PCR PROTOCOL (E. STRAND 2017)

Set-up: Turn on PCR machine and start pre-heating without starting the protocol.

1). Make the "Master Mix" in a single centrifuge tube labeled "M" based on # of samples and content below. Wait to add the Taq Polymerase until DNA has been placed into PCR tubes.

"8 + 1" = 8 samples + 1 negative

"Negative" (-) is a validity measure that refers to a PCR tube that contains NO DNA, only Master Mix. If a band shows up in the "negative" column on the gel, we can't trust our DNA sample columns to be correct either.

Component	**Volume (ul)**	**8 + 1**	**16 + 2**	**24 + 2**	**32 + 2**	**40 + 2**
5% DMSO	6.25	62.5	112.5	175	212.5	262.5
H2O	2	20	36	56	68	84
10x Buffer	1.25	12.5	22.5	35	42.5	52.5
MgCl2 (50 mM)	0.625	6.25	11.25	17.5	21.25	26.25
Primer Mix F	0.125	1.25	2.25	3.5	4.25	5.25
Primer Mix R	0.125	1.25	2.25	3.5	4.25	5.25
DNTP (10 mM)	0.0625	0.63	1.13	1.75	2.13	2.63
Taq	0.0625	0.63	1.13	1.75	2.13	2.63
DNA	2	2	2	2	2	2
total	12.5	100	200	300	400	500

10.5 ul "Master Mix" + 2 ul DNA = 12.5 ul reactions

25 ul reactions – calculate concentrations accordingly; 23 ul "Master Mix" + 2 ul DNA

5% DMSO - binds to the DNA at the Cytosine residue and changes its conformation which makes the DNA more labile for heat denaturation. Since most of the primers are GC (guanine-cytosine) rich, DMSO indirectly facilitates the annealing of primers to the template (this enhances the amplification).

10x Buffer – Creates optimal conditions for activity of Taq DNA polymerase (based on contents of buffer).

MgCl2 (50 mM) – Helps with the removal of beta and gamma phosphate from DNTP by binding to the alpha phosphate group of DNTP (breaks down DNTP so Taq can bind them to the new strand of DNA).

Primers (Forward & Reverse) – used to determine the DNA fragment to be amplified by PCR. We're always trying to isolate a specific gene so before starting PCR, we have to either design our primers (you can do this via Blast) or find them from a previous paper.

- ex): For Baitfish Fall 2017, we are isolating a universal fish gene COI. This gene is very commonly used for genetic barcoding across all organisms.

dNTP (10 mM) – deoxynucleotide triphosphates; single units of the bases A, T, G, and C that are used to make the new DNA strand.

Taq Polymerase – An enzyme that can withstand protein-denaturing conditions (high temp) required to run PCR. Attaches nucleotides to a DNA template, thereby coping the DNA.

2. Add 2 ul of DNA to its corresponding PCR tube. Label the tops and sides of the tubes in case the heat from the PCR machine rubs off the top label. You can never label enough!

3. Add the Taq polymerase to the "Master Mix" and flick the tube a few times to mix the solution. Taq is sensitive and will start to work right away so we wait as long as we can before adding it to the mix to preserve its function.

4. Add 10.5 ul of "Master Mix" to each PCR tube. At the end each sample tube should have 12.5 ul total (10.5 ul MM + 2 DNA) and the negative tube should have 10.5 ul MM only.

** Make sure there are no bubbles or solution along the sides of the PCR tube, fix carefully with a pipette**

5. Place in PCR machine and start the following protocol (at this point it should already be inputted as a program in the machine):

TEMPERATURE °C	**TIME**	**CYCLES**
95	2 MIN	1
94	30 SEC	35
52	30 SEC	
72	1 MIN	
72	10 MIN	1
4	hold	

1. Denaturation (94°C)

The hydrogen bonds holding complementary strands of DNA together broken

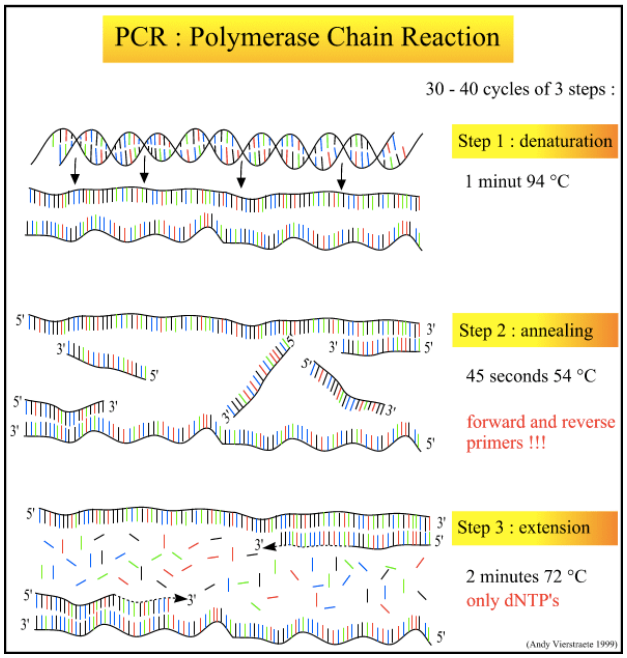
2. Annealing (52 °C) *

The primers (forward and reverse) attach to the DNA template

3. Extending (72 °C)

The new strand of DNA is made by Taq polymerase enzyme moving in both forward and reverse directions.

* 52 °C doesn't work perfectly for all primers. When you get a new set of primers you should set up a gradient of temperatures to test the optimum (46 °C – 56 °C ish). Usually the optimum is between 50 °C -54 °C.



Troubleshooting at the end of this packet.
Also, some labs use "GreenTaq" which is a solution you can buy that is pre-mixed with buffer, dNTPs, etc. In that case, "Master Mix" just needs GT, F/R Primer, and H2O.

VISUALIZING YOUR PCR PRODUCT

Spectrophotometer – (or nanophotometer) measures the amount of photons (intensity of light) absorbed after it passes through a solution so the amount/concentration of a known chemical substance can be measured. Nucleic acids absorb (UV) light due to heterocyclic/aromatic rings (not sugar – phosphate backbone, but AGCT). **Usually pay the most attention to 260/280 ratio.

- see directions of the instrument wherever you are, all of them are slightly different

Ng/ul: [DNA]
260/280 Ratio: assess the purity of your DNA sample. Pure RNA is ~2.0 but a range of 1.7 – 2.0 is good.
260/230 Ratio: if this ratio is too high or too low usually your blank measurement was messed up.

GEL ELECTROPHORESIS PROTOCOL:

A gel matrix that contains an electrical field used to separate DNA strands based on size. The bigger strands move through the gel slower and tend to stay closer to the wells compared to the small strands that will move faster and end up closer to the end of the gel.

"Run to red" – the black (-) (top of the rig next to the wells) and red (+) (bottom of the rig near the end of the gel) cords of the gel rig. DNA bands are negatively charged (because of the phosphate group) so they will run to the bottom of the gel (red).

Making the gel:
Make sure to use graduated cylinders and flasks that are already labeled TAE and used only for TAE.
Place the gel "boat" and "comb" into the rig so that it remains level, the silicon sides do not create gaps, and the "comb" rests in the indents along the "boat" wall.

Add 100 mL 1x TAE buffer + 1.2 g agarose powder to a flask
Heat the mixture for 1 minute 30 seconds
Add the heated mixture to the "boat" and leave to harden (time will vary based on size of gel)
The "comb" must be resting in the liquid so that when the gel hardens and comb is removed, deep wells will appear.

Loading the gel: Using a well plate (or parafilm)
The first well will contain the "ladder": set of standards used to approximate size of DNA bands.
1 ul of ladder + 1 ul 6x loading dye + 4 ul H2O

The next wells will be DNA samples: (this combination can very b/w labs)
2 ul DNA + 3 ul H2O + 1 ul 6x loading dye

The last well will be the negative:
2 ul "negative" + 3 ul H2O + 1 ul 6x loading dye

6x loading dye: causes the sample to sink to the bottom of well so the sample will run properly. Your sample will not run without dye.

Take your full well plate to the gel rig and carefully draw up the 6 ul well and disposit into its corresponding well. Always put the ladder in the first well, then DNA samples, and finally the negative. Once all wells are properly loaded, fill the gel rig with excess 1x TAE buffer, the gel must be fully submerged in 1x TAE buffer.

Running the gel: (differs between labs and what you're running)
at 80V for 1 hour and 30 minutes. Or at 120 V for 30 minutes. Make sure there are bubbles coming from both ends of the rig, if there are no bubbles it means your gel rig isn't working. After about a minute, you should start to see the DNA sample separate from the dye, a darker band will be left behind as a light blue band moves down the gel.

Staining the gel: To see the DNA strands under UV light, we must stain the gel with EtBr. This causes any ladder or DNA strands in the gel to appear white. Each lab does this process differently, some labs add EtBr to the gel and sample itself while loading and others will place the entire gel in a EtBr "bath" for about 45 minutes and a DI "wash bath" for about 10-15 minutes. Be careful not to stain for too long, this will cause the gel background to become "noisy" and it will harder to see the bands.

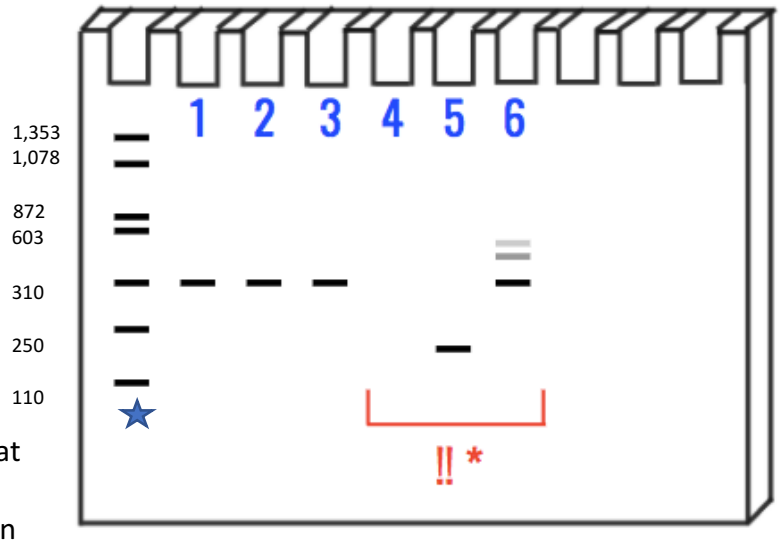
Taking a photo of the gel: Each lab will have a different instrument, but essentially each one will expose the gel to UV light and DNA bands will appear on the gel (in a photo on the computer, not physically on the gel).

★ LADDER

1, 2, 3 = worked perfectly, yay! ☺
We want dark, single bands at the correct base pair length

4, 5, 6 = didn't work, boo ☹
Now we must go through a series of troubleshooting. The problem could be either with DNA extraction, PCR, or gel loading.

"primer dimer" – cloudy, blurry, non-distinct band at the very end of the gel, even below the end of the ladder; primers bind to themselves and shows up on the gel.



*These numbers are just examples, each lab will use a different ladder: 50 bp (base-pair), 100 bp, Phix, etc.

TROUBLESHOOTING: the super fun part! (if all else fails, google PCR troubleshooting, lots of resources)
Change one thing at a time so you know what change affects what outcome.
PCR:
Annealing temperature – try a gradient of temperature/MgCl2 (try this first, best bet) (6)
+ / - [DNA] to the PCR tube (BUT be careful sometimes too much DNA can be inhibitory, dilute) (4)
+ / - [primer] , [additives] (buffer, dNTP, etc but most often primer is more effective) (4)
Human error (i.e. pipetting issues), just try it again without any changes
Reconsider primer design if other troubleshooting doesn't work (5)

Gel:
+ / - [EtBr]
Human error—loading (annoyingly common) (4)

DNA extraction: normally not the issue but it can be; need to re-do the extraction but don't need to change the protocol, usually human error. This is a last resort effort, try PCR and gel before extraction changes.
+ / - [tissue] (4)