DNA Barcoding

DNA Barcodes are used to identify and classify living things, and allow non-experts to objectively identify species – even from small, damaged, or industrially processed material. Just as the unique pattern of bars in a universal product code (UPC) identifies each consumer product, a "DNA barcode" is a unique pattern of DNA sequence that identifies each living thing. Molecular biology techniques, including DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, and nucleotide sequencing can be employed to generate data for phylogenetic analysis through the DNA Subway “Blue Line”.

Barcoding is made possible due to the variability in the mutation rate in genes. For example, the chloroplast gene ribulose-1,5-bisphosphate carboxylase (rbcL) and the mitochondrial cytochrome-c oxidase subunit I (COI) has regions that are highly conserved across species and other regions that highly variable. Similarly, regions that code for the 16S ribosomal RNA could be used. Using primers to the highly conserved region we can amplify the variable region. The sequence of the variable region will help us identify which species your tissue sample was derived from. This analysis has been done to show that the species of fish advertised in the fish market is not always the true species.

DNA barcoding is used to identify the species an individual belongs to, this applies to both flora and fauna. We will use it to identify the species of fish that is commercially available in Riverside. Some sample prep prior to the lab is required and is described below:

1. All participants will supply their own experimental materials by obtaining and gathering tissue samples from various fish/plants available at your local market/restaurants/neighborhood. You need a piece of fresh or frozen (has to be uncooked) tissue no bigger than a dime. It is important that you obtain as much information as possible such as the name of the organism, if available and location. No live fish will be killed during the course of this experiment.

2. Place your tissue in a zip-top bag and label the bag with your name and include as much information as possible such as where you obtained it, what type of fish/plant it is presumed to be, date, etc. Keep it in a freezer/ice until for later use.

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DNA Isolation

We will isolate DNA from tissue and test the quality of that DNA isolate

**DNA Extraction**
Work in pairs. One person will attempt to extract DNA from plant tissue, the partner will isolate DNA from animal tissue.

**Materials list:**
- Tissue (plant or animal)
- Extraction Buffer
- 10% SDS (sodium dodecyl sulfate)
- 5M KOAc (Potassium Acetate)
- 4 pieces of Miracloth (filter cloth)
- 100% Isopropanol
- 70% Ethanol
- Re-suspension Buffer
- Sterile water
- Freezer block
- Ice
- 65°C bead block
- 1.5 ml Tubes
- blue micro-pestle

**Method:**

1. Label two of the 1.5 ml tube with the tissue names you are using and your initials. Label it with the type of tissue you are using.

2. Cut a piece of tissue about the size of a lentil (or the equivalent of two rice grains) and place in its corresponding tube (one for the 1.5 ml tube another for the 2 ml tube). Add 750 µl of Extraction Buffer to the 1.5 ml tubes and begin to grind the tissues in the 1.5 ml tubes using the blue micro-pestle. The Extraction buffer contains EDTA a chemical that removes magnesium ions from solution and prevents DNases from destroying the DNA. The buffer keeps the pH slightly basic to further block enzyme function.

3. Add 120 µl of 10% SDS. Mix by inverting. SDS is a detergent that denatures and binds to the hydrophobic portion of protein and lipids. SDS is the main detergent in shampoo where it does the same thing to clean hair.

   Prepare all samples to this step. Keep them on ice until all are ready for step 5.

4. Place tubes in a 65°C bead block for at least 20 minutes.

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5. Add 225 µl 5M KOAc. Mix well by inverting several times (important!), then place on ice 5 minutes. *KOAc causes the SDS to precipitate along with the proteins and lipids. The solution will turn cloudy.*

6. Spin for 5 minutes at top speed in a centrifuge. Label a set of new tubes (initials, type of fish).

7. Squirt about 700 µl of the supernatant through miracloth funnel into the second tube. The instructors will demonstrate how to make a funnel out of the miracloth. *This step removes solids that did not pellet during the centrifugation.*

8. Add 600 µl of ice-cold isopropanol. Mix the contents thoroughly by inverting. *The isopropanol (rubbing alcohol) causes a change in the hydrophobicity of the solution and the DNA precipitates. DNA precipitate may or may not be visible at this point; don’t worry if you don’t see much. Potential stopping point.*

9. Spin for 5 minutes at top speed. *Look for a small glassy pellet in the bottom of the tube that may be visible. The instructors will help you.*

10. Carefully pour off supernatant. Then use a P20 set to 20 µl to remove the last drops.

11. Add 500 µl of 70% ice-cold ethanol and flick until the pellet comes off the bottom. *The ethanol removes residual isopropanol and salts from the pellet. Potential stopping point.*

12. Spin 5 minutes.

13. Pour off the ethanol. Then use a P20 set to 20 µl to remove the last drops. Make sure the pellet stays in the tube!

14. Place the tube in the bead bath with the top open for 5 minutes to allow the remaining ethanol to evaporate.

15. Resuspend the DNA in 50 µl Re-suspension Buffer. Let sit at RT for about 5 minutes. Depending on amount of starting material may need to be diluted for PCR.

16. We will use a nanodrop (spectrophotometer) to measure your nucleic acid concentration. Your table facilitator can help you.

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Polymerase Chain Reaction

**How it works:**
PCR is a powerful technique used to isolate and amplify specific regions of DNA. The Actin gene that is the focus of this activity is about 2 kb and is one of over 20,000 genes in the genome. PCR is a remarkable technique because a scientist can use it to isolate a single gene from the entire genome. Figure 1 describes the steps in PCR.

**Figure 1.** The polymerase chain reaction specifically copies a target DNA sequence. (a) Double-stranded DNA containing the target sequence. (b) Two primers have sequences complementing primer-binding sites at the 3’ ends of the target gene on the two strands. The strands are separated by heating (called denaturation); then cooled to allow the two primers to base pair to the primer-binding sites (called annealing). Together, the primers thus flank the targeted sequence. (c) After the temperature is raised, Taq polymerase then synthesizes the first set of complementary strands in the reaction (called extension). These first two strands are of varying length, because they do not have a common stop signal. They extend beyond the ends of the target sequence as delineated by the primer-binding sites. (d) The two duplexes are heated again, exposing four binding sites. After cooling, the two primers again bind to their respective strands at the 3’ ends of the target region. (e) After the temperature is raised, Taq polymerase synthesizes four complementary strands. Although the template strands at this stage are variable in length, two of the four strands just synthesized from them are precisely the length of the target sequence desired. This precise length is achieved because each of these strands begins at the primer-binding site, at one end of the target sequence, and proceeds until it runs out of template, at the other end of the sequence. (f) The process is repeated for many cycles, each time creating more double-stranded DNA molecules identical with the target sequence.
We will test the quality of your DNA extractions using the Polymerase Chain Reaction (PCR). You and your partner will share the DNA you extracted plus an unknown sample. You will need a negative control as well (qH2O). We will use 16S primers. If available, COI primers can be used.

1. Label a 1.5 ml tube with your initials and MM.
2. Label a strip of 0.2 ml tubes.
3. Fill in the table below with the values on the white board.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1 reaction (µl)</th>
<th>4 reactions (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.5</td>
<td>6.0</td>
</tr>
<tr>
<td>2X MyTaq</td>
<td>7.5</td>
<td>30.0</td>
</tr>
<tr>
<td>Primer F</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Primer R</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Total</td>
<td>10.0</td>
<td>40.0</td>
</tr>
<tr>
<td>DNA</td>
<td>5.0</td>
<td>-</td>
</tr>
</tbody>
</table>

4. Pipette 15.0 µl into each small tube in the strip.
5. Add 5.0 µl DNA to each sample tube (4 samples, one from each group member).
6. Add 5.0 µl of water to the last tube.
7. Place strip in thermocycler.
   Run program “FISH BRCD”
   
   98°C  3 min  
   98°C  20 sec 
   52°C  20 sec 
   72°C  40 sec 
   72°C  5 min 
   12°C  ∞
   
   35 cycles    primers (16S)

   16Sar-5′  CGCCTGTATTATCAAAAACAT
   16Sbr-3′  CGGTTCTGAACTCAGATCAGCT

**Gel Electrophoresis**

Pour a 1.5% agarose gel. **WEAR GLOVES!!**

1. Put 0.8 g of agarose in a boiling flask.
2. Fill the flask with 1X TAE to the red line.
3. Microwave for 1.5 mins.
4. Add 2.0 µl of Ethidium Bromide.
5. Swirl and pour into the gel rig.
6. After PCR is finished.
a. Load 5.0 µl of ladder into the first well.
b. Load 12.0 µl of each reaction into the other wells.
c. Run the gel at 170V for 20 min.

7. Photograph the gel using the gel imager.

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**Learning Goals**

Students will:
1. Be able to calculate dilutions.
2. Be able to properly dilute common reagents.
3. Be able to use a micropipette to accurately measure liquids.