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MIRANDA LAB PROTOCOL
318 New Science Building
Texas Southern University

The laboratory is designed for molecular systematics, evolution and ecology. The facility is designed for enhanced biodiversity research and training of undergraduates and graduate students at Texas Southern University.

We currently focus on the use of molecular biological data, specifically nucleic acid sequences (i.e. DNA). With the relative ease in generating sequence data at present, coupled with accelerated development in biocomputing, TSU students can avail of powerful tools to address scientific questions from a diversity of endeavors such as evolutionary biology, medicine, forensics, ecology, and conservation biology, and even space science. We commonly call the practice of molecular data analysis as bioinformatics, but in our lab, we specifically refer to our practice as phylogenetics and evolution. Our thrust is not only focused on molecular evolution but also how it explains the evolution of other traits (such as morphology and behavior) and adaptation.

Work in the lab is mainly divided into two major parts: lab work and sequence analyses. For vertebrates, lab work proceeds in the following sequence; (A) DNA isolation, (B) gene amplification using polymerase chain reaction (PCR), (C) DNA purification, and (D) DNA sequencing. DNA sequence analysis proceeds as follows: sequence retrieval, contig assembly, alignment, and phylogenetic analysis using a variety of optimality criteria.

A. Genomic DNA Preparation (Under Preparation) .

1. Qiagen Tissue Kit

2. MO BIO Microbial DNA Kit

3. Whatman FTA cards

B. The Polymerase Chain Reaction

The polymerase chain reaction is a method to amplify DNA *in vitro* without having to use bacteria and viruses as cloning vectors. It uses oligonucleotide primers, loose dNTPs, and DNA polymerase in a DNA thermal cycler. When you are doing P.C.R., you should keep the following general considerations in mind:

PCR TEN COMMANDMENTS

1. Always work on a clean, DNA-free surface.
2. Always wear clean gloves when handling any PCR reactants, tubes, etc.

3. Use dedicated DNA-free pipettors. Use the positive displacement pipettor for adding DNA.
4. Thaw your reagents, then place them on ice. **Do not allow reagents to reach room temperature.**
5. Always run a negative control with every PCR and a positive control with any new reaction (i.e., new primers, dNTPs, enzyme, etc.).
6. Never store your PCR reagents with your DNA samples or amplified products.
7. Briefly vortex and touchspin reagents prior to use.
8. Aliquot reagents out into small quantities (about 100 ul total for primers), so that in case of contamination the tube can be thrown out.
9. **Carefully label all reaction** tubes and take careful notes of what you did to avoid repeated mistakes.
10. If at first you don't succeed, try, try again is not a good philosophy in PCR. If your reactions fail for any non-obvious reason, you must find out why. Usually it's for one of three reasons: 1.) DNA prep is no good 2.) primers don't work on that taxon 3.) Enzyme has lost all activity.

Rapid Cycler Capillary System The Rapid Cyler from Idaho Technologies is not as popular as the heating block system but it is much faster, can be as effective, if not more.

The Veriti Cycler. For detailed information, see Applied Biosystems Veriti Thermal Cycler User Guide. The two enzyme systems used in this lab are 1) Amplitaq Gold PCR Master Mix and 2) GeneAmp Fast PCR Master Mix (2X). The Master Mix provides more consistent result with the Veriti but run takes longer.

Equipment Needed:

1. DNA thermalcycler.
2. Microcentrifuge.
3. DNA-free pipettors (0.4-10 μl, 10-50 μl, and 40-200 μl).
4. Positive displacement pipettor
5. Vortexer
6. Ice bucket.
7. 0.2 ml thermal reaction tubes (do not substitute).
8. UV light box.
9. Mini agarose gel rig and comb.
11. 0.2 ml Eppendorf tubes.

Solutions Needed:

1. Sterile ddH_2O (nuclease-free) .
2. Amplitaq Gold Master Mix
4. 10 mM dNTP mix.
5. 12 picomole (12μM) oligonucleotide primers (Optimal primer concentration can vary from 5 μM to 20 μM) .

7. Sample DNA.
9. 1-2% HMP agarose in TAE w/ ethidium bromide.
11. 1XTAE buffer.

Amplitaq Gold Master Mix Protocol:

1. Place all frozen materials (except for the Amplitaq Gold on long-term storage which should stay at -20deg C) on ice and allow them to thaw.
2. Label N+1 reaction tubes, where N is the number of DNA samples you have. The extra tube will be your negative control. ALWAYS USE A NEGATIVE CONTROL
3. You should select two primers to use in the amplification. One primer must be a light strand primer and the other must be a heavy strand primer downstream to the right (for example on the 12S rRNA gene: L821 and H1194).
4. Make a reaction "cocktail" by adding the following to the negative control tube for each reaction tube [e.g. for 4 reactions (3 DNAs plus control), you should add 4 times the volume shown:

   2.0 µl ddH_2O
   1.0 µl L-Primer
   1.0 µl H-Primer
   5.0 µl Amplitaq Gold Master Mix

   Vortex for about 10 seconds and touchspin in the microcentrifuge.

5. Place the tube on the UV light box (when available), cover the top of the box with foil, and expose to UV for 5 minutes to eliminate any contamination.
6. Aliquot out the cocktail into every tube in equal volumes.
7. Add 1 µl of sample DNA using the positive displacement pipettor to the appropriate tubes and vortex. Your positive control DNA should be one that has worked with the primer pair before. **DO NOT ADD DNA TO YOUR NEGATIVE CONTROL**.
8. Touchspin.
9. Program the thermalcycler to the desired parameters (see appendix).
10. Put the tubes into the thermalcycler, make sure they are seated into the wells all the way, and start the run.
11. Run out 5-10 µl of reaction product and 1 µl of 1X dye on an agarose w/ ethidium bromide gel (see appendix for gel loading). Make sure you run a molecular size marker. We usually use phiX174 HaeIII. Visualize under UV or gel documentation system and take a picture. The relative brightness of the band should provide a rough estimate of the quantity and concentration of DNA. A more accurate estimate of concentration can be done if the gel documentation system has a software for quantitative analysis.

12. If you have reaction products of the right size and concentration and there are no bands in the negative control lane, repeat the PCR using the same parameters but with total volume 5 times more. Run a test gel again of 1 ul sample. Clean the DNA for sequencing using Wizard PCR Preps DNA Purification System (Promega) if results are favorable You are now ready to clean the DNAs for sequencing

C. Agarose Gel Electrophoresis

Agarose gel electrophoresis is the commonest way of separating and analyzing DNA. The DNA or PCR product is visualised in the gel by addition of ethidium bromide (EB). This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. Care should be taken in handling EB since it is a known mutagen or carcinogen.

We usually use 1.0% gel for a 500 -1.0 kb sized fragment. For avian introns, and fungal ITS fragments less than 400 kb, a 2-3 % gel is better. Low percentage gels are softer and may break when you try to lift them. High percentage gels are often brittle and do not set evenly.

1. Making the gel : Weigh out 3.0g of agarose into a 500 ml Erlenmeyer flask. Add 300mL of 1X TAE, swirl to mix.

Microwave for to dissolve the agarose. The agarose solution can boil over very easily so keep checking it. It is good to stop it after 45 seconds and give it a swirl. It can become superheated and NOT boil until you take it out whereupon it boils out all over you hands. So **wear gloves** and hold it at arms length.

Leave it to cool on the bench for a few minutes. If you had to boil it for a long time to dissolve the agarose then you may have lost some water to water-vapour. You can weigh the flask before and after heating and add in a little distilled water to make up this lost volume.

2. Get the gel tank ready, on a level surface. Pour 30 mL of dissolved 1X TAE (or TBE) into a Falcon tube. Add 5 µL of ethidium bromide (….mg/mL) and swirl to mix
The reason for allowing the agarose to cool a little before this step is to minimise production of ethidium bromide vapour. Ethidium Bromide is **mutagenic** and should be handled with extreme caution. Dispose of the contaminated tip into a dedicated ethidium bromide waste container. 10mg/mL ethidium bromide solution is made up using tablets

(to avoid weighing out powder) and is stored at 4°C in the dark with TOXIC labels on it. Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip. Insert the comb and double check that it is correctly positioned.

The benefit of pouring slowly is that most bubbles stay up in the flask. Rinse out the flask immediately. Leave to set for at least 30 minutes, with the lid on if possible.

Once the gel solidifies, remove the comb (I usually use the 1.0 mm sized comb for midi-gels). Use the first and the last lane for molecular markers. So if you have 10 samples, use the land 1 and lane 12 for the markers. Pour 1X TBE buffer into the gel tank to submerge the gel to 2–5mm depth. This is the running buffer. For a small quantity 'test' PCR, load 10 ul of sample plus 1-3 ul of blucose dye.

Continue loading the samples and finish of with a final lane of marker

I load gels from right to left with the wells facing me. This is because gels are published, by convention, as if the wells were at the top and the DNA had run down the page. If this seems confusing then you can load left to right with the wells facing away from you. Close the gel tank, switch on the power-source and run the gel at 5V/cm.

For example, if the electrodes are 10cm apart then run the gel at 50V. It is fine to run the gel slower than this but do not run any faster. Above 5V/cm the agarose may heat up and begin to melt with disastrous effects on your gel's resolution. For the lab's midigels, we run the gels at 60-70V.

Check that a current is flowing.
You can check this on the power-source, the milliamps should be in the same ball-park as the voltage, but the the best way is to look at the electrodes and check for bubbles. If not then check the connections, that the power-source is plugged in etc. Make sure you use running buffer instead of water (some people always do).

Monitor the progress of the gel by reference to the marker dye.

Stop the gel when the bromophenol blue has run 3/4 the length of the gel, or ¾ of half of the if you are running two rows

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Switch off and unplug the gel tank and carry the gel (in its holder if possible) to look at on the UV light-box or Gel Logic 212 Documentation System (Kodak) .

UV is **carcinogenic** and must not be allowed to shine on naked skin or eyes. So wear face protection, gloves and long sleeves. NEVER OPEN THE GEL DOC BOX IF UV IS ON.

Loading buffers

The loading buffer gives colour and density to the sample to make it easy to load into the wells. Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis. This allows you to monitor the progress of the gel. The most common dyes are bromophenol blue (Sigma B8026) and xylene cyanol (Sigma X4126).

Molecular Size markers

There are lots of different kinds of DNA size markers. In the old days the cheapest defined DNA was from bacteriophage so alot of markers are phage DNA cut with restriction enzymes. Many of these are still very popular eg, lambda HindIII, lambda PstI, PhiX174 HaeIII. These give bands with known sizes but the sizes are arbitrary. Choose a marker with good resolution for the fragment size you expect to see in you sample lanes. For example, for tiny PCR products you might choose PhiX174 HaeIII but for 6kb fragments you would choose lambda HindIII. More recently, companies have started producing ladder markers with bands at defined intervals, eg. 0.5, 1, 1.5, 2, 2.5kb and so on up to 10kb. If you know the total amount of DNA loaded into a marker lane, and you know the sizes of all the bands, you can calculate the amount of DNA in each band visible on the gel. This can be very useful for quantifying the amount of DNA in your sample bands by comparison with the marker bands. It is good to load two markers lanes, flanking the samples. Lots of companies sell DNA size markers.

D. DNA Purification

The following instructions are modified from The Promega system (Cat # A2180) Technical Bulletin with added details. The kit includes the following:

250ml Wizard PCR Preps DNA Purification Resin
25ml Direct Purification Buffer
250 Wizard Minicolumns

250 syringe barrels

Direct Purification of DNA from PCR Amplifications

1. Prepare three(3) 1.5 ml Eppendorf tube for every DNA sample. Cut off the cap of the first two 1.5 tubes LABEL ALL tubes appropriately on side, or both cap and sides of the 3rd one.

1. For each completed PCR amplification, transfer the aqeous (lower) phase to a clean microcentrifuge tube. Avoid getting too much of the mineral. This is no problem when using modern cyclers such as Veriti.

2. Aliquot 100 ul of Direct Purification Buffer into the 1.5 tube with DNA. Vortex briefly to mix.

3. Add 1ml of resin and vortex briefly 3 times over a 1-minute period. If using a vacumm manifold proceed to B, if not proceed to A below.

I. <u>Purification without a vacuum manifold.</u>

Materials

80% isopropanol (2-propanol, reagent grade)
deionized water or TE-buffer
disposable 3ml Luer-Lok syringes.

One disposable 3 ml Luer-Lok syringe is required for each PCR Prep.

1. For each PCR product or gel sample, prepare one Wizard Minicolumn. Remove and set aside the plunger from a 3 ml disposable syringe (The kit does not include the plunger anymore). Attach the syringe barrel to the Luer-Lok extension of each minicolumn.

2. Pipet the resin/DNA mix into the Syring Barrel.

3. Bring the Luer-Lok-syringe assembly to the fume hood and insert the tip into the vacuum assembly. Turn the vacuum (yellow) on.

4. When slurry is pumped, pipette 2 ml of 80% isopropanol into the syringe, and turn the vacuum again to wash the column.

5. Remove the syringe and transfer the Monicolumn to the first capless 1.5 microcentrifuge tube. Centrifuge the Minicolumn (attached to the minicentrifuge tube) for **2 minutes** at 10,000 x g to dry the resin.

6. Transfer the Minicolumn to the 2nd capless microcentrifuge. Apply 50 ul of water or TE buffer to the Minicolumn and wait 1 minute. Make sure no droplet of water

is on the inside surface of the column. Otherwise, tap the column a few times on the table. Centrifuge the Minicolumn for **20 seconds** at 10,000 x g to elute the DNA fragment.

7. Remove and discard the Minicolumn. Store the purified DNA at 4°C or -20° C.

II. Purification Using a Vacumn Manifold... Coming Soon.

4. After DNA purification, run a test gel of 1 ul of your purified DNA plus 3 – 4 ul of blue dye to check

E. Preparing PCR Products for Sequencing

Visit http://www.seqwright.com/shippinginfo/shippinginfo.html

F. Recipes and Chemistry Calculations (WORK IN PROGRESS)

Primers
Molecular Marker
dNTPs
Ethidium bromide
Agarose
TAE buffer solution
Primer concentration for AB sequencing
DNA sample for AB sequencing