the pipettor ends with 70% ethanol frequently to remove contamination.

Wear gloves when setting up PCR reactions.

Make small aliquots of solutions used for PCR and discard aliquots after each use. Never use PCR reagents or pipettes for other purposes.

Do not work with tubes containing amplified DNA in the same area that the PCRs are set up.

Agarose Gels

Gel electrophoresis is one of the most widely used techniques in molecular biological research. It allows the separation of charged macromolecules in an electric field based on the size and charge of the molecules. DNA molecules have a negative charge and thus move towards the + pole in an electric field. In gel electrophoresis, the DNA samples are separated in a gel made of agarose. The DNA molecules migrate through the pores in the gel depending on their shape and size as well as charge. Small molecules move more rapidly than larger molecules with a loose conformation. The size of an unknown DNA molecule is determined by comparing its rate of migration with DNA molecules of known sizes (the marker DNA, a 1 kb ladder). After a given period of time (40 min to 2 hr), the location of the DNA molecules is visualized by staining the DNA with a dye, ethidium bromide, which makes the DNA molecules fluorescent when they are irradiated with ultraviolet light. These gels are then photographed to provide a permanent record.

One way to imagine this process is to visualize a hallway filled with netting (agarose). A mouse, cat, dog, and horse (representing different sized DNA fragments) are released at one end of the hallway. The small mouse moves very quickly through the netting. The larger cat moves more slowly because it can not fit through the holes as easily. The even larger dog moves very slowly because it can not fit through most of the holes in the netting. The horse is not able to move through the netting at all and makes no progress down the hallway.

To examine PCR products, an agarose gel is prepared in a running buffer (usually TBE or TAE; see below). The agarose gel has wells on one end that DNA is loaded into. The DNA products from the PCRs are mixed with a loading buffer that contains dyes to follow the progress of the electrophoresis and glycerol to help the DNA solution sink to the bottom of the gel wells.

After the gel has been placed in running buffer in an electrophoresis unit and been loaded with the PCR products, an electric current is applied using a power source and the DNA moves through the gel. The smaller the DNA fragments, the faster they move through the gel. When electrophoresis is complete, the DNA is visualized by staining it with a fluorescent dye (usually ethidium bromide). (CAUTION! Ethidium bromide is a carcinogen. Do not expose skin to ethidium bromide. Check with institutional safety officer to determine what precautions must be taken before disposing ethidium bromide at your facility.) and examining the gel on a UV light box. (CAUTION! Exposure to UV light may cause blindness and eye protection must be worn.) The DNA fragments are compared to a commercial size standard to determine what size DNA fragments have been amplified by the PCRs.

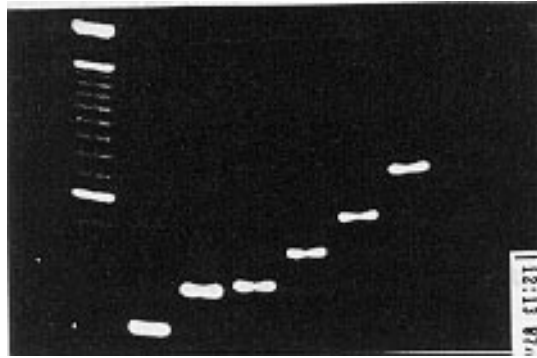


Figure. Photograph of an electrophoresis gel separating DNA fragments of different sizes. The DNA fragments are the light bands on the black background. Lanes 2-7 each contain DNA from a different polymerase chain reaction. Lane 1 has a mixture of DNA fragments of known size. These differ in size by 100 nucleotides or "letters" and the smallest one is 200 nucleotides. Compare the sizes of the DNA fragments in lane 2 and lane 3. They are different, so these two fungi, the unknown #7 and *S. borealis*, are different. Lane 1 = the known sizes of DNA used as a standard, Lane 2 = ITS A DNA from *S. borealis*, Lane 3 = ITS A DNA from unknown #7, Lane 4 = ITS B DNA from *S. borealis*, Lane 5 = ITS B DNA from unknown #7, Lane 6 = ITS1-4 DNA from *S. borealis*, Lane 7 = ITS1-4 from unknown #7.

PCR— Extraction of DNA from plant samples

*****PCR is a very sensitive technique. It is important to wear gloves and take care to reduce the chance of contamination at each step.*****

Heat Extraction with DELLAPORTA

(see Potter et al. 2003. Plant Disease 87:1205-1212.)

DNA EXTRACTION FROM PLANT CELLS AND PROTOPLASTS

This is a modification of a Dellaporta procedure obtained from Cooley and Russell (Agracetus inc.).

For plant tissue; Obtain a small amount of tissue (2-3 cork borer #4 circles for fresh or frozen tissue, 5mg for dry tissue).

For protoplasts; Resuspend sample in 500 µl of Dellaporta sample buffer.

1. Add 500ul Dellaporta extraction buffer:

150 ul BME (beta mecaptoethanol)	10mM BME
5 ml 1M Tris pH 8.0	100mM Tris pH 8.0
5 ml 0.5M EDTA pH 8.0	50mM EDTA
5 ml 5M NaCl	500mM NaCl
<u>34 ml dds H2O</u>	
50ml total	

2. For dry tissue: leave on ice for ~20 minutes with 500ul Dellaporta.
3. Grind tissue using a Kontes pestle.

4. Incubate at 65°C for 5 min
5. Spin for 10 minutes at 14,000 rpm centrifuge
6. Remove Supernatant to new tube. Discard tissue debris.
7. Dilute 1:10 and 1:100 for PCR. Use without dilution for hybridization
8. Store at -20 C.

DELLAPORTA

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For plant tissue; Obtain a small amount of tissue (2-3 cork borer #4 circles for fresh or frozen tissue, 5mg for dry tissue).

For protoplasts; Resuspend sample in 500 µl of Dellaporta sample buffer.

1. Add 250 I Dellaporta extraction buffer:

150 ul BME (beta mecaptoethanol)	10mM BME
5 ml 1M Tris pH 8.0	100mM Tris pH 8.0
5 ml 0.5M EDTA pH 8.0	50mM EDTA
5 ml 5M NaCl	500mM NaCl
<u>34 ml dds H2O</u>	
50ml total	

Grind tissue using a Kontes pestle. Add 250 I more Dellaporta buffer and mix.

Optional step: leave on ice for ~20 minutes with 500 I Dellaporta, then grind with pestle.

2. Add 33 µl of 20% SDS (10g/50ml ds H2O) and invert tube to mix. Incubate at 65°C for 10'.
3. Add 160 µl of 5 M potassium acetate (245g KoAC/ 500ml dsH2O), vortex and spin for 10' at 10,000- 12,000 rpm.
4. Transfer 500-600 µl of supernatant to a clean tube. Add 160ul of %M KoAC, vortex, spin at 12,00 rpm for 5 minutes. Remove 700ul of supernatant to new tube.
Optional step for dirty pellets, add 700 µl of PCI, shake for 5' then spin for 5' at 10,000 rpm. Take top aqueous phase to new tube (~ 500-600 ul).
5. Add 0.5 volume (350 µl) of isopropanol, ice cold. Mix well and centrifuge for 10' at 12,000 rpm.
6. CAREFULLY, Aspirate supernatant (easier with pipette). Beware large pellets will slide to bottom of tube or dislodge and become aspirated. Leave 10-15ul S/N behind if necessary.

7. Wash with 500ul of 70% ethanol, spin for 5 minutes at 12,000 rpm, carefully remove as much S/N as possible by pouring.
8. Air dry for 1 hour or use speed vac for 5 minutes.
9. Resuspend in 500 µl of H₂O or TE. Use 1 to 20 ul in PCR. Store at -20 C.

PUREGENE DNA Purification Kit (commercial kit)

DNA Purification From 10-20 mg Dried or 20-60 mg

Fresh or Frozen Leaf Tissue

Expected Yield Range 3-30 µg DNA

Cell Lysis

1. Add 10-20 mg dried tissue (finely ground), 20-60 mg frozen tissue (may be finely ground with a mortar and pestle in liquid nitrogen), or 20-60 mg fresh leaf tissue (2-5 disks) to a 1.5 ml tube. A leaf disk (7 mm diameter) may be prepared by placing the leaf between the microfuge tube and its cap and then snapping the cap closed. Work quickly and keep tissue cold to minimize DNase activity. Note: it may be necessary to vary the amount of starting material depending upon the species, age, tissue preparation and genome size.
2. Add 600 µl **Cell Lysis Solution** to the leaf tissue. For dried tissue, vortex 1-3 seconds to wet the tissue. For unground tissue, homogenize using 30-50 strokes with a microfuge tube pestle.
3. Incubate cell lysate at 65°C for 60 minutes. After 30 and 60 minutes invert tube 10 times.

RNase Treatment (Optional)

1. Add 3 µl **RNase A Solution** to the cell lysate.
2. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15 minutes.

Protein Precipitation

1. Cool sample to room temperature.
2. Add 200 µl **Protein Precipitation Solution** to the cell lysate.
3. Vortex vigorously at high speed for 20 seconds to mix the **Protein Precipitation Solution** uniformly with the cell lysate. Alternatively, invert rack containing the samples 150 times (approximately 2 minutes) to mix the **Protein Precipitation Solution** uniformly with the cell lysate. For species with high polysaccharide content, it may be necessary to incubate sample on ice for 5-15 minutes.
4. Centrifuge at 13,000-16,000 x g for 3 minutes. The precipitated proteins should form a tight, green pellet. The supernatant may range in appearance from brown to green depending on the sample. If the pellet is not tight, repeat Step 3, followed by incubation on ice for 5 minutes, and then repeat Step 4.

DNA Precipitation

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a 1.5 ml centrifuge tube containing 600 µl **100% Isopropanol** (2-propanol).
2. Mix the sample by inverting gently 50 times.
3. Centrifuge at 13,000-16,000 x g for 1 minute. The DNA will be visible as a pellet that ranges in color from off-white to light green.
4. Pour off supernatant and drain tube briefly on clean absorbent paper. Add 600 µl **70% Ethanol** and invert tube several times to wash the DNA pellet.
5. Centrifuge at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol. *Pellet*

may be loose so pour slowly and watch pellet.

6. Invert and drain the tube on clean absorbent paper and allow to air dry for 10-15 minutes.

(Over)

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DNA Hydration

1. Add 100 μ l **DNA Hydration Solution** (100 μ l will give a concentration of 100 μ g/ml if the total DNA yield is 10 μ g).
2. Rehydrate DNA by incubating sample 1 hour at 65°C and/or overnight at room temperature. If possible, tap tube periodically to aid in dispersing the DNA.
3. If particulates are present in the rehydrated DNA sample, centrifuge at 13,000-16,000 x g for 5-10 minutes and then transfer the supernatant containing the DNA to a clean tube. Store DNA at 4°C. For long-term storage, store at -20°C or -80°C.

Extraction of DNA from *Bemisia tabaci*

Mehta, et al

1. Place 1-5 whiteflies in a microfuge tube containing 50 μ l of STE (0.1 M NaCl, 10 mM Tris-Cl [pH 8.0], 1mM EDTA [pH 8.0]).
2. Grind using a plastic pestle.
3. Immediately after grinding, centrifuge the tubes for 10 min in microcentrifuge at 10,000 rpm.
4. Collect the supernatant and use immediately for PCR.

Zhang, et al

1. Kill insects by freezing in microfuge tube.
2. Add 10uL 0.5M NaOH to each tube.
3. Crush insect with pipette or Kontes pestle for 30 seconds.
4. Add 30uL 1M Tris-HCl buffer (containing 1% SDS and 20mM EDTA, pH8.0), mix well.
5. Incubate solution at 65 C for 15 minutes, centrifuge at 12,000 rpm for 10 minutes.
6. Transfer 30uL of supernatant to new tube.
7. Add 30uL 95% ethanol, mix, and freeze for 30 minutes at -20 C.
8. Precipitate DNA by centrifugation at 12,000 rpm for 10 minutes.
9. Dry pellet at 72 C and resuspend in 30uL 1X TE.

Preparation of a PCR reaction.

(Step two should be done using an ice bucket with ice to keep all components as cold as possible.)

PCR components may be purchased separately or pre-mixed. If possible, use pre-mixed reagents to reduces error and save time when preparing PCRs. We will be using two forms of pre-mixed PCR reagents: Promega Master Mix and Amersham PCR beads.

The PCR mixes are a combination of all ingredients necessary to run a PCR except for the sample DNA and the primers.

1. Nucleotides dATP, dCTP, dGTP, and dTTP. The 'd' identifies the sugar deoxyribose (sugar molecule of DNA). ATP, CTP, etc. provide the adenine, cytosine, guanine, and tyrosine.
2. 2.5 mM Magnesium chloride (MgCl₂).

3. 10X buffer.
4. Taq polymerase (heat stable enzyme needed to build a DNA molecule).

The Promega Master Mix is in liquid form and varying volumes can be used depending on the final volume of PCR desired. The beads contain dehydrated reagents and the same volume must always be used.

To use the Promega Master Mix for PCR, simply add the correct volume of Master Mix, primers, and template DNA to a PCR tube. Master Mix should be stored at -20°C .

Ready to go PCR Beads (commercially available)

1. Cut the required number of tubes from the 96 tube setup.
2. Check that each tube has a bead and tap the tube so the bead is forced to the bottom.
3. Remove the paper/ foil lid from the tube and place on ice. Keep on ice until ready to place in the PCR machine.
4. The PCR reactions are 25 μl .
5. Make up a master mix of 5' and 3' primers (at 5-50 picomoles) and sterile distilled H_2O , add this to each tube.
6. Gently flick the tube to dissolve the pellet
7. Depending on the complexity of the DNA (plasmid 50 pg, genomic DNA 50-100ng), add a volume of the DNA that will give you a final volume of 25 μl .
8. Add mineral oil if required.
9. Apply bubble caps
10. Place in thermocycler and use a standard cycling protocol

Notes: The standard MgCl_2 concentration is 1.5mM, this can be adjusted using sterile 25 mM MgCl_2 . A positive control is provided which gives a 500 bp product.

Set up PCRs with Promega Master Mix.

Component	Volume
PCR Master Mix	25 μl
Primer #1	1 μl
Primer #2	1 μl
DNA from Boil Preparation	5 μl
Sterile Water	8 μl
Final Volume	50 μl

Component	Volume
PCR Master Mix	25 μl
Primer #1	1 μl
Primer #2	1 μl
DNA from InstaGene Prep.	13 μl
Sterile Water	0 μl
Final Volume	50 μl

Step 3: Gel Electrophoresis

Electrophoresis is a technique that allows for the separation of DNA fragments by size by utilizing the negative charge of DNA and the properties of electricity. PCR products are placed into wells at the top of an agarose gel. Electrodes are then placed at the top (negative electrode) and bottom (positive electrode) of the gel. When electricity is applied, the negatively charged DNA will migrate towards the positive electrode. The speed and distance a DNA fragment will migrate depends on the size of the fragment and how well it can move through the agarose. When the electricity is turned off, the DNA will cease to migrate. Gels can then be stained with ethidium bromide (a mutagen that binds to DNA allowing it to fluoresce under UV light) and size of DNA can then be determined.

Exercise: Preparation of a PCR reaction and an agarose gel for electrophoresis to identify an unknown bacterial potato pathogen.

Step 3:

Materials

1. 1% agarose gel
2. Gel box, gel combs, and voltage box.
3. 1 X TAE buffer (TAE = Tris-Acetate-EDTA).
4. 20 μ l pipetteman and a box of yellow tips.
5. One square piece of Parafilm.
6. 6X loading dye
7. PCR products.
8. 100 bp ladder and 1 kb ladder (a liquid that contains different DNA fragment of known size used to determine the size of DNA fragment from a PCR product) in 6 X loading dye.
9. Ethidium bromide (EtBr).
10. InstaStain Sheets.

Protocol

1. Heat a flask containing 1 % agarose gel in TAE buffer until the agarose is completely dissolved.
2. Allow liquid to cool until you can hold the flask in your hand without burning it.
3. Place gel comb at the top of the gel box.
4. Gently pour melted agarose gel into box until the liquid is approximately halfway up the teeth of the comb. Avoid getting air bubbles in the gel.
5. Let gel solidify for approximately 15 minutes.
6. Gently remove comb and cover gel with enough 1 x TAE buffer to cover the gel by about 2 cm.
7. Remove PCR tubes from thermocycler. If mineral oil was used, remove all traces of mineral oil using a pipette. (Hint-Place tubes into a -20 C cooler for 20 minutes. PCR products will freeze but the mineral oil will not allowing it to easily be removed with out loosing any of your product. Melt samples again by holding tube in your fingers for about 1 minute). Set tube aside.
8. On a piece of parafilm, place five 5 μ l drops of 6x loading dye. Make sure each drop is separated from one another.
9. Open each sample tube and remove 10 μ l of product. To one of the 5 μ l drops of loading dye, gently add the 10 μ l of PCR product. Mix dye and product together by drawing liquid back into pipette tip and expelling it back onto the parafilm. Finally, draw the 10 μ l of liquid and place it in one well of the agarose gel.

10. Repeat step 9 for the remaining samples and for the 100 bp standard and 1 kb standard.
11. Slide lid of gel box on so that the black (-) electrode is at the top and the red (+) electrode is at the bottom. Plug cords into voltage box and turn on. Adjust voltage to 100 V. Allow gel to run for 30 minutes to 1 hour or until the dark blue dye is 2 cm from the end of the gel.
12. Turn off voltage box and remove lid. Carefully remove gel from box and place InstaStain sheet over gel. (Caution – the Ethidium Bromide stain is a carcinogen, do not touch without wearing gloves!) (Alternatively, gels may be stained for 15 min in 1 µg/ml Ethidium Bromide in water)
13. Let gel stain for 15 minutes.
14. View gel under UV light.

100 bp DNA ladder

100 bp DNA Ladder is suitable for sizing double-stranded DNA from 100 to 1,500 bp. Prepared from a plasmid containing repeats of a 100-bp DNA fragment, the ladder consists of 15 blunt-ended fragments ranging in length from 100 to 1,500 bp, at 100-bp increments, and an additional fragment at 2,072 bp. The double-stranded ladder can be visualized on 1 to 2% agarose gels after ethidium bromide staining. For easy reference on agarose gels, the 600-bp band is two to three times brighter than the other bands in the ladder. This ladder can be stained with ethidium bromide or radiolabeled using T4 polynucleotide kinase or T4 DNA polymerase.



1 µg/lane, 1.5% agarose gel stained with ethidium bromide

Recipes for Buffers used with Agarose Gels.

Tris Acetate EDTA (TAE) Gel running buffer
 1x working solution 1 liter of 50x stock solution
 40 mM Tris-acetate 242 g Tris base
 1 mM EDTA 57.1 ml glacial acetic acid (Hazardous, open in fume hood)
 100 ml of 0.5 M EDTA pH 8.0

Tris Borate EDTA (TBE) gel running buffer
 0.5x working solution 1 liter of 5 x stock solution
 45 mM Tris-borate 54 g Tris base
 1 mM EDTA 27.5 g boric acid
 20 ml of 0.5M EDTA pH 8.0

Gel Loading Buffer
 0.25% bromophenol blue
 0.025% xylene cyanol FF
 15% Ficoll (Type 400, Phamacia) in water

Both TAE and TBE buffers work well for gel electrophoresis. Gels may be run at a slightly higher voltage with TBE buffer, thus the results are obtained more quickly. However, the 5x stock solution often forms a precipitate, making it difficult to store for long periods. The TAE buffer does not precipitate and 1 liter of 50x stock can be used for many gels.

It is important that the agarose gel and running buffer both be the same (do not run a TAE gel in TBE buffer!) It is also important that the buffer lot used to make the gel be identical to the running buffer because subtle differences in concentrations between lots may adversely affect agarose gels. For example, do not prepare an agarose gel from one stock of TAE and use a different stock of TAE as the running buffer for the gel.

Interpretation of gel:

How are the sizes of DNA molecules determined?

What are the sizes of your fragments?

What can you conclude about your unknown sample?

How could you use PCR to determine if a sample was infected with *Ralstonia solanacearum*?

PLACE YOUR PHOTOGRAPH HERE:
(Label photograph)

Sequencing

Direct sequencing of PCR products:

Cut and Kill PCR reaction

1. 2-5 ul PCR reaction mixture
2. 1 ul of Shrimp Alkaline phosphatase (1 units/ul)

3. 1 ul of exonuclease I (20 units/ul)
4. Run in Cut and Kill program in PCR (30' at 37 C, and 15 min at 75 C)

Sequencing Reaction

1. In each tube place the following:
 - a. 2 µl Big Dye
 - b. 3 µl Big Dye Buffer
 - c. 7 ul from Cut and Kill PCR reaction
 - d. 10 pmol primer (10 pmol/µl is equal to 10µM) for specific primer reactions and 2 to 4 x this amount (20 to 40 pmol) for general, degenerative primer (general about half the amount used in the PCR reaction)
 - e. Water to a vol of 20 µl
2. Run in MJ DNA Engine PTC200 thermocycler with program ABISEQ and with heated lid option.

Clean Up of Sequencing reaction Mixture: magnetic beads –

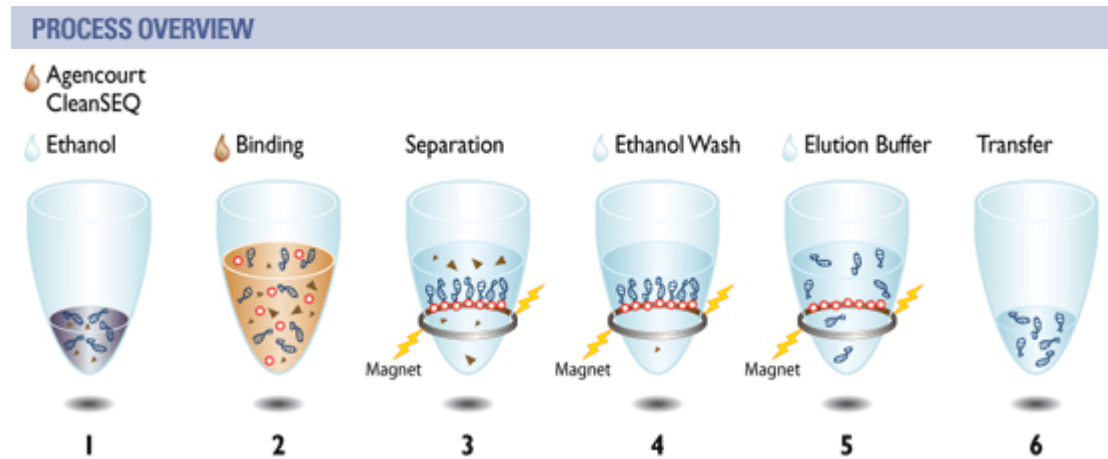
Agencourt Bioscience Corporation
 500 Cummings Center
 Suite 2450
 Beverly MA, 01915

Agencourt CleanSEQ Features:

Long Phred 20 read lengths
 Increased fluorescent signal intensities
 Easily automated
 Compatible with most common dye-terminators and sequencing instrumentation

Agencourt CleanSEQ Benefits:

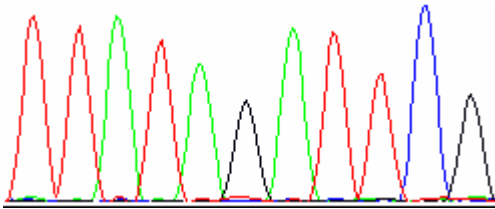
Produces higher quality sequencing data
 Reduces sequencing costs
 Enables increased BigDye® dilution
 Saves time



1. Add Agencourt CleanSEQ reagent and ethanol to sequencing reaction 2. Bind sequencing products to magnetic beads 3. Separate sequencing products from contaminants with magnetic field 4. Wash with ethanol 5. Elute from magnetic particles 6. Transfer away from magnetic beads

Sequence done by Commercial Company or BioTechnology Center

Abi files are returned, which must be viewed with a software, such as Chromas



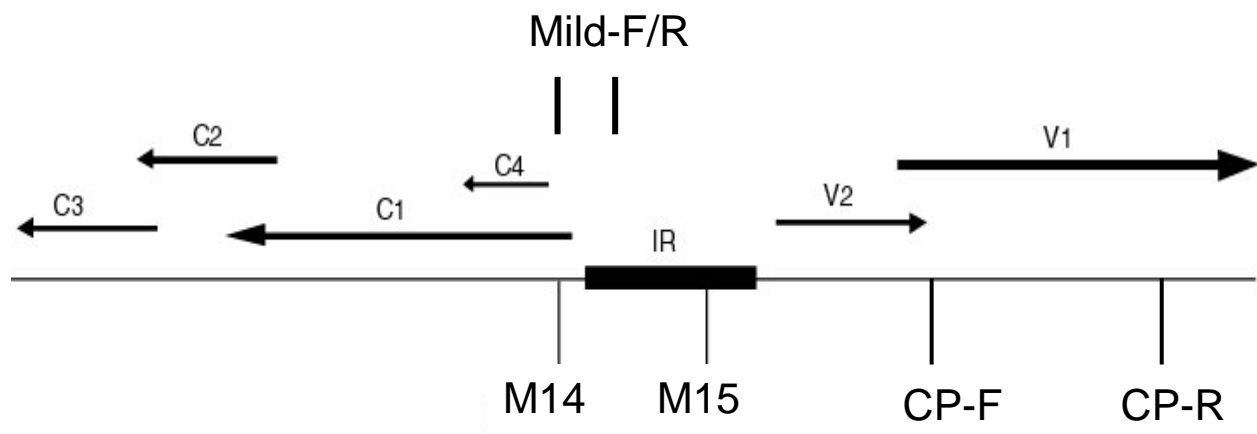
Sequence manipulation – many softwares (DNAMAN, inexpensive)

GenBank (free Internet Access)

www.ncbi.nlm.nih.gov/

Primers for Detection of Geminiviruses:

Genome of monopartite geminivirus, about 2.75 kb



General Primers:

CP-F (coat protein gene forward)
CP-R (coat protein gene reverse)
PCR fragment size: 480 bp

Specific primers:

Specific for TYLCSV

TY-sar-F (anneal in C1 gene) (M14 primer)

TY-sar-R (anneal in IR gene) (M15 primer)

PCR fragment size: 380 bp

Specific for TYLCV-mild strain (a recombinant virus)

TY-mild-F (anneal in C1)

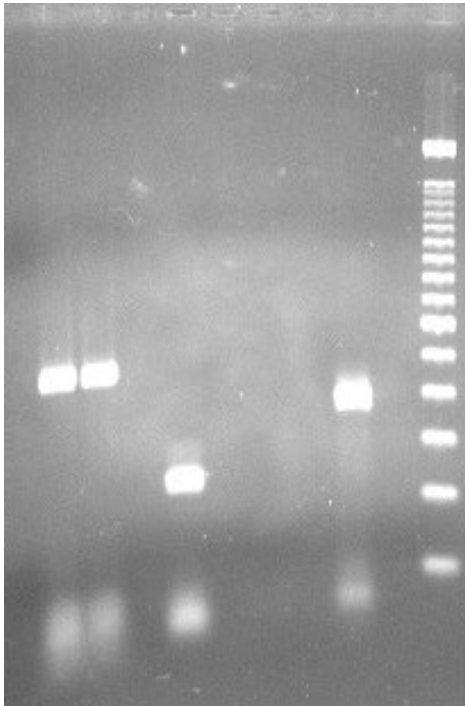
TY-mild-R (anneal in IR)

PCR fragment size: 260 bp

(Anfoka, G.H., M. Abhary, and M. K. Nakhla. 2005. Molecular identification of species of the *Tomato yellow leaf curl virus* complex in Jordan. *Journal of Plant Pathology* 87:61-66)

Agarose Gel electrophoresis:

1 2 3 4 5 6 7 8 9 10



Lane 1, Plant 1 with General Primers

Lane 2, Plant 2 with General Primers

Lane 3, symptomless plant, General Primers

Lane 4, Plant 1 with TY-sar Primers

Lane 5, Plant 2 with TY-sar Primers

Lane 6, symptomless plant, TY-sar Primers

Lane 7, Plant 1 with TY-mild Primers

Lane 8, Plant 2 with TY-mild Primers

Lane 9, symptomless plant, TY-mild Primers

Lane 10, 100-bp marker (Invitrogen) (count from bottom, 100, 200, 300 etc)

What are the sizes of the fragments for each PCR reaction?

What can you conclude from these PCR data?

How would you sequence these PCR fragments?

What would be the management strategies for control of this tomato disease?

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