Protocols for clearing and staining of delicate roots

For examining roots for mycorrhizal infection.
(courtesy Jennifer Parke)

1. Wash roots free of soil, chop into approx. 1 cm segments. (It's a whole lot easier than looking at tangled up roots.)
2. Clear in 10% KOH (standard protocol is overnight in a 55 C water bath for your roots I'd try 30 min to 1 hr. at room temperature, then look at them under the scope and see if they look cleared - if not, try some gentle heat treatment or longer at room temperature.
3. Rinse 2-3x in tap water.
4. Acidify in dilute HCl (I don't remember the exact concentration - will look it up for you if you need it)
5. Stain in trypan blue in lactoglycerin - I'd probably just do this at room temp for 10 min. instead of the 15-20 min. at 55 C as called for in the protocol.
6. Destain in lactoglycerin (can store cleared and stained roots in lactoglycerin for months).

This is essentially the Phillips and Hayman (1970)(TBMS 55:158-160) original protocol except that lactoglycerin is substituted for lactophenol.

Protocol for Freezing Cultures in the Gilbert/Parker Labs

1. Grow your culture on a petri plate. It is best to have spores or new growing mycelium because they will revive easier.
2. Materials needed:

-disposable pasteur pipettes in cardboad cylinder or in beaker with foil cover.
-Malt Extract Broth/Glycerol (3.75 g malt extract broth + 125 mL diWater + 125 mL glycerol; autoclave)
-Blue pipette tips
-an unused petri plate or small dish
-Cryogenic Vial (2mL).

Freezing Protocol:

-Working in the biosafety cabinet, fill a small beaker with 70% ethanol, and soak several fine metal spatulas.
-Pour a few mL of the Glycerol/MEB into the sterile Petri plate so that you can cap the bottle and save it for later.
-Label Cryovials as needed, on side and on cap. , I always put some sort of distinguishing label on the top too because that is all you see when you open the box.
- Grab a sterile pasteur pipette by the tip and without touching anything, use it like a cookie cutter to make ~5-6 disks from the growing edge of mycelium.
-Flame the alcohol off a spatula, and us it to carefully lift each disk one by one off of the plate and place it in the cryotube. Replace the spatula in the alcohol and cap the tube.
-Get a 1000µL Pipette with a blue tip and use it to add MEB/Glycerol mixture to tube. No exact amount is needed, just cover up the disks. Avoid splashing and touching the tip to the vial and you can use it for the next one.
-Close the vial tight, and place in a labeled storage box and then in the -80 freezer..

Protocol for connecting digital camera on the microscope to the imac

1. Turn on imac.
2. Connect myVideo cable (yellow end at one end) from Video input composite (on myVideo) to video out (on side of Coolpix camera).

3. Make sure myVideo is connected to iMac with a USB cable.
4. Launch "EskapeTV Viewer" on imac (alias on desktop; program in myVideo folder).
5. Turn on camera.
6. Find the specimen you want to view in the microscope.
7. Pull out the knob on the right of the scope so that the light goes up to the camera. You should now be able to see the image both on the camera screen and on the computer screen.

To capture the image
1. Choose Record Frame from the Control menu (or type command-S).
2. This saves the image automatically as "EscapeTV#.pic" in the folder "myVideo Folder" on Maduro. The # indicates a number that will increase with each photo taken.
3. The image is a pict file. Please move your images to a folder outside of the "myVideo Folder" location.
4. You can open this file in just about any application (Word, Powerpoint, Photoshop, etc.)

Protocol to Balance – Computer Interface in Parker Lab
Protocol Written by Julie Beckstead

What you need?
Lab PC w/ BalanceLink software; Mettler balance AT201 Parker Lab, connection cable (in balance drawer if not connected

Setup
1. Connect connection cable. Start with computer and balance off.

Open software
1. Turn on balance
2. Open BalanceLink software on PC: click window explorer; local disc (C:); Program Files; Balance software; BALINKE.EXE
3. If you get a message that there is no balance connected, then first check to see that the cord is connected properly. If yes, then continue.
4. Open Excel file: go to your prepared excel spreadsheet or open a blank one (start; programs; excel)
5. In Excel place the cursor where you want to begin.

Get Ready to Weigh
1. In Excel place the cursor where you want to begin.
2. Weigh sample: place sample on scale (tare if needed; then sample); push the print button on the balance when you are ready to enter data.
3. Repeat 5 & 6 to weigh all samples. Note when data is entered in excel the cursor doesn't return to the next line. BECAREFUL! The program doesn't delete your previous measurement, but it will create a messy string of values.
4. For help information and options to modify setup see Balinke.txt. Note: the above problem can be corrected but you will need to figure out how. Follow the directions in the Balinke.txt file.

Finished
1. Save & backup excel file; quit programs and shut down computer; turn off balance;
2. Lastly, disconnect cable connector when computer and balance are off.

Protocols for Fungal DNA extraction for PCR
Use DNA extraction kit: Extract-N-Amp Plant PCR Kit from Sigma-Aldrich Lab.
Product codes XNAP2, XNAP2E and XNAR

Fungal strains from which you are isolating must be grown on Malt Extract Agar (with or without chloramphenicol) and they must be pure strains!!!

DNA extraction
In the DNA extraction kit there are two little bottles one is labeled Extract Solution and the second is Dilution Solution. Both of these are what we refer to as Extract solution and Dilution solution in the following DNA extraction protocol.

1. Obtain sterile 2.0 ml collection tube and label all the tubes with their corresponding name.
2. In the biosafety cabinet using sterile technique cut a piece of mycelium (a couple mm diameter) from the single strain fungus that has been growing on Malt Extract Agar. As long as there are hyphae growing on the media it is ok to isolate from it. Take as little agar as possible.
3. It is very important that the piece of mycelium you select is small enough so that it will be completely submerged in the Extraction solution (100 µl). This is also important since the agar does not disintegrate in the process of extraction, so if there is not enough liquid it can be difficult to extract DNA for PCR later in the process.
4. Place the selected piece of mycelium in the corresponding clean sterile 2.0 ml collection tube.
5. Add 100 µl of the Extraction solution to the collection tube containing the mycelia sample. Close the tube and vortex briefly. Make sure the mycelium is completely submerged by the Extraction solution!
6. Incubate at 95 °C in the Fisher Scientific dry bath incubator block located the lab bench in 478 NS2 for 10 minutes. Note that the fungal tissue usually does not appear to be degraded after this treatment. It is ok. Take back to the biosafety cabinet.
7. Add 100 µl of the Dilution solution to the 2 ml tube; close and vortex to mix.
8. Store the extracted DNA solution at 2-8 °C. (Samples are viable for up to one year under these conditions).
9. After DNA has been extracted, run a 1% agarose gel to check for the presence of DNA. Gel preparation for 1% agarose gel is listed below (Agarose mass varies depending on the agarose gel concentration. A higher agarose concentration leads to a thicker gel.). Note that you don't need to do this for all your extractions – just check to make sure it is working on a few.

Preparation of TBE Buffer
To prepare 2 L of 10X TBE Buffer:
216g Tris base
110g Boric acid
80mL 0.5M EDTA (pH 8.0)

autoclave for 20 min
Dilute to 1X TBE for use (1:10 10XTBE:Sterile millipure water) for use

Preparation of Agarose Gels
IF YOU HAVE NEVER PREPARED A GEL BEFORE,PLEASE HAVE SOMEONE WITH EXPERIENCE GUIDE YOU.
1. For 1% agarose gel, take a 250 Erlenmeyer flask and place 1 g agarose and 99 mL of 1x TBE solution into it. Swirl and place in microwave.
CAUTION: YOU MUST WEAR EYE GOGGLES AND INSULATED GLOVES. SOLUTION IS VERY HOT
2. Select one minute and heat the solution, carefully watching the beaker in the microwave to see when it begins to boil.
3. As soon as it begins to boil, stop the microwave. When safe remove from microwave and swirl gently. If all the agarose has not melted and you still have some agarose crystals in the solution re-insert into the microwave and heat for a few seconds at a time. Stop the microwave every few seconds and swirl the flask until a clear, colorless, crystal-free solution is achieved.
4. Wait until the gel-solution prepared above has cooled to "baby-bottle" temperature to pour into the gel mold.
Excel calculator for determining concentrations for PCR reactions download here

Reconstituting PCR Primers
Primers currently available in the lab were ordered from Invitrogen and located in the 478 NS2 freezer in a labeled box. If additional primers are needed one can order them from Invitrogen's web page (http://www.invitrogen.com). One has to navigate to: custom primers: DNA list form: enter research name: scale of synthesis (means the quantity of primer needed) = 50 nmole: Purity: desalted is good for PCR.
The primers can be bought reconstituted (liquid form ready to use) or dry. In the lab we currently have dry primers and to be used they must be reconstituted. The primer concentration comes written on the small tubes where the lyophilized primers come in and this concentration will be needed to reconstitute the dry primers into solution.
You will need 1xTE Buffer to reconstitute your dry primer. First search in the lab to see if there is some already made. If there is none available you need to make it. To make 1xTE Buffer follow the recopies at the end of this protocol.
The dry primer comes labeled with the quantity of primer in nmole on the tube.
To prepare a 100µM mother solution, it must be diluted by ten times that number of µL of TE buffer.
That is:
100µM Mother Solution = p nmole dry primer + p*10 µL 1XTE buffer.

For example, if there is 32.64 nmole primer in the tube, then diluting this with 326.4 µL of 1xTE buffer will produce a 100µM Mother Solution.
If you want to recheck the calculations for the Primer Mother Solution, use the calculator in the PCR recipes.xls file, available in the Lab Stuff link on the lab web site (http://people.ucsc.edu/~ggilbert/) or on the computer in 490 NS2, under Gilbert Lab Projects: Fungal Collection PCR: PCR recipes.xls. Use the Primer Solutions tab.
For PCR reactions, you will use a 10µM primer Working Solution. To make it
1. Take 10 µl of primer Mother Solution and put into a centrifuge tube.
2. Add 90 µl of 1xTE buffer to centrifuge tube and vortex.
3. Don't forget to label your tubes.
NOTE: the Extract-N-Amp PCR ready kit contains JumpStart Taq antibody for specific hot start amplification. Therefore, PCR reaction can be assembled at room temperature without premature Taq DNA polymerase activity.

PCR Recipe
NOTE: For PCR reactions use autoclaved ultra-pure water from Shannon lab in 424 NS2.
Calculations for the Master Mix
1. To calculate needed concentrations for the Master Mix, use the Excel calculator in the PCR recipes.xls file, available in the Lab Stuff link on the lab web site (http://people.ucsc.edu/~ggilbert/) or on the computer in 490 NS2, under Gilbert Lab Projects: Fungal Collection PCR: PCR recipes.xls. Choose the MasterMix tab.
2. On the left hand side of the chart you have the recommended volumes needed for each reagent for each sample.
3. In the yellow box type in the number of reactions you will use in a single run, which should be # Fungal samples + 1 negative control + 1 positive control.
4. The green boxes on the right then show the adjusted volumes for each reagent you need for that run, including enough for 2 "extra" samples as a buffer.
5. Now you are ready to prepare your master mix.
Preparing the Master mix: (best to work in Biosafety Cabinet)
1.Take a 2-ml centrifuge tube and label it "master mix". (Select a tube big enough to hold the entire content of your master mix
2.Pippette into labeled tube the total volume of ultra pure water needed for your total run.
3. Into the same tube add the given volume of Extrac-N-Amp Mix
4. Pipette into it the given volume of 10-µM forward primers.
5. Pipette into it the given volume of 10 µM reverse primer.
6. Close centrifuge tube and Vortex
7. Label your PCR reaction tubes accordingly. Remember you must be able to distinguish among strain samples and the tubes you are inserting them in. Rxn tubes are really small. It is up to you how you label, but it works well to use 1,2,3... and record in your lab notebook what number corresponds to what strain.
8. Into each PCR reaction tube pipette 16 µl of master mix.
9. Take your extracted DNA samples and briefly centrifuge to collect all the fungal debris at bottom.
10. To the corresponding PCR reaction tube, pipette 4.0 µl of your isolated DNA sample (make sure you only take the supernatant) and close the PCR reaction tube to avoid contamination.

11. Once you have done all of your samples for this run make sure all of your tubes are completely sealed so they don't evaporate while in the thermal cycler.
12. Don't forget to run a negative control (blank meaning no DNA added) and a positive (DNA sample that you know has worked on the PCR before).
12. Place samples into the thermal cycler.
13. Run your samples at the recommended cycle for the primers employed.
14. When done, store your amplified DNA samples in the freezer.

PCR Thermocycling times and temperatures
(Launching Program 1 on Techne Genius in 478 NS2 gives you this)

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	94	3 min	1
Denaturation	94	30 sec	34
Annealing	54	30 sec	
Extension	72	1min	
Final Extension	72	10 min	1
Hold	4	Indefinitely	

Running the gel to check for quality of PCR products
Run PCR products on a 1% agarose gel. (see Preparation of agarose gel, above).
Pour 1xTBE Buffer into gel box and place your cold 1% agarose gel inside.
Now you need to prepare your PCR samples for loading.
To add the Dye to your PCR product take a large piece of parafilm, big enough for well-spaced drops for each of your samples in the run. (Dye is in the freezer).
In an ordered fashion place 1-µl droplets of Dye to parafilm. You will need as many dye droplets as you have samples plus 1 for ladder.
Then add 5µl of PCR sample to one dye droplet. Mix by drawing into pipette. Repeat this step until all the samples have dye, with new tip for each sample.
Include a Ladder in your gel run. (There are ladders that came with the primers and located in the freezer in 478-NS2).
Now load your samples onto gel. Make sure you know the order in which you load the samples, always placing your ladder in the first well and each additional sample into the following wells.
Run gel at 96 Volt for 40 min.
Stain gel with SYBR gold for 15 min. (OR with GelRed)
Take a picture of your gel and label picture appropriately.

Sybr gold must be diluted 35µl into 350 mL 1xTBE; it will keep about a week.

Cleaning the PCR product
2 x 5 µl of PCR product + 2 µl of Exosap-IT (from USB corporation)
Incubate 37 °C for 15 minutes; then 80 °C for 15 minutes.
- To prepare the samples to send to Berkeley lab mix 12.2 µl of the sample + 0.8 µl of diluted primer
- To dilute primer if initial primer concentration was 10 µM dilute 10 times to take to 1 µM.

Reagent and Gel Recipes

Before you make any of these reagents make sure that there is none available in the lab!
Wear protective eye gear and gloves at all times!
Work with acids and bases in the fume hood.
NOTE: For PCR reactions use autoclaved Ultra-pure water.
Ultra-pure water can be obtained from the Shannon laboratory in 424 NS2.
0.5 M EDTA, pH 8.0
In a 250 ml Erlenmeyer flask place:

18.6 g EDTA ($C_{18}H_{11}N_2Na_2 \times 2H_2O$)
70 ml Ultrapure H_2O

Stir contents in the flask until all of the solid has dissolved then add approximately 5.0 ml of 10N NaOH, 1-mL at a time until a pH of 8 is reached. Pour solution into a 100 ml volumetric flask and bring the volume up to 100 ml with Ultrapure water. Transfer to a storage bottle and autoclave.
10N NaOH (Sodium hydroxide) (OjO – Goggles, gloves, fume hood!)
In a 250 ml Erlenmeyer flask place:

40 g of NaOH pellets (Sodium Hydroxide)
80 ml of Ultra-pure water
Stir and heat until all of the NaOH has dissolved. Solution heats quickly, use caution)
Cool solution in fume hood.

Place the cooled solution in a 100 ml volumetric flask and bring the solution up to 100 ml. Store solution at room temperature and in a plastic bottle; it will erode glass.
1M Tris-HCl (OjO – Goggles, gloves, fume hood!)
A bottle of 1M Tris-HCl is available in cabinet C in 478 NS2. You will need to bring the pH of this solution to 7.4 by adding HCl. You will only need a very small quantity of this solution therefore place 25-50 ml of 1M Tris-HCl into a small storage bottle and add small aliquots of HCl until a pH of 7.4 is achieved.
TE Buffer (10mM Tris-HCl, 1mM EDTA) (Work in Biosafety Cabinet)
To a 50-ml volumetric flask pipette:
0.5 ml 1M Tris-HCl, pH 7.4 (sterile) 0.1 ml 0.5M EDTA, pH 8.0 (sterile)
Bring the solution to 50 ml with sterile Ultrapure water. Transfer the solution into a sterile storage bottle.