Enhanced Laboratory learning with Instructional Protocols for Safe Molecular Technology to fit PSPP departmental needs

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Katherine Mohatt and Dr. Cathy Cripps
Plant Sciences and Plant Pathology Department
Montana State University, Bozeman MT 59717
Contact: CCripps@montana.edu

Molecular Tools in the Classroom

Introduction

These laboratory exercises were originally developed to identify fungi using common molecular tools. These same protocols can also be applied to plants for identification.

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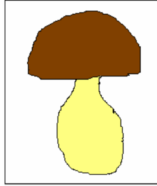


Lab 1: Introduction to the Molecular Biology Laboratory

Introduction: Analysis of the cell component DNA involves the use of specialty equipment, techniques, and protocols. Standard equipment used in the molecular biology laboratory includes pipettes, centrifuges, as well as other tools that will be introduced in future lab exercises. In this lab exercise, students will learn the basic techniques involved in pipetting and correct use of a centrifuge while learning to follow a simple protocol.

Goal: To familiarize students with techniques used in the molecular biology laboratory through practice of proper pipette technique, centrifuging, and following a protocol. Gloves are used with hazardous material.

Materials:

1. A set of 3 pipettes per group (2 - 20µl, 50-200µl, and 200 – 1,000µl)
2. Three boxes of corresponding pipette tips per group
3. 1 box each of latex and nitrile gloves
4. 12 centrifuge tubes per person (in tube rack)
5. 4 mixtures (sand, flour, salt, cloves)
6. Centrifuge
7. Sharpies
8. Protocol
9. 1 labeled jar per group for pipette tip waste

Technique (Read first!):

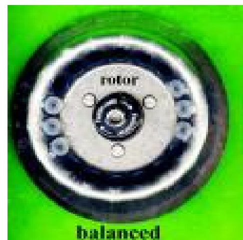
PIPETTING – Choose the most appropriate volume pipette (ex. If you want to pipette 300µl, use the 200-1,000µl pipette) and corresponding tip (push pipette into pipette tip without touching tip). Adjust the volume on the pipette by turning the knob to the desired volume (for the above example, the volume should read 030 NOT 300). Depress the pipette until you feel resistance (don't force it). Insert pipette tip into liquid and then release*. Depress the pipette again to discharge the liquid being careful not to contaminate the tip during transfer. Release pipette tip into desired tube by pressing white button. NEVER go beyond the volume capacity specified on the pipette.

***Note:** To prevent contamination of your samples and materials, keep lids covering pipette tips and sample tubes closed as much as possible and avoid contact with the pipette tip when transferring liquid. It is helpful to have tubes and mixtures ready before you prepare pipette tip. Also, wear gloves when doing any work in the lab.

CENTRIFUGE – Place centrifuge tubes directly across from each other in the centrifuge. For example, if the centrifuge has 18 slots and you have 6 tubes, place three tubes in slots 1-3 and three tubes in slots 10 – 12. It is CRITICAL that the centrifuge is balanced (see below). Make sure that the tubes have similar volumes of sample and an even number of tubes. You may have to add an extra tube with water to balance the centrifuge. Alternatively, if you can coordinate with another group to combine tubes in order to balance, this would save time. Replace spray guard (if present), close lid, and set desired rpms and time (this is usually specified in protocols).

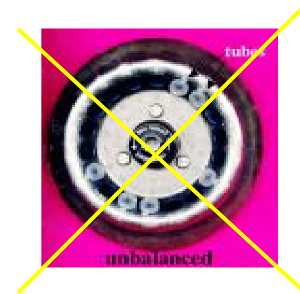
Correct!

Balanced



Incorrect!

Unbalanced



PROTOCOL – A protocol is a detailed set of instructions used to perform a specific task. Before starting any procedure, it is highly recommended that you read the protocol in entirety first to make sure you have all necessary materials and understand the procedure. Also, reading the protocol first will help you determine the most efficient and time saving methods for completing the task.

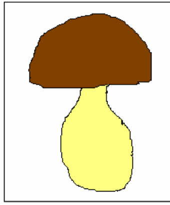
Protocol for separating clear fluid from a cloudy mixture (Pipette & Centrifuge Practice)

1. Swirl mixtures and then aliquot (pipette a specified amount) 500µl from each mixture into labeled centrifuge tubes.

2. Centrifuge at 12,000 rpms for 1 minute (BALANCE!).

3. Carefully pipette off as much supernatant (liquid) as possible into a fresh labeled tube. *Hint: Start with a large volume pipette with the tip held close to the surface (AWAY from the pellet). As you make your way to the pellet at the bottom, use smaller volume pipette and tips (with smaller openings) to avoid picking up debris.

4. Repeat steps 2 - 3 with the supernatant obtained until you have a clear liquid (it may be colored, but should be free of particles). Repeat for all tubes.



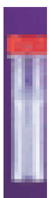
Lab 2: DNA Extraction from Fungal or Plant Tissue

Introduction: DNA extraction is the first step in many protocols when the ultimate goal is to analyze a portion of the DNA. Careful extraction technique will result in pure, clean DNA that can be readily analyzed by a variety of methods. In this lab, we will use two kits to extract DNA from plant or fungal tissue. The first step in this process breaks the cell walls of the organism to release the DNA. We will use a detergent to dissolve the lipid bilayer of the membrane and mechanical agitation to fully disrupt the cell walls. Once the cell contents are released, the DNA will be bound to a filter that can then be washed to remove impurities. Bound DNA is later released into a buffer for storage and future use.

Goal: To extract DNA from fungal or plant tissue using the simple FastDNA® extraction kit and Qiagen spin columns*.

Materials:

1. A set of three pipettes per group (2-20µl, 50 – 200µl, 200 – 1,000µl)
2. Three boxes of corresponding pipette tips per group
3. 1 box each of latex and nitrile gloves
4. 6 -8 sterile 1.5 microcentrifuge tubes per group
5. Sharpies
6. FastDNA® extraction kit
7. Qiagen kit containing Spin Columns (any will do)
8. Centrifuges
9. 1 tube rack per group
10. 1 labeled jar for pipette tip waste per group
11. 1 labeled beaker for flow-through waste per group

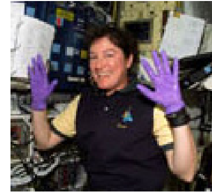


= Lysing Matrix A Tube

= Qiagen spin column

= 1.5 ml microcentrifuge tube

DNA Extraction Protocol:


gloves?

Breaking cell membranes to release DNA

1. Pipette: 800 µl of CLS – VF buffer and
   200µl PPS into Lysing matrix A tube (see above)

2. Place tissue sample from specimen into the labeled Lysing Matrix A tube with buffers.

3. Homogenize the samples in FastPrep® instrument for 30 seconds at a speed setting of 4. Be sure to balance the instrument as you would a centrifuge (your instructor might do this part).

4. After the samples have been homogenized, centrifuge them at 14,000 rpm (or max) for 10 minutes (you might want to coordinate with other groups for this).

Binding DNA to spin column

5. Carefully remove samples from the centrifuge being careful not to agitate the pellet. Slowly pipette off 600 µl of the supernatant (which contains your DNA) into a Qiagen spin column (see photo) by using the 200 µl pipette and taking 200 µl at a time to avoid transferring debris. Note: The purple part is spin column, clear part is collection tube. Discard lysing matrix tube with pellet in waste jar.

6. Let supernatant stand for 1 minute in the spin column then centrifuge for 1 minute at 6,000 rpm's.

7. **DNA is now bound to the filter in the purple tube**. Decant (by pouring out) liquid in collection tube (flow-through) into designated waste container.

Washing bound DNA to purify

8. Add 750 µl of wash solution (80% EtOH) to the spin column. Let stand 5 minutes.

9. Centrifuge at 14,000 rpms (or max) for 1 minute. Decant flow - through, then centrifuge again for 1 minute at 14,000 rpms.

Releasing bound DNA into storage solution

10. Place purple tube (spin column) into a fresh labeled micro-centrifuge tube.

11. Pipette 50μl of elution buffer (EB) into the spin column. Let stand 1 minute.

12. Centrifuge for 1 minute (BALANCE!) at 6,000 rpm's. **DNA is now in the bottom of the tube!!**

13. Remove spin column and store at - 20°C.

Next lab: We will use the extracted fungal DNA, add enzymes and primers, and place in a thermocycler to initiate Polymerase Chain Reaction (PCR). This will allow us to make many copies of just one particular fragment of our fungal or plant DNA.

NOTE: This protocol is modified from the FastDNA® Manual and QIAquick® Spin Handbook and is more expensive but far less time consuming than using the FastDNA protocol alone. If on a budget, the FastDNA protocol alone is equally effective with sporocarp or plant tissue samples.

References:

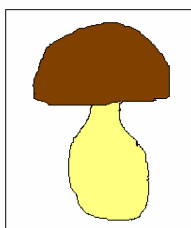
FastDNA® Kit Application Manual. Revision # 6540-400-4H01 Catalog # 6540-400.

Lee, S.B., and J.W. Taylor. 1990. Isolation of DNA from fungal mycelia and single spores. *In* PCR Protocols: A Guide to Methods and Applications. Edited by M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White. Academic Press, San Diego, CA pp 282-287.

Ola, K., M. Jonsson, and K. Ihrmark. 1999. Cook-Book for DNA Extraction, Restriction analysis (RFLP), Agarose Gel Electrophoresis, PCR, and Sequencing. In ICOM pre-conference workshop for DNA-based methods for identification of ectomycorrhizae.

QIAquick Spin Handbook for PCR Purification. July 2002.

Rogers, S.O., and A.J. Bendich. 1985. Extraction of DNA from milligram amounts of fresh, herbarium, and mummified plant tissues. Plant Molecular Biology 5: 69-76.



Lab 3: Amplification of the Internal Transcribed Spacer Region (ITS) from extracted DNA using Polymerase Chain Reaction (PCR)

Introduction: Genomic DNA from an organism is too large to analyze. It is therefore necessary to specifically target a small region within the DNA for analysis. The ITS region is universally present in plants and fungi and is useful for identification and elucidating species relatedness. Use of Polymerase Chain Reaction (PCR) allows researchers to target a specific region (like the ITS) and make millions of copies of it for analysis. This is achieved by using specially designed primers which bind to the DNA at both ends of the target sequence. Nucleic acids are then added to compliment a target sequence. Repeated cycles of this process, carried out in a thermocycler, result in an exponential increase in copies of the target sequence. In this lab, we will use our extracted DNA from the previous lab in a PCR reaction with primers designed to target the ITS region.

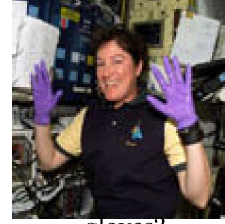
Goal: To amplify the ITS region from DNA extracted using PCR.

Materials:

1. A set of two pipettes per group (2-20µl, 50-200µl)
2. Two boxes of corresponding pipette tips with aerosol barriers per group.
3. 1 box each of latex and nitrile gloves
4. 1 strip of sterile PCR tubes and lids per group
5. 1 tube rack per group to accommodate PCR strips
6. 1 ice bucket per group
7. 1 1.5 ml tube with 50µl of Jumpstart Ready Mix® per group
8. 1 1.5 ml tube with 8µl of each primer (ITS1-F and ITS4 for fungi or ITS1 and ITS4 for plants)
9. 1 1.5 ml tube with 50µl of molecular biology grade H_2O
10. DNA extracted last week
11. Jar for pipette tip waste per group

Protocol:

For each sample, pipette the following into one 1.5 ml tube in a tube rack:


gloves?

1. 25µl of Jumpstart *
2. 4µl of primer mix *
3. 18µl of sterile water *
4. 3µl of DNA that was extracted last week *

   50µl reaction mix *
5. Place PCR mixes in ice bucket or (TA will) put in thermocycler using the program described in Table 1.

* Keep on ice at all times

Table 1. PCR program used to amplify ITS region.

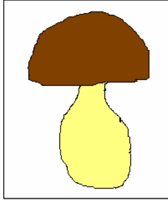
	Denaturation	**Annealing**	**Extend**	**Final**	**Store**
°C	95	55	72	72	5
Time (min)	2	0.5	2	10	∞
# of Cycles	40	40	40	1	1

Next lab: We will load these samples into an agarose gel and then perform electrophoresis to see if PCR was successful and prepare sample for sequencing.

References:

Gardes, M. and T.D. Bruns. 1996. ITS-RFLP Matching for Identification of Fungi. Methods in Molecular Biology 50: 177-186.

Ola, K., M. Jonsson, and K. Ihrmark. 1999. Cook-Book for DNA Extraction, Restriction analysis (RFLP), Agarose Gel Electrophoresis, PCR, and Sequencing. In ICOM pre-conference workshop for DNA-based methods for identification of ectomycorrhizae.



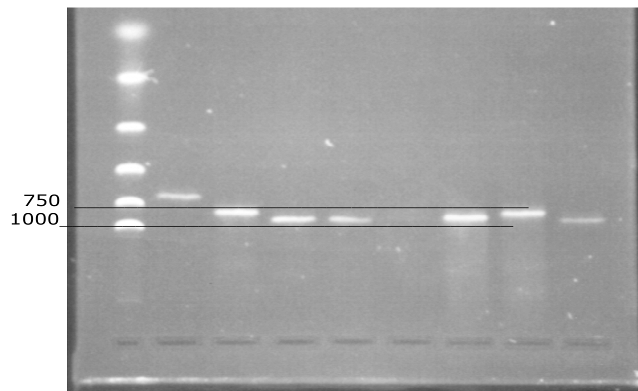
Lab 4: Gel Electrophoresis and purification of PCR product

Introduction: Gel electrophoresis is a common method used to visualize, quantify, and measure the length of DNA fragments in a sample. To determine if PCR was successful, a portion the reaction is ran through an agarose gel using an electrical current (DNA is negative and is "pulled" towards the positive end). A molecular marker is loaded next to the sample so that the length of the DNA fragment can be determined (smaller fragments travel faster). The expected length of a PCR product is usually known (the ITS region should be between 650 – 800 base pairs), therefore a "band" of that length is evidence that PCR was successful. For this lab exercise, we will analyze our PCR products by direct nucleotide sequencing. Samples of our PCR products will be sent to an automated sequencing facility and therefore need to be purified to eliminate reagents used in the PCR reaction.

Goal: To ensure a PCR product is obtained and to purify the PCR product for sequencing.

Materials:

1. A set of three pipettes per group (2-20µl, 50-200µl, 200-1000µl)
2. Electrophoresis grade agarose (weighed out on scale)
3. TBE buffer (1X)
4. Centrifuges
5. 6 sterile microcentrifuge tubes per person
6. DNA stain
7. 50 ml graduated cylinder
8. Gel lane sheet
9. 2 electrophoresis boxes
10. Power source
11. PCR marker
12. Qiagen® PCR purification kit



Example of DNA bands migrating in an agarose gel. Numbers indicate length of bands in base pairs based on molecular marker length.

Protocol:

<u>Gel Preparation</u> **(Optional or done by TA)***

1. Weigh out enough agarose to make a 1.5% gel. Quantity of agarose depends on the gel box capacity. For example, if the gel box normally holds 40ml, then 0.015 x 40 = 0.6g agarose.

2. Measure appropriate volume of 1X TBE Buffer using a graduated cylinder (this would be 40 ml for our example).

3. Combine agarose and TBE buffer in a 100ml Erlenmeyer flask and briefly swirl. Heat in a microwave or hot plate until all flakes of agarose are dissolved. If using a microwave, heat on high for 10 seconds, then swirl, and repeat until all flakes of agarose are dissolved.

4. Pipette enough Ethidium Bromide (CARCINOGENIC!) to comprise 0.003% of the gel (less than 1 µl)**. Alternatively, use approximately 3 drops of Carolina Blue DNA Stain (this is the safe alternative, but see critique**).

5. Pour into gel tray making sure it is level and no air bubbles are present (use a pipette tip to pop them). Place comb into gel.

6. Let gel harden for approximately 10 – 15 minutes or until opaque. Remove comb and position gel in box. Add enough 1X TBE Buffer to cover the gel.

* **Note:** Gel preparation adds approximately 30 – 60 minutes to the lab exercise. To save time, the instructor or TA can prepare the gel using EtBr prior to lab time (see below).

****Note:** Ethidium Bromide is HIGHLY carcinogenic, and it is *not* advised for student use. EtBr results in a better visualization of DNA than Carolina Blue Stain, but must be used in conjunction with a Transilluminator versus a simple light box. In addition, using EtBr instead of the Carolina Blue DNA Stain will save approximately 30 minutes in the final staining and de-staining procedures.

<u>PCR Product Visualization and Purification</u> **(students start here)**

1. Load 20µl (ONLY!) of your PCR product into an available lane in agarose gel using the appropriate pipette; make sure you indicate on sheet WHICH LANE your product is in. Replace the lid on the gel box,

making sure the electrodes are connected. Turn on power supply and adjust the voltage to 75. Run gel for 1 hour.

2. While a portion of your PCR product is running on the gel, take the remaining product (approx. 30µl) and pipette into a fresh 1.5ml labeled microcentrifuge tube.

3. Add 125µl Buffer **PB** to your sample (from Qiagen kit).

4. Pipette Buffer PB and your PCR product into labeled Qiagen spin column and let stand for 1 minute. From here on this procedure should be familiar, it's the same technique we used to isolate and purify your DNA!

5. Centrifuge spin columns for 1 min at 6,000 rpm's. Your PCR product is now **bound in the filter**.

6. Discard the liquid in the collection tube (flow-through)

7. Add 750µl of Wash solution to spin column and let stand for 5 minutes.

8. Centrifuge for 1 minute at 14,000 rpm's.

9. Discard flow-through in collection tube by decanting, then centrifuge again at 14,000 rpm's for 1 minute.

10. Add 40µl of Buffer **EB** (from microcentrifuge tube) making sure the buffer covers the filter and is not on the sides of the tube. Let stand for 1 minute.

11. Centrifuge for 1 minute at 6,000 rpm's.

12. Place tube in black ice bucket at the front of the room. Your sample is now ready to be sequenced.

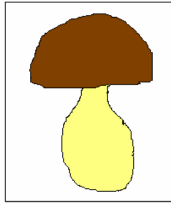
13. After 1 hour, your DNA will have "run out" on the gel, turn off the power supply and remove the lid.
    - If your gel was stained using the Carolina Blue DNA stain, carefully remove it from the box (in a tray) and place it in the final stain solution for 10 minutes. De-stain gel for another 20 – 30 minutes by placing it in deionized water and swirling. Replace the water every 5 minutes to hasten the process.
    - If your gel contains EtBr, a TA will transport and photograph the gel under a UV transilluminator.

14. If your PCR product is present, it will be visualized as a single band in the 650-800 base pair size range in the lane that you loaded your sample. Record its presence on the sheet provided and indicate the intensity of your band relative to the marker (ex. A bright band might be 3x brigher that the band in ladder).

References:

QIAquick Spin Handbook for PCR Purification. July 2002.

Ola, K., M. Jonsson, and K. Ihrmark. 1999. Cook-Book for DNA Extraction, Restriction analysis (RFLP), Agarose Gel Electrophoresis, PCR, and Sequencing. In ICOM pre-conference workshop for DNA-based methods for identification of ectomycorrhizae.



LAB 5: ANALYSIS OF FUNGAL SEQUENCE USING BLAST SEARCH

Introduction: Basic Alignment Search Tool (BLAST) is a free online search tool that allows researchers to compare sequences from their unknown organisms or genes to those from known sources in the GenBank database. GenBank contains sequences from thousands of plants and fungi, and can potentially be a powerful tool for identifying unknown organisms. The program works by performing a multiple sequence alignment of your sequence with other similar sequences. A list of the sources of sequences that most closely match your own is also provided. The strength of a match can be determined from the Score value (the higher the better) and Blast Expected value (the lower the better). As a cautionary note, ANYONE can submit sequences to the GenBank database; therefore it is essential that the source of the sequence can is deemed credible to be certain of the resulting identification.

GOAL: To determine the identity of fungus or plant by submission of the ITS sequence to BLAST search for comparison to known taxa.

Materials:
1. Sequence
2. Computer with internet access

Protocol:

1. Open your sequence saved as a word document.

2. Select the sequence and right click to "Copy"

3. Go to www.ncbi.nlm.nih.gov/BLAST. Under the Nucleotide heading (first box on left), choose Nucleotide – nucleotide (blastn)

4. Right click to "Paste" your sequence into top box labeled "Search"
5. Click "BLAST!"

6. Then click "FORMAT!"

7. The results of your BLAST search will now be displayed. A list of the closest matches (with scores) and BLAST expected values will indicate the fungus (or plant) with a sequence most similar to yours starting at the top. Scrolling down this list you will see the details of each match starting with the closest match. The percent identity tells you how similar your sequence is and it also gives you the number of base pairs that are identical to your sequence. If you click on the ascension number of that fungus, it will tell you who submitted it (ANY ONE can submit a sequence, so you must check this) and see if it's been published.

References:

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic alignment search tool. Journal of Molecular Biology 215: 403 – 410.

MOLECULAR BIOLOGY GLOSSARY

http://seqcore.brcf.med.umich.edu/doc/educ/dnapr/mbglossary/mbgloss.html