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DNA Barcoding of Fish Eggs Methods

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Protocol status: Working
We use this protocol and it's working

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Funders Acknowledgements:
Florida Institute of Oceanography's RESTORE Act Centers of Excellence Program
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Abstract

This DNA barcoding of fish eggs method is used to identify fish eggs down the the species level. Our lab has been using this method for about 10 years with good results and many publications. It is a destructive sampling method, meaning you will only have one chance to extract the DNA from the fish eggs. Once extracted, fish egg DNA samples have lasted in our -20 degree C freezers for over 5 years with good quality DNA. Many of the reagents used can be swapped for your preference.

Protocol materials

- Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #**BP2818500
- Water DNA Grade DNASE Protease free **Fisher Scientific Catalog #**BP24701
- 10 M NaOH **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**72068
- 0.5 M disodium EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4055
- 1 M Tris-HCl **Fisher Scientific Catalog #**BP2473-1
- Agarose powder **ApexBio Technology Catalog #**20-101
- Ethidium Bromide (1% Solution/Molecular Biology) **Fisher Scientific Catalog #**BP130210
- Flat PCR Caps, strips of 12 **Fisher Scientific Catalog #**AB0851
- AlumaSeal II Sealing Films **Fisher Scientific Catalog #**AF100
- BSA 20 mg/mL **Fisher Scientific Catalog #**FERB14
- Dntps **Genesee Scientific Catalog #**42-403
- Red Taq & Buffers (NH4, MgCl2) **Genesee Scientific Catalog #**42-401R
- GeneRuler DNA Ladder **Fisher Scientific Catalog #**SM0334

Picking Eggs

1 Prepare X amount of 1.5 mL screw cap tubes (1 per site)

Equipment	
Screw cap tubes and caps	NAME
Tube	TYPE
Genesee	BRAND
21-262, 21-266A	SKU

2 Label each tube with site #

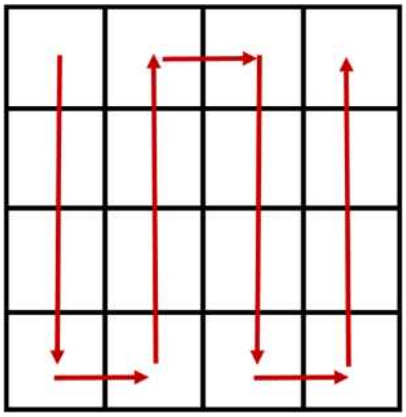
3 Pipette about 1 ml 70% ethanol into each tube
-Make 70% ethanol in a falcon tube (7 parts ethanol to 3 parts DI water)
Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #**BP2818500

4 You will be using a dissecting scope with dark field illumination, turn on the light

5 Pour all contents of one vial of CUFES plankton into a square, gridded petri dish
-Use disposable pipette to rinse out eggs stuck in vial with excess ethanol in plate
-Don’t worry about getting all the plankton out, just the eggs

Equipment	
Square petri dish	NAME
Petri dish	TYPE
Andwin Scientific	BRAND
70691CS	SKU

6 Using the petri dish grid, go from left to right, up and down searching for eggs (see figure)
-This is to prevent bias of picking large or small eggs
-Go through whole plate or until you have reached 100 eggs
-Count the remaining eggs in the sample and record the number



Petri dish diagram

7 Pick up eggs up using forceps (PL-30 size) and place into screw cap tube
-Be careful to not pick up other plankton or pop eggs
-Push each egg into the ethanol so they do not desiccate

8 Use a counter clicker to keep track of how many eggs you have collected

9 When you have finished the grid or collected 100 eggs, place a clean petri dish under the vial (to catch spills)

10 Carefully pour the CUFES contents from the picking petri dish into the original vial
-Use a disposable pipette and excess ethanol in vial to rinse off dish to gather all plankton back into vial
-Use additional 70% ethanol to rinse off dish if needed
-Do not overflow vial
-If any contents spilled onto the extra petri dish, also add those to the vial

11 Store screw cap tubes and vials at room temp

12 Rinse off plate with water to remove excess ethanol & wipe off forceps with ethanol

13 Turn off microscope

HotSHOT extraction

14 Follow the HotSHOT method

Citation

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT).
Biotechniques.

https://doi.org/10.2144/00291bm09 LINK

This method was applied to zooplankton eggs in 2008

Citation

Montero-Pau, Javier, Africa Gómez, and Joaquín Muñoz (2008)
. Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs.
Limnology and Oceanography: Methods .

https://doi.org/10.4319/lom.2008.6.218 LINK

This method was applied to fish eggs in 2018

Citation

Makenzie Burrows, Jeremy S. Browning, Mya Breitbart, Steven A. Murawski, Ernst B. Peebles
(2018). DNA barcoding reveals clear delineation between sp. Fisheries Oceanography.

https://doi.org/10.1111/fog.12404 LINK

15 Make Alkaline lysis buffer
-Add 25 ml of PCR water
Water DNA Grade DNASE Protease free **Fisher Scientific Catalog #**BP24701
-Add 62.5 µl of 10 M NaOH (base cabinet)
10 M NaOH **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**72068
-Add 10.0 µl of the 0.5 M disodium EDTA (chemical cabinet-right side)
0.5 M disodium EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**4055
-Make fresh every 1-2 months
-Stored at room temperature

16 Make Neutralization reagent
-Add 24 ml of PCR water
Water DNA Grade DNASE Protease free **Fisher Scientific Catalog #**BP24701
-Add 1 ml of 1 M Tris-HCl
1 M Tris-HCl **Fisher Scientific Catalog #**BP2473-1
-Stored at room temperature

17 Pick individual eggs into individual PCR tubes (use PCR strips) using a sterile pipet tip (10-100 ul pipette depending on size of egg), remove excess ethanol

This can be difficult! Tips and tricks:
-You need to pick the right size tips to get the eggs. Pick a tip that is slightly larger than the eggs you are trying to pick up
-The whole process is done with pressure
-Push out all the air in the pipette tip before sucking up the egg
-Keep pressure on the egg or it will come off the tip as you pull it from the tube
-If you accidentally suck up an egg within the tip, don't panic!
-Gently push the egg out of the tip by flushing it with ethanol up and down slowly to avoid popping it
-If it is really stuck, you can cut off the tip of the tip and try again

Equipment

PCR tubes	NAME
Tubes	TYPE
Genesee	BRAND
22-705A	SKU

18 Add 50 µl of alkaline lysis buffer and crush egg against side of tube using a sterile toothpick or pipette tip
-Sterilize toothpicks in plastic small tri-pour on dry cycle in Autoclave
- If you struggle to pop the egg, try removing the liquid, popping the egg, then re-adding the liquid

19 Incubate tubes at 95 C for 30 minutes
-Turn on thermocycler (back right) or any block incubator
-Click incubate – set 95 C plate and 56 C top without timer (infinity sign)
-Make sure tube caps are tight! They will pop off it they are not secured
-Set a timer for 29 minutes and 30 seconds so you have time to take it out

20 Cool tubes on ice for 3-4 minutes, then centrifuge briefly to spin down any condensation

21 Add 50 µl neutralizing reagent to each tube

22 Vortex the tubes to mix and centrifuge briefly. This is your DNA template, use 2 µl for PCR or freeze at -20C for long term storage
-To store in the freezer, label a box with the year, your initials, and fish eggs

PCR (Polymerase Chain Reaction)

23 Fish egg primer cocktail sequences:
VF2_t1 - TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC
FR1d_t1 - CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA
FishF2_t1 - TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC
FishR2_t1 - CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA

New primers that need to be reconstituted
- Add x µl of DNA free water (equal to the number of ng DNA on each primer tube) to dry primer powder
- Vortex until powder dissolved, spin down

Primer dilution
- Add 100 µl of each primer to a 1.5 ml tube, then add 600 µl DNA free water
- This can be done with smaller aliquots if you are low on primer stock
- Ex: Add 25 µl each primer and 150 µl water (Makes 10 µM primers)

Citation

Natalia V. Ivanova, Tyler S. Zelmak, Robert H. Hanner, Paul D. N. Herbert (2007)
. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes.

https://doi.org/10.1111/j.1471-8286.2007.01748.x LINK

24 Reagents needed:

- BSA 20 mg/mL **Fisher Scientific Catalog #**FERB14
- Dntps **Genesee Scientific Catalog #**42-403
- Water DNA Grade DNASE Protease free **Fisher Scientific Catalog #**BP24701
- Red Taq & Buffers (NH4, MgCl2) **Genesee Scientific Catalog #**42-401R

25 Turn on thermocycler & prepare program
(Makenzie's folder - "Fish45")
94 C for 2 min
45 cycles of
94 C for 30 sec
52 C for 40 sec
72 C for 1 min
72 C for 10 min

26 Bleach & UV hood, pipets, and rack

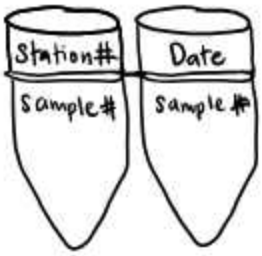
27 Fill small cooler 1/3 full of ice

28 Defrost DNA on top of ice (may need to flick to mix occasionally)
Put Red Taq buried in ice

29 Defrost other reagents on bench until completely defrosted - primers, dNTPs, NH4, MgCl2, BSA
Optional - roll reagents in hands to warm faster

30 Vortex and spin down all reagents EXCEPT DNA (flick and spin) & Red Taq (only spin down)

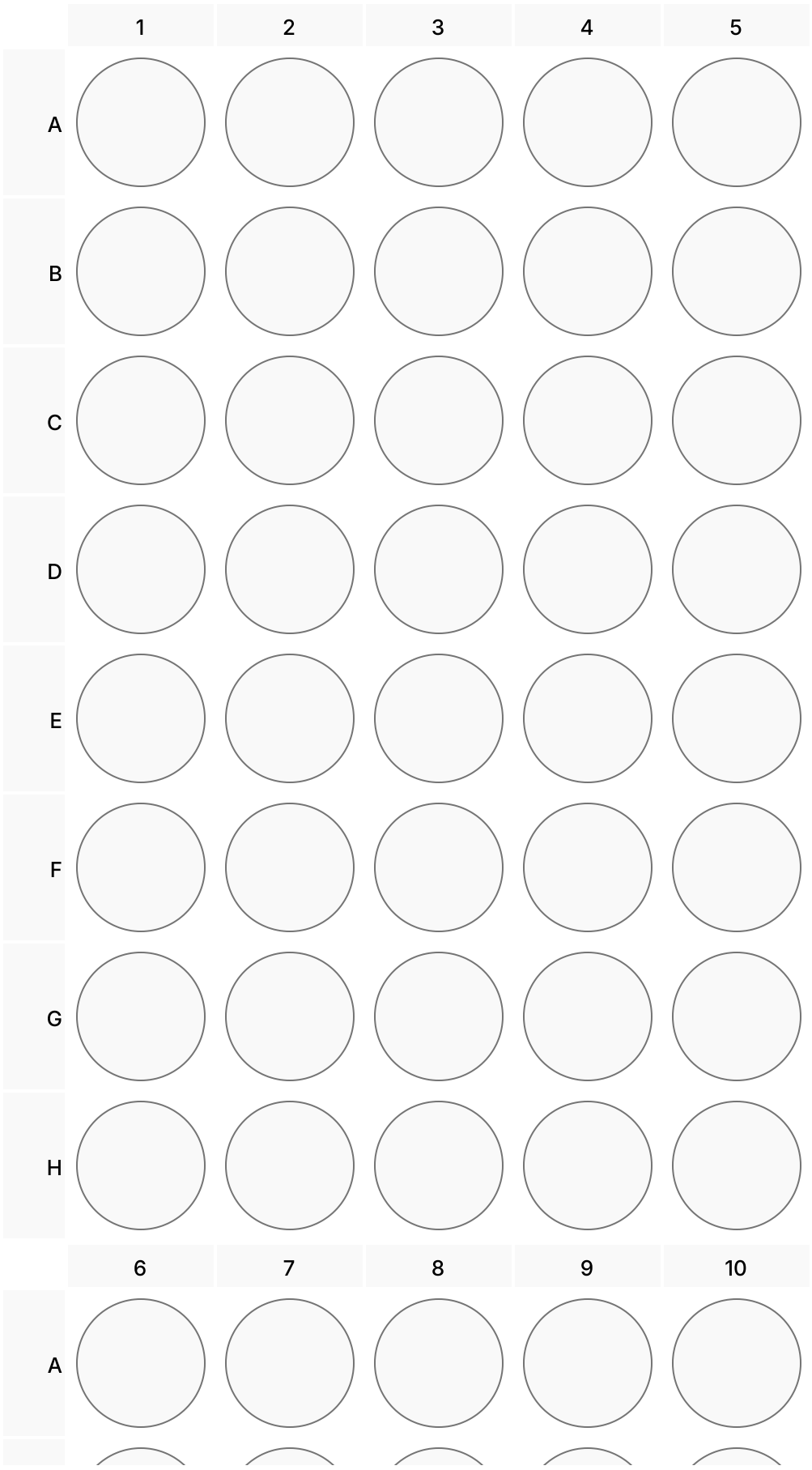
31 While waiting for defrosting to happen, label all tubes in numerical order and include a negative control (-)
PCR tubes are small. Write station number and date at the top of the tubes, then sample numbers on middle of tubes.



Tube label example

Equipment	
PCR tubes	NAME
Tubes	TYPE
Genesee	BRAND
22-705A	SKU

32 If using a 96 well plate, label the side with the date, stations, initials. Fill out a form like below to orient the order of your samples.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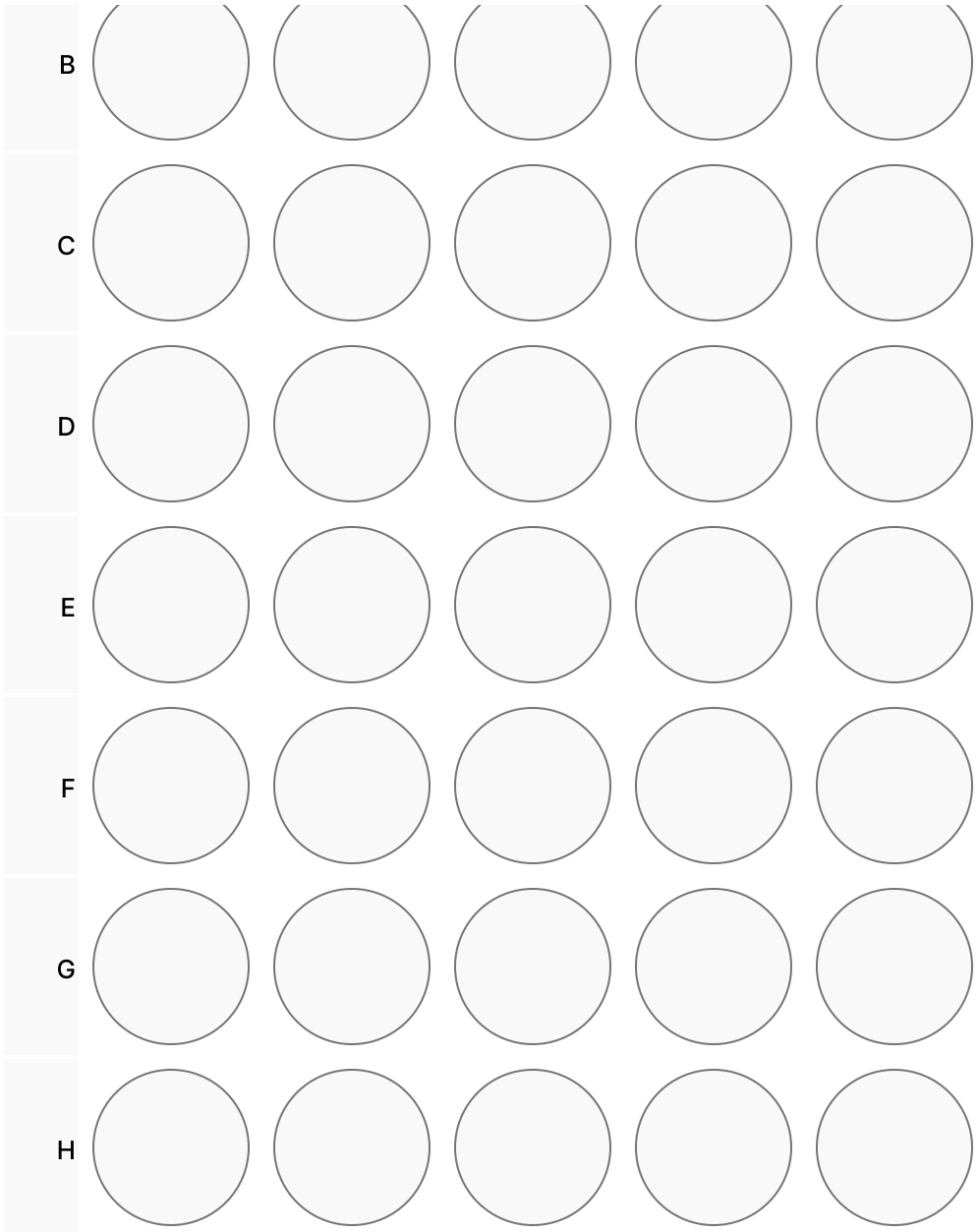
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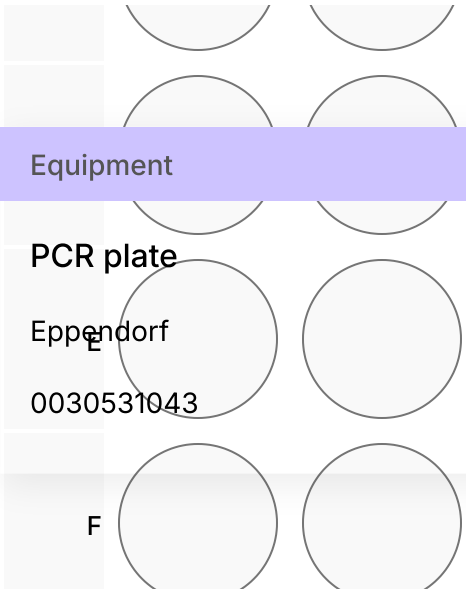


Equipment

NAME	BRAND	SKU
PCR plate	Eppendorf	0030531043

33

Note

Work quickly! Temperature sensitive products (DNA & taq) need to be kept on ice

Prepare master mix for x templates, 1 negative, and 1 extra
For 96 well plates, use 100x to account for loss in the multichannel pipette reservoir

Example for 50 µl PCR reaction (1x)

Water - 38 µl
10X NH4 Buffer - 5 µl
50 mM MgCl2 - 1.5 µl
BSA - 0.5 µl
10 µM Primers** - 1 µl
10 mM dNTPs - 1 µl
Taq - 1 µl
Template - 2 µl (for each tube with different template DNA)

34 Mix all reagents together in appropriate size tube (except DNA)

35 Pipette 48 µl of master mix into each PCR tube

36 Add 2 µl DNA template into each PCR tube (matching up egg numbers) except the negative control

37 Flick tubes and spin down in centrifuge
(PCR plates need to be covered with foil and spun down as well)

38 Put everything in a cooler and bring to the PCR room (only bring tubes inside room)

39 Add samples to thermocycler & begin cycle (double check it is running before you leave)

40 Take off your gloves, retrieve cooler/racks and clean up in main lab (put away reagents, dump ice, bleach/UV hood)

41 02:00:00
Incubation time

42 Taking out samples:
Press Enter to exit reaction (make sure it is on your program – view at top of screen)
- If it is not on your program, use the arrow keys to get to the bottom and press enter to select your thermocycler
- Open thermocycler and remove your products
- Turn off thermocycler (unless it says otherwise)!

43 Place PCR tubes in baggie or rack to store in -20 C freezer or run a gel immediately

Gel Electrophoresis

44 Make gel while waiting for thermocycler to finish

Note

- Gel is good for up to 4 days
- Change TAE buffer if needed (check for goop and particles to see if clean/clear)

45

Agarose Gel Protocols

Agarose (g) to Add for Gels			
	250 mL	350 mL	630 mL
1.0%	2.5	3.5	6.3
1.5%	3.75	5.25	9.45
2.0%	5	7	12.6
EtBr (µL)	12.5	17.5	31.6

Agarose (g) to Add for Gels			
	100 mL	120 mL	150 mL
1.0%	1	1.2	1.5
1.5%	1.5	1.8	2.25
2.0%	2	2.4	3
EtBr (µL)	5	6	7.5

Agarose gel concentration, size, and measurements

Choose your gel rig size and 1.5% agarose gel. Follow measurements on diagram above. Your size of gel depends on how many samples you are running. Small rig (150 mL) for just a
couple strips, larger rig (350 mL) for full PCR plate.

46 Add selected measurement of
Agarose powder **ApexBio Technology Catalog #**20-101 and 1X TAE buffer (mL) to Erlenmeyer flask (size depending on how much liquid you add) and swirl gently.
Do not fill more than halfway or it will overflow when boiling.

Note

Making 50X and 1X TAE
Ingredients:
Tris Base, 242g (Fisher #BP152-1, 1kg), Tris – EDTA 1x solution, pH 8.0, 100 mL (Fisher #BP2473-500, 500 mL), 100% Glacial acetic acid, 57.1 mL (Fisher #BP1185-500, 500 m)
50X TAE Buffer Protocol (concentrate):
1. In main lab, pour acetic acid into two falcon tubes using hood, then bring to PCR room
2. In PCR room, measure 500 mL of DI water in graduate cylinder, then pour into large, clean bottle
3. Weigh out 242 g of Tris base using large weigh boat
4. Add Tris base to bottle and shake to dissolve powder. NOTE: This takes a long time! Shake it, wait an hour, shake, wait an hour, etc (about 2-3 hours).
5. When Tris base is fully dissolved, pour 100 mL Tris EDTA into the bottle using a graduated cylinder
6. Add acetic acid to bottle and mix gently (it will warm up a bit)
7. Pour solution into 1 L graduated cylinder, then fill to 1 L using DI water
Pour back into bottle, label and date it

1X TAE Buffer Protocol (big carboy):
1. Fill empty carboy to 19.6 L line with DI water
2. Add 400 mL of TAE 50X Buffer
3. Mix carboy by gently rocking on edge of counter
4. Leave top slightly unscrewed to allow for flow out of spigot
5. Date and initial with lab tape
Calculation: C1V1 = C2V2
(50x) V1 = 1X (20 L)
V1 = 400 mL of TAE 50X Buffer
V2 = 20 L – 400 mL = 19.6 L DI Water
Sources: Sambrook & Russell, Molecular Cloning, Vol. 1, 3rd edition, 5.8

47 Microwave solution for 1 minute

48 Wearing heat gloves, swirl flask gently

49 Microwave again for 45 sec (or until boiling), swirl again

50 Set up gel siding and combs (tape sides if needed)

51 Once flask has cooled for a few minutes and is cool enough to touch the bottom for 10 sec, add

Ethidium Bromide (1% Solution/Molecular Biology) **Fisher Scientific Catalog #**BP130210

(follow diagram in step 45)

Warning - Ethidium bromide is cancerous. Be careful to open lid and do not breath in fumes.

52 Swirl gently and pour immediately into gel holder

53 Pop or move any bubbles with a pipette tip

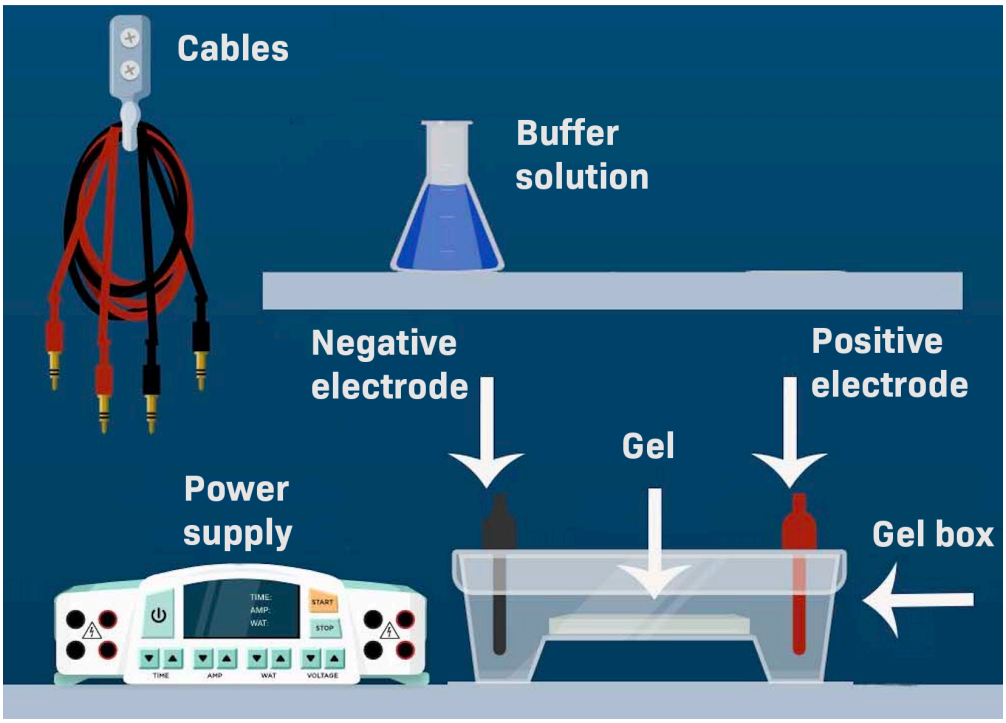
54 Add desired amount of combs with room between rows for gel to run

55 Wait 00:20:00 for gel to harden

56 Pull gel combs directly up to remove and remove siding or tape

57 Add 1X TAE buffer to cover gel in gel box at least 1/2 inch

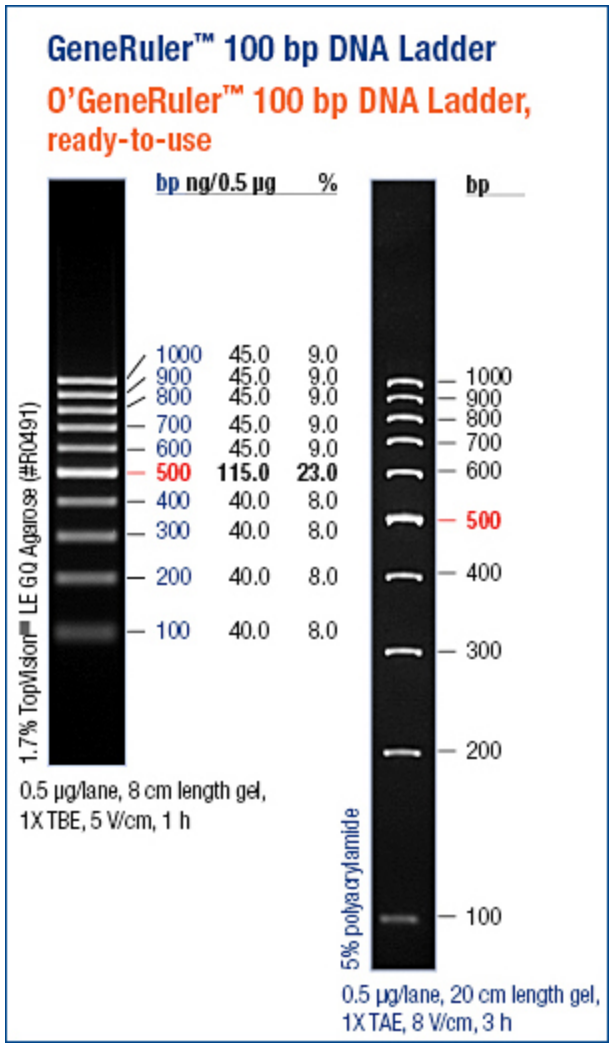
58 Place lid on gel rig, check connection of leads is in correct direction (black/neg on left and red/positive on right) before plugging into the power supply



Gel rig diagram

59 Gather your thawed PCR products and DNA ladder (fridge with pink cap)

GeneRuler DNA Ladder **Fisher Scientific** **Catalog #**SM0334



Ladder diagram

60 Add ladder to edges and your samples in the middle of each row

61 Turn on power supply (back or side)
Set machine to 120V

62 Run for 01:00:00
Check to make sure bubbles are running up both sides

63 Turn off power supply

64 Cut gel piece out with razor blade if needed or remove full gel to bring to imager

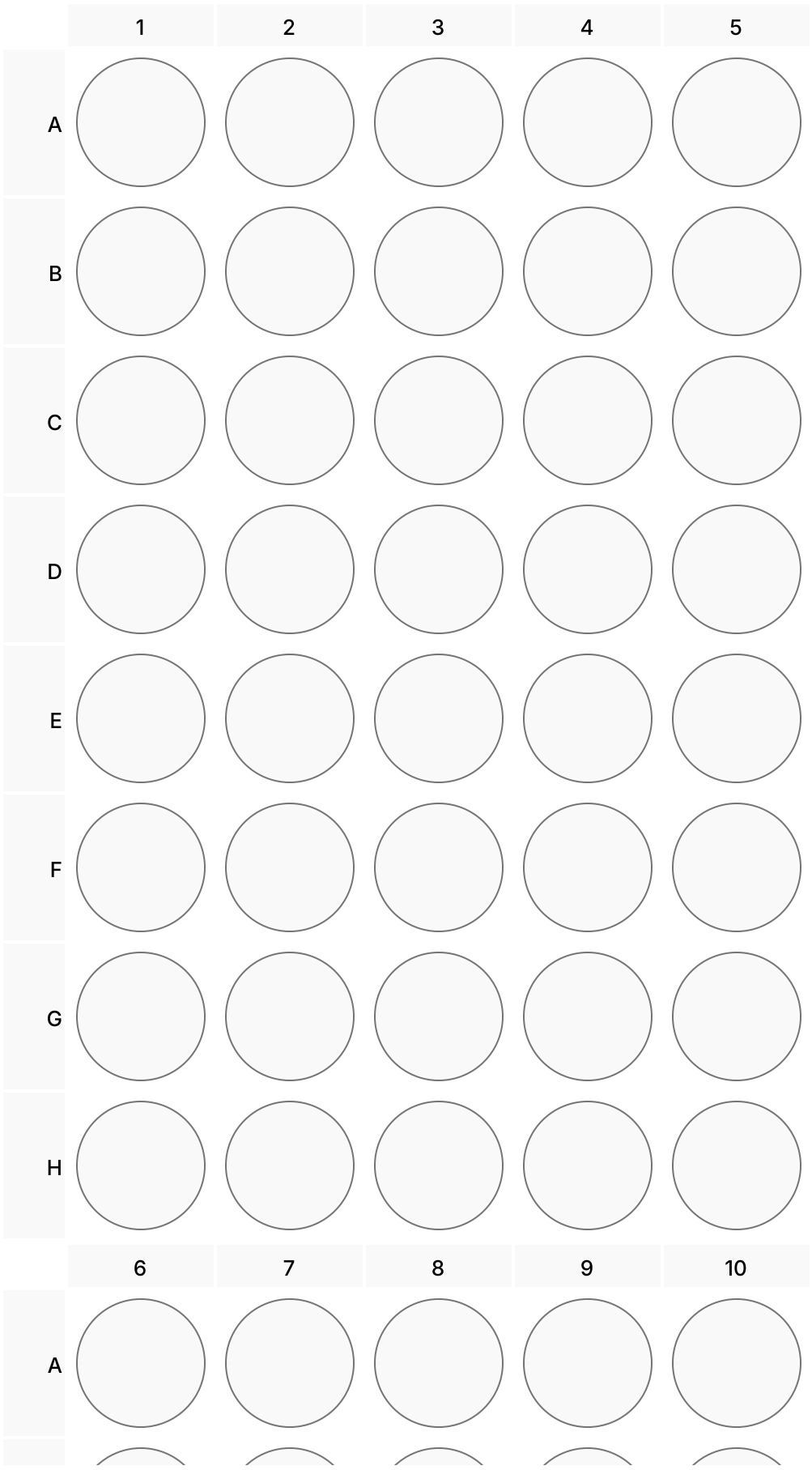
65 Take your gel photo
Clean gel imager base with Ethanol and Kim wipes before placing gel
- Turn on UV light with door open
- Add gel with wells towards back and centered
- Open imager program
- Adjust lens at top if need to zoom in or out
- Acquire image
- Edit for better color
- Print to Mitsubishi
- Take out gel with gloved hand
- Turn off machine and keep door open
- Save picture to your computer folder with date

66 Throw gel away in biohazard

67 Label your gel image and place it in your notebook
- Use sharpie to label numbers, ladder and negative control
- Mark positives (about 600-700 bps long)
- Note - all wells should have primer dimers at the bottom (about 100 bp long)

Sending samples for sequencing

68 Prepare a 96 well plate with uncleaned PCR products in any order you choose, preferably
all the same site per plate, make sure to write this order down in a table like this: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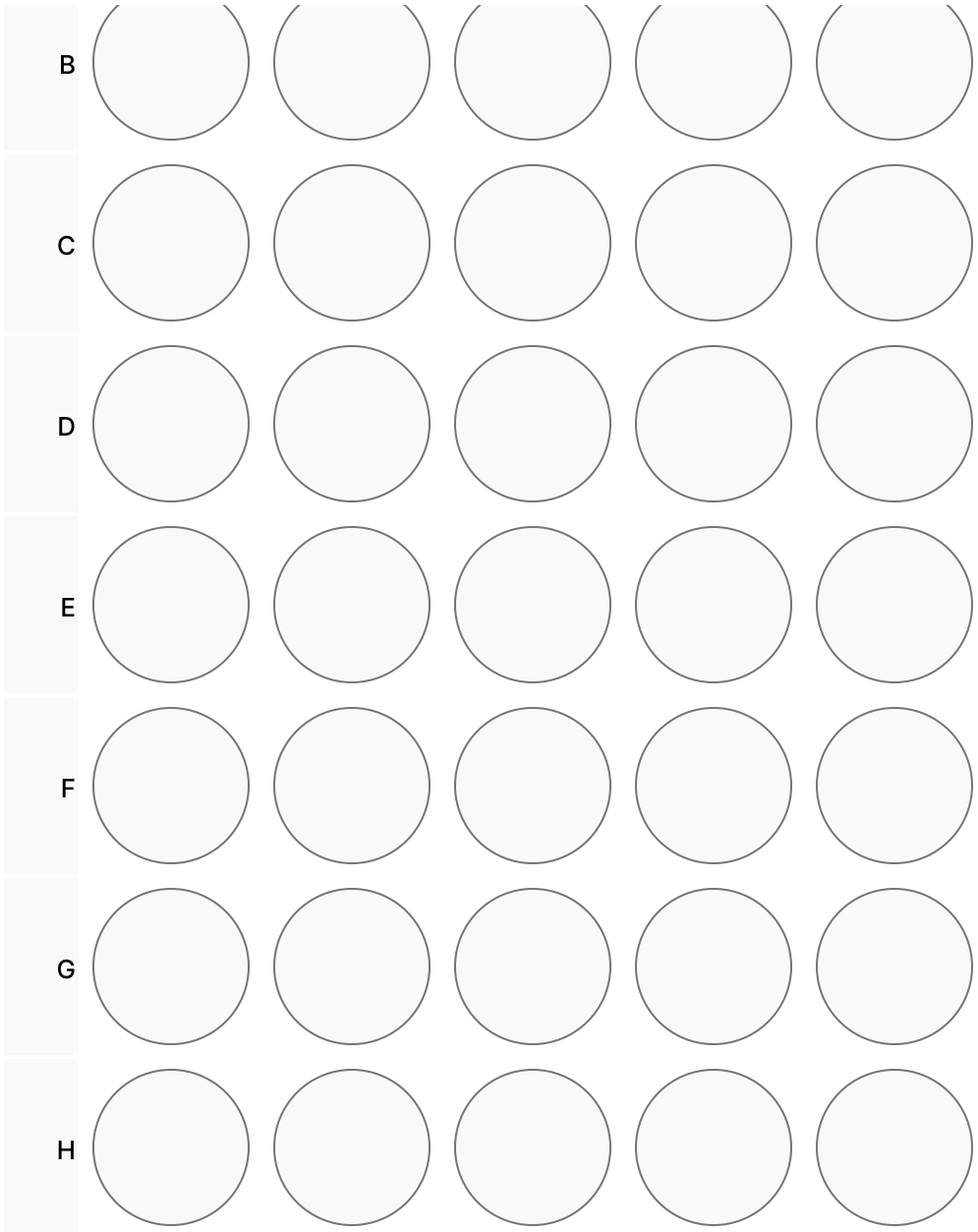
B
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12
A
B
C

69 Place shipping caps Flat PCR Caps, strips of 12 **Fisher Scientific Catalog #**AB0851 on plate, then add foil sticker
AlumaSeal II Sealing Films **Fisher Scientific Catalog #**AF100
Label with date and name matching to your form.

> Note
>
> This can be a pain… use a tape dispenser or sharpies to help push them down

70 Send an email with DNA order forms filled out to the best of your ability (TacGen), making sure it
says unpurified PCR under “type”, primers M13 F (-20), 700-800bp

71 Ship samples overnight in a box with bubble wrap or stuffing & the printed form with samples

> Note
>
> Your samples do not need to be cold.
> Do not ship on a Friday unless you have okayed it with TacGen

72 Get account number and shipping information from Lab Manager.

Sequence data processing

73 Open program: Geneious 7.1.9

74 Create folder or continue under your name folder

75 Right click “local” and create new folder

76 Drag and drop or import your sequences (.ab1 files) into your new folder
(Ctrl a to select all, ctrl c to copy, ctrl v to paste)

77 Once sequences imported, select all (ctrl a)

78 Select Annotate & Predict tab at top
- Trim ends...
- Select the following only:

Remove new trimmed regions from sequences
Trim primers: Allow Mismatches: 5
Minimum match length: 5
Error Probability Limit: 0.05
Trim 5′ End – At least 3 bp
Trim 3′ End – At least 3 bp
OK

79 Click off the sequence names to save

80 Reselect all sequences (cnrl a)

81 Select Align/Assemble program at top left
- De Novo Assemble
- Rename “Assembly name”
- Select the following:

Assembly by 1st part of name, separated by – (Hyphen)
Assembler: Geneious
Sensitivity: Custom Sensitivity
Do not trim
Save assembly report
Save list of unused reads
Save in sub-folder
Save contigs
Allow gaps – max per read 5%
Minimum overlap – 100
Word length – 10
Maximum mismatches per read – 1%
Maximum gap size – 5
Minimum overlap identity – 99%
Index word length – 10
Reanalyze threshold – 0
Maximum ambiguity – 16
Speed 5

OK

82 How to save sequences:
Go to new folder created

83 Select all Contigs

84 File – Export – Consensus sequences
Highest quality, assign quality – total
OK
Create sequence list
Save as FASTA

85 Select unused reads
File – Export – Selected Documents
Save as FASTA

86 Create a spreadsheet with the following columns:
Station, Egg Number, Sequence name, Scientific Name, Number of eggs, Probability of Placement, Notes, Sequence

87 Go to BOLD Systems Database
(https://bench.boldsystems.org/index.php/IDS_OpenIdEngine)

88 Click Identification tab - select species level identification

89 Copy and paste TXT files into BOLD box

Note

BOLD will not work on sequences less than 80 bp. You can try to identify these in NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This can take some time. Email me results box is an option.

90 Download results summary

91 Populate your spreadsheet with the scientific name of the best identification and probability of placement

Note

Scroll down on BOLD to check species IDs are accurate - sometimes they can be off due to private identifications.
Add additional species IDs in alphabetical order using a “/” if the match does not state “This
identification is solid unless there is a very closely allied congeneric species that has not yet been analyzed.

92 Optional: add common names in your spreadsheet from FishBase (https://www.fishbase.se/search.php)

93 NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) any sequences that were not found in BOLD
Click blast, put the top hit in the spreadsheet and add in percent identities, make note it was "blasted"

94 Once you have finished matching IDs, make a summary chart
Do not include species IDs less than 97% (not considered trusted)

95 Add sequences to your spreadsheet from the TXT file by copying and pasting

Citations

Step 14

Makenzie Burrows, Jeremy S. Browning, Mya Breitbart, Steven A. Murawski, Ernst B. Peebles. DNA barcoding reveals clear delineation between sp
https://doi.org/10.1111/fog.12404

Step 23

Natalia V. Ivanova, Tyler S. Zelmak, Robert H. Hanner, Paul D. N. Herbert. Universal primer cocktails for fish DNA barcoding
https://doi.org/10.1111/j.1471-8286.2007.01748.x