

Jul 31, 2024

## SANGER Sequencing EFGL

DOI

[dx.doi.org/10.17504/protocols.io.yxmvmew25g3p/v1](https://dx.doi.org/10.17504/protocols.io.yxmvmew25g3p/v1)

EagleFish GeneticsLab<sup>1</sup>

<sup>1</sup>Eagle Fish Genetics Lab



EagleFish GeneticsLab

Eagle Fish Genetics Lab

---

OPEN  ACCESS



DOI: [dx.doi.org/10.17504/protocols.io.yxmvmew25g3p/v1](https://dx.doi.org/10.17504/protocols.io.yxmvmew25g3p/v1)

**Protocol Citation:** EagleFish GeneticsLab 2024. SANGER Sequencing EFGL. [protocols.io](#)

<https://dx.doi.org/10.17504/protocols.io.yxmvmew25g3p/v1>

**License:** This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

**Protocol status:** Working

We use this protocol and it's  
working

**Created:** July 11, 2024

**Last Modified:** July 31, 2024

**Protocol Integer ID:** 103236

## Abstract

This protocol describes how the Eagle Fish Genetics Lab (EFGL) prepares extracted DNA samples for the ABI 3500xL Genetic Analyzer, and how we run and collect data from this machine. This process is achieved by PCR amplification, ExoSAP-IT purification, E -Gel confirmation, Qubit quantification, BigDye sequencing, CleanSeq clean up, set up of the 3500xL Genetic Analyzer (3500), and finally the collection of results. This should result in easy-to-read nucleotide peaks that are exported as an ABI file.

## Materials

### General Materials

- Pipettes
- Pipettes tips
- Gloves
- Clean Work Bench
- Heat Seal
- PCR Plate Heat Sealer
- Vortex mixer
- Mini centrifuge
- Plate centrifuge
- Thermocycler

### PCR Amplification of Target Sequence

- Forward and reverse primers
- 1.5mL vials
- PCR master mix reagents/kits
- DNA tray(s)
- Unskirted PCR plate(s)

### EGel

- Ladder
- E-Gel cassette
- Lab grade water
- Electrophoresis Device
- USB

## SECTION 1 – PCR Purification with ExoSAP-IT

- Forward and reverse primers
- 1.5mL vials
- PCR master mix reagents/kits
- DNA tray(s)
- Unskirted PCR plate(s)

## SECTION 2 – Quantify Purified PCR Products with Qubit

- Qubit dsDNA high sensitivity kit
- Qubit assay tubes
- 2mL vials
- Purified PCR products
- Ice block(s)

## SECTION 3 – BigDye Sequencing Cycle

- Forward and reverse primer (10 $\mu$ M)
- BigDye and BigDye Terminator v3.1 5X sequencing buffer
- 2 1.5mL vials to dilute forward and reverse primer to 1.6 $\mu$ M
- 2 1.5mL vials for forward and reverse master mix
- 2 unskirted PCR plates (if only running <48 samples, could use same plate)
- p10 multichannel pipette with a box of tips
- Repeater pipette, and 0.1mL combitip
- Ice block(s)
- Ice cooler

#### **SECTION 4- Post Cycle Sequencing Clean-up wit CleanSeq**

- Agencourt CleanSeq beads
- 85% ethanol
- 50mL falcon tube(s)
- ABI plate(s)
- Multichannel and single pipettes, and corresponding tips (~6 boxes for 48 samples)
- Repeater pipette – 5mL combitip
- 25mL reservoir
- Magnetic plate
- Nexttec adhesive seal
- Rubber seal

#### **SECTION 5 – 3500 Sequencing Run**

- ABI Plate in light protected container
- USB stick
- 3500xL Genetic Analyzer

#### **SECTION 6 – Collect Results**

- USB stick

## PCR Amplification of Target Sequence

- 1 Materials needed:
  - Forward and reverse primers
  - Heat Seal
  - 1.5mL vials
  - PCR master mix reagents/kits
  - DNA tray(s)
  - Unskirted PCR plate(s)
  - Pipette and tip
- 2 **To note:** As there are several PCR kits our lab uses for this section, below is a generic protocol for amplifying PCR products for Sanger sequencing. Refer to the Appendix for our working recipes/programs.
- 3 Obtain sample list from lead biologist or data coordinator, and print tray maps if necessary.
  - a. If this is your first attempt, start small (ideally 8-12 samples – as Step 4 in Post cycle sequencing clean-up can be challenging).
- 4 Consult the lead biologist on the master mix recipe and cycling program to use.
  - a. **Appendix section's recipes and programs** have been successful at the Eagle Fish Genetics Lab,  
but please double check if those are the right ones for your lab.
  - b. Depending on the species and PCR product size, the PCR kit(s) could vary and/or be used in combination.
- 5 Locate forward and reverse primers stock (usually at 100 µM concentration) located in  -20 °C freezer and dilute each individually to a working concentration of  10 µM .
  - a. In a 1.5mL vial, add  20 µL of 100µM primer to  180 µL of water.  
Repeat for the other primer.
  - b. Store the 100µM stock back in lab  -20 °C freezer and keep 10µM working stock in lab fridge.
- 6 Prep PCR master mix in a 1.5mL vial. All reagents and/or kits are in our lab mini  -20 °C freezer
- 7 In a new unskirted PCR plate, dispense master mix into each well.

- 8 Using a multi-channel pipette, add DNA samples into each well.
- 9 Heat seal, gently vortex and quick spin  down.
- 10 Load the unskirted plate and run the appropriate thermal cycler program.  
(In most cases, these PCR runtimes can range between  04:00:00 **or longer**, so plan accordingly)  
4h
- 11



One of our EFGL's thermal cyclers

- 12 Run **1% e-gel** to confirm the successful amplification of PCR products. Follow e-gel protocol found in:  
S:\Eagle Fish Genetics Lab\LAB PROTOCOL BOOK\05 - DNA quantification\E-Gel  
or on protocols.io under "**E-Gel Protocol EFGL**"

- a. Save e-gel images in a project folder
  - b. If successful, and depending on the sample size of the project, consider using double comb gels if
    - possible (more samples and less time)
- 13 Repeat steps 7 to 13 for the remaining samples for your project.
- a. It is NOT recommended to PCR all samples at one go, especially for a large project and the
    - availability of 3500xL Genetic Analyzer is entirely dependent on the Wildlife lab's schedule.
    - They have dibs.
  - b. Stagger the sections, if possible.

## SECTION 1 – PCR Purification with ExoSAP-IT

- 14 Materials needed:
- ExoSAP-IT Express reagent (pre-aliquot strip tubes in  -20 °C small freezer)
  - PCR product plate
  - 1 unskirted PCR plate
  - p10 multichannel pipette and 2 boxes of tips
  - Ice block (in  -20 °C small freezer)
- 15 Vortex ExoSAP-IT Express reagent and keep on an ice block (ExoSAP-IT doesn't freeze).
- 16 Aliquot  5 µL of PCR product into a new unskirted PCR plate.
- 17 Add  2 µL of ExoSAP-IT Express to each PCR sample (ExoSAP-IT it is quite viscous, pipette slowly).
- 18 Heat seal, vortex gently, and quick spin  down
- 19 Run ExoSAP-IT program on thermal cycler. Runs for about  00:05:00 .  
a. User name: General  
b. General → Sanger → Exo sapit  
c. The program is currently only on our labs machine 1's and machine 10's groups as the Eagle Fish Genetics Lab
- 20 Samples are now PCR purified

5m

The unskirted plates are to be **placed on ice blocks for downstream steps.**

21 (If not using immediately)

Heat seal and store in  -20 °C freezer. (Samples are still stable and usable after a month [tested in May 2022])

22

ExoSAP-IT Express Program	i. 37 degrees, 4min
	ii. 80 degrees, 1 min
	iii. 4 degree hold

## SECTION 2 – Quantify Purified PCR Products with Qubit

23 Materials needed:

- Qubit dsDNA high sensitivity kit
- Purified PCR products
- p10 multichannel pipette and box of tips
- 1 unskirted PCR plate
- Ice block(s)

24

Sequencing products	Amount of DNA required
100-200bp	1-3ng
200-500bp	3-10ng
500-1000bp	5-20ng
1000-2000bp (mitochondrial sequences)	10-40ng

25 **Note:** Remember that you will end up needing to sequence forwards and reverses separately (

 1 µL of

purified PCR product each), and that after Qubit there will only be  5 µL of purified PCR product left. If the concentration is too low to do a forward and reverse run, you will need to ExoSAP more PCR product, and qubit them again to make sure you have enough.

- 26 If the purified PCR product was previously frozen, it is OK to quick spin  to thaw (~  00:00:05), gently vortex, and quick spin  down again. Remember to place plate back on ice block immediately after.
- 27 Obtain the average qubit scores for each sample. Depending on the length of your sequencing products, refer to the table above to determine the required amount of DNA.
- 28 In some cases, these purified PCR products are often higher than 60ng/ $\mu$ L. (Qubit machine will read:  
"Too high".)
- For mitochondrial sequences:
    - Recommended to try a 1:10 dilution prior to Qubit, most samples should stay within 10-30 range after dilution.
    - 1:10 dilution: Add  1  $\mu$ L of purified PCR product in  9  $\mu$ L of water in a new unskirted PCR plate.
    - Keep diluted plate on ice block as well
  - If 1:10 dilution doesn't fall within the required range, calculate out the actual DNA present, and either change the dilution factor or proceed with undiluted purified PCR products
- 29 Heat seal, and store the original purified PCR product plate back in  -20 °C freezer, if not using.
  - If you need to go back to these samples, note that the diluted samples may drop in DNA concentration. These are still viable for sequencing after a month in  -20 °C freezer.  
(tested in May 2022)
- 30 When proceeding to the next step, purified PCR products should stay on ice block.

## SECTION 3 – BigDye Sequencing Cycle

- 31 **Note:** Reach out to Wildlife lab to make sure 3500 machine is available for use, prior to step 3. It is ideal to do STEP 3-5 on the same day. If sequencing within next 1-3 days, it is OK to stop at either the end of step 3 or step 4.
- 32

Sequencing Reaction Program	i.	96 degrees, 1 min
	ii.	96 degrees, 10 sec
	iii.	50 degrees, 5 sec
	iv.	60 degrees, 2 min
	v.	Return to step ii 24 times
	vi.	Ramp rate in steps ii-iv should be 1 degree per sec
	vii.	Hold at 4 degrees

33 Materials needed:

- Forward and reverse primer (10µM concentration)
- BigDye and BigDye Terminator v3.1 5X sequencing buffer
- 2 of 1.5mL vials to dilute forward and reverse primer to 1.6µM concentration
- 2 of 1.5mL vials for forward and reverse master mix
- 2 unskirted PCR plates (if only running <48 samples, could use same plate)
- p10 multichannel pipette with a box of tips
- Repeater pipette, and 0.1mL combitip
- Ice block(s)
- Ice cooler

34 Dilute both forward and reverse primers to 1.6µM.

- Full tray:  $\text{PCR tube} 16 \mu\text{L}$  of 10µM primer, with  $\text{PCR tube} 84 \mu\text{L}$  of water =  $\text{PCR tube} 100 \mu\text{L}$  primer at 1.6µM
- Small sample size (up to 40 samples): Add  $\text{PCR tube} 6.4 \mu\text{L}$  of 10µM primer, with  $\text{PCR tube} 33.6 \mu\text{L}$  water =  $\text{PCR tube} 40 \mu\text{L}$  primer at 1.6µM

35 Thaw BigDye and keep in ice cooler.

- a. There are usually several aliquots of  $\text{PCR tube} 20 \mu\text{L}$  BigDye in 1.5mL vials. Use those first, unless more is required
- b. BigDye is susceptible to multiple freeze/thaw. Aliquot out more in  $\text{PCR tube} 20 \mu\text{L}$  (or higher, full tray) needs  $\text{PCR tube} 100 \mu\text{L}$  ) when required. **\*Avoid exposure to light during storage.**

36 Prep 2 master mixes, one for forward sequencing, and one for reverse sequencing.

We'll be doing eighth reactions in a total volume of  $\text{PCR tube} 10 \mu\text{L}$  (This is a deviation from the manufacturer's protocol).

37

Reagent	Amount
BigDye	0.5µl
BigDye Terminator v3.1 5X Sequencing Buffer	2µl
Forward or Reverse Primer (1.6µM)	1µl
Water	5.5µl
<b>Total</b>	<b>9µl</b>

\*Multiply volumes by number of samples, make sure to account for pipetting error.

- 38 **Note:** If the purified PCR from ExoSAP-IT does not have sufficient DNA concentration, add more purified PCR product and remove water. If going this route, you will need a different master mix ratio.
- 39 Mix with pipette or vortex gently, and spin down.
- 40 Use repeater pipette to aliquot out  9 µL of forward Master Mix into each well on new unskirted PCR plate.
- a. Repeat step for reverse Master Mix into separate wells. This can be on the same plate as the forward sequences if it all fits to a single plate. If not, use a separate unskirted PCR plate.
- 41 Add  1 µL of purified PCR product into each well that has the forward Master Mix, and repeat for wells with reverse Master Mix.
- 42 Heat seal plate(s), vortex briefly and spin  down (Do not worry about bubbles).
- 43 Run BigDye sequencing thermal cycler program. Runs for about  01:30:00 . 1h 30m
- a. User name: General
- b. General → Sanger → BigDye sequencing
- i. Go to "Edit", to make sure that it has an asterisk beside step 2-4. This means the temperature is ramped up by 1 degree per second
- c. Program is only on machine 10's group in our lab
- 44 This can be left overnight, but do not delay too long (no more than a week). If delay is needed, store in  -20 °C freezer, and protect from light.

- 45 Prep electronic sample sheet for 3500 xL Genetic Analyzer machine while waiting for run to complete
- Go to LAB PROTOCOL BOOK → 13 – Sanger Sequencing
  - Open Genemapper Plate Template.xlsx
  - Make edits to:
    - Plate Name (cell A4)
    - Sample Name (cell B6-B102) – leave blank if empty, max 96 samples per spreadsheet. If more than 1 plate, do each plate separately
  - The following stays the same:
    - Assay: Genetics\_Lab\_Fish\_Seq
    - File name convention: Carp
    - Results Group: Carp
  - Save file(s) as Text(tab delimited) on both project folder, and flash drive.

46

3500 Plate Layout File Version 1.0							
Plate Name	Application Type	Capillary Length (cm)	Polymer	Number of Wells	Owner Name	Barcode Number	Comments
Whitefish_06172024	Fragment	50	POP7	96			
Well	Sample Name	Assay	File Name Convention	Results Group	Sample Type	User Defined Field 1	User Defined Field 2
A01	PwiCLWR20C_0006_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
B01	PwiCLWR20C_0008_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
C01	PwiCLWR20C_0011_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
D01	PwiCLWR20C_0012_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
E01	PwiCLWR20C_0013_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
F01	PwiGALL20C_0001_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
G01	PwiGALL20C_0013_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
H01	PwiGALL20C_0015_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
A02	PwiGRC023C_0055_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
B02	PwiGRC023C_0056_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
C02	PwiGRC023C_0064_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
D02	PwiLOGN20C_1006_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
E02	PwiMDSN20C_0008_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
F02	PwiMDSN20C_0009_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
G02	PwiTRUC23C_0102_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse
H02	PwiTRUC23C_0103_R	Genetics_Lab_Fish_Seq	Carp	Carp	Sample		Reverse

Example of our Genemapper Plate Template.xlsx

- 47 **\*\*Note\*\* 3500 machine does injections in columns of 3.** If you have a partial tray that the sample

2h

size is not a factor of 3 columns, or a full column → Fill it with water.

For example:

- If you have 4 columns of samples, fill column 5 and 6 with water.
- If you have 2 columns + 7 samples, add water to the 8th well in the 3rd column.
- Each injection takes ~  02:00:00

## STEP CASE

Cleanseq Cleanup    58 steps

- 48 CleanSeq <– Yields better results

Materials needed:

- Agencourt CleanSeq beads
- 85% ethanol
-  50 mL falcon tube(s)
- ABI plate(s)
- Multichannel and single pipettes, and corresponding tips (~6 boxes for 48 samples)
- Repeater pipette
-  5 mL combitip
-  25 mL reservoir
- Magnetic plate
- Lab grade H<sub>2</sub>O

49 **Not recommended to do more than 48 samples at a time, to avoid overdrying of beads mid clean-up**

50 Make 85% ethanol in a  50 mL falcon tube. You will need a total of  242 µL of 85% ethanol per sample for this section. Multiply the volume per sample and accounting for pipette error.

a. For a full tray -->  21250 µL 100% ethanol +  3750 µL water =  25 mL

b. For 16 samples --> (16 (samples) + 3 (pipetting error)) X  242 µL =

 4598 µL of 85%

ethanol

i.  4598 µL of 85% ethanol -->  3908.3 µL pure ETOH and

 689.7 µL water

51 Add  10 µL of CleanSeq beads into each well with a repeater pipette, aim on the high side of the well to prevent contamination.

52 Pipette out the required ethanol ( 42 µL each sample) in a  25 mL reservoir, and use a multichannel pipette to add  42 µL of 85% ethanol to each well

- Pipette up and down 5-10 times to mix solution, discard tips after each use.
- No incubation needed after mixing

53 Place plate on magnetic plate, and wait  00:05:00 at room temperature. Magnetic beads will form a ring (or 4 lines down the wells, pattern usually alternates on columns depending on the

5m

magnetic plate  
you're using)

54



Example of EFGL magnetic plate

55 **\*\*Note\*\* Steps 56- 59 can be challenging, but you must be relatively quick, as stalling can cause beads to dry out which will affect the end result. If just starting out, do 2 wells at a time, and slowly ramp up until you are comfortable with 8 wells.**

56 Set multichannel pipette to ~  65  $\mu\text{L}$ , and take out all the supernatant. Avoid touching or aspirating the beads as much as you can.

- a. It is easier to do 4 at a time or less, until you are comfortable
- b. Lower the pipette tip to the bottom of the well to aspirate all of the ethanol

57 (Plate stays on magnet)  
Once all supernatant is removed, use a  5 mL combitip and dispense  100  $\mu\text{L}$  of 85% ethanol to each well. Wait  00:00:30.

30s

58 (Plate stays on magnet) Use a multichannel pipette and set to  110  $\mu\text{L}$  to remove all ethanol.

a. Lower the tip all the way to the bottom of well and aspirate

59 Repeat step 57 and 58 once.

60 Air dry beads at room temperature for  00:05:00, avoid overdrying (if cracks are seen, it is overdried).

6m

You will want the beads to look matte)

a. **About**  00:01:00 **into drying:** Remove the plate from magnet. Check to ensure there is not an

excess of ethanol left in the wells. Pipette out any remaining ethanol. Discard tip after each use.

b. Leave plate off of the magnet for the remaining time to dry.

61 Use a multichannel pipette to dispense  40 µL of water into each well

a. Remove any air pockets formed at the bottom of well(s) with a p10 pipette

b. Tap plate gently on bench a couple of times, so beads will slide down and go back into the solution for an easier elution.

62 Incubate plate at room temperature for  00:05:00, while off magnet.

5m

63 Place plate back on magnet for  00:05:00.

5m

The unskirted PCR plate should sit on the face of the magnet plate inbetween the holes, not down in the holes.

a. This creates bead pellets at the bottom corner of the well instead of a ring, for easier elution.

64 Use a multichannel pipette, and transfer  35 µL of the solution into a new ABI plate. Avoid transferring beads!

2d

a. If there is **more than**  24:00:00 **delay** to sequencing, transfer to non-skirted PCR plate

instead of ABI plate. Heat seal, and store in dark in  -20 °C freezer.

(good up to a month – not tested)

i. If delay is less than  24:00:00, store in dark in  4 °C freezer.

b. It is suggested to transfer 2-4 wells at a time by lowering tip to the opposite end of the pellet

c. If beads get into your pipette tip, place the solution back into the well and wait for solution to

clear up.

- 65 Seal ABI plate with Nexttec adhesive seal and bring to Wildlife lab for processing in a light blocking container.
- 66 Switch out Nexttec seal with the rubber seal and load it on the 3500 xL Genetic Analyzer machine to sequence.

## SECTION 5 – 3500 Sequencing Run

- 67 Materials needed:
  - ABI Plate in light protected container
  - USB stick
- 68 Make sure both computer and 3500 are turned on.
  - a. If 3500 is off, and computer is on. The computer needs to be restarted.
    - i. Log in under Administrator, password is Administrator
  - b. Follow the instructions (bottom left corner) on 3500 to sync 3500 and computer.
    - i. Data collection is the software that you launch to start the run. So, donot open the software on the computer until you see the green check mark.
  - c. If 3500 is on, but you're experiencing issues with the 3500 data collection software, reboot the computer. **HOWEVER!** Please make sure whoever is using that computer has their GeneMapper results saved! Please check with the techs in the lab.
- 69 Once it's all green, plug in your flash drive (there are more USB slots at back of the tower if the front ones are occupied)
- 70 Open the 3500 data collection software (icon looks like the 3500 machine)
- 71 Load plate(s) onto 3500.
  - a. Press "Tray" button and wait for machine to bring out the tray platform forward for loading.
  - b. Open the door and load plate(s).
- 72 Go back to the computer, you should be on the "Dashboard" tab on top left.
- 73 Under "Consumables Information", make sure there's sufficient samples left to run your samples. If you are about to run out, reach out to the lab manager to get a new one.
- 74 Go back to "Dashboard" tab, and select "Create New Plate"

- 75 Enter your plate name, it is recommended to use the same plate name in your .txt file.
- No other changes to settings
  - Click "Assign plate contents"
- 76 Click "Import" to import your .txt file
- It might give you some warnings to the import, Click on "Proceed" anyway
  - The screen should now show you a map with all your samples to be run
- 77 If you have your template set up right, you should see samples highlighted with a blue dot, and all 3 boxes below the tray map are checked with "Genetics\_Lab\_Fish\_Seq", "CARP", CARP".
- If not, do the following:
    - In Assay box, if it is empty or not the right assay. Select "Add from library".
    - Under filter, select "Sequencing" → Select "Genetics\_Lab\_Fish\_Seq"
    - The other 2 boxes, select "Add from library", and select "CARP".
    - Highlight all samples, and check the "Genetics\_Lab\_Fish\_Seq" box, along with both "CARP" boxes. Your samples should now be highlighted in blue. Empty wells should remain blank/un-highlighted.
    - To un-highlight the empty wells, select them and uncheck all 3 boxes
- 78 Click "Link plate for run"
- If you only have 1 plate → wait  00:02:00 → run window appears → Click on "Start run"  
or go to step "b" to add plate B.
  - If you have 2 plates, click on "Assign plate contents" on the left column
    - Repeat steps 9-13
    - It'll ask to overwrite plate A, or assign to plate B. Assign it to plate B.
    - Wait a  00:02:00 , then click "Start run"
- 79 It will take  00:02:00 before the run is initiated, and the estimated runtime is on the top right of the screen.
- Each injection takes about  02:05:00 regardless the number of samples within each injection.
  - You'll want to wait for at least  00:05:00 to make sure the timer continues to countdown  
(it refreshes every  00:00:05 ). At the  01:58:00 mark, there'll be movement in the

4m

4h 10m 5s

machine where the first injection is initialized. If the timer continues to countdown after, the run has successfully begun. You can walk away now.

- 80 Make sure to check back on the machine  01:00:00 later regardless, as the data collection software might crash.

1h

- a. If it does (timer stops counting down, or the software has quit itself), and no error message

is given: re-start the plate loading process, and start the run again.

- i. Start from the beginning if no data present, or till where it stopped last. Updated: 2/2/2024

1. To check: Go to Computer → Data → Applied Biosystems → 3500 → Data → Carp → the last run folder → check each injection (successful AB1 file should be ~300kB in size)

ii. If injection folders are empty, none of the samples sequenced

- b. If there is an error message, reach out to Wildlife Lab to troubleshoot if necessary.

i. Note down the error message and timestamp

81 **Things to note/Troubleshooting steps:**

34m

- Polymer pouch count goes down each time the run is restarted, even though it has not been used technically. If you are certain you have sufficient remaining samples to run, proceed to run anyway – despite the error message.

- There are 2 reasons to why the timer stop might stop. If timer stops at about  00:05:00 into the run, chances are the oven is not up to temperature (oven needs to be at  60 °C )

- o Go to "Dashboard", "oven temp" should be green, and at about  60 °C

- o Then, check "EPT" tab in the Workflow window, under "Instrument Run Views" and "Flags"

- o Check to see if lines are still climbing. Takes about ~  00:10:00 since you've clicked "Start Run", and timer should countdown at that point.

- o There'll be movement at  00:02:02 (switching between buffer A and B, and at about

-  00:01:58 for first injection)

- o Overall, this could take up to  00:15:00 from the time you click "Start Run" even if the oven is at temp. So, be patient.

- If everything seems to be right, and the timer is still not counting down, and you feel you have given sufficient time.

- o It might be a software issue, there is no known reason for the cause, or error messages

- to tell you it is not running. ↓ last fixed by defragmentation of the computer 6/21/22
- o Click on “Terminate Run”, abort the entire injection list.
- o Close the Data collection program, then turn 3500 off.
- o Shut down the computer.
- o Then follow instructions to start both the computer and the 3500 back up.
- o Load the plates on the software again, they should have already been saved. You can load by clicking on “Existing plate” rather than “Create new plate” on Dashboard.
  - Filter: Sequencing
  - Type in your plate names, assign plate A (and B)
  - Start run
- If new polymer pouch is required:
  - o In the Dashboard homepage, go to Wizards. Select “Refresh polymer”
  - o Go through each step shown on the screen
    - The lever to load/unload the polymer pouch can be “sticky”, so some force is required
    - Use a kimwipe with a bit of distilled water to clean the spout.
    - Check for bubbles, and if all is well, you are set to run the plates
  - o Close the 3500 machine door
- If you keep getting errors with setting up your assays, and that the oven is not pre-heating. The 3500 data collection software may have glitched out. You will have to reboot the computer while leaving the 3500 turned on.
  - o After it reboots, everything should go as planned.

## SECTION 6 – Collect Results

- 82 Go to “Computer” → Select “Data (D:)” → Applied Biosystems → 3500 → Data → Carp
- 83 The run folder will be listed according to their start date/time, or look for the latest.
- 84 Copy the entire run folder to your flash drive, and load to your project folder on S: drive.
- 85 Remove your trays from the machine.
  - a. Remove them from the “jacket” and place the trays in the pile of used trays.
  - b. Wildlife lab will autoclave the rubber tops to reuse, so do not toss them.
- 86 Review quality score on Sequencher. (Ideal is 70% or higher)
  - a. If lower than 60-70%, and scores do not improve after tweaking the sequences, you

may  
need to re-run those samples

## Appendix – Successful PCR recipes

87 **TaKaRa LA Taq** (Cat# RR002A) -  20 µL reactions

87.1 Redband ND1\_ND2 region: ND12L and ND12H primers

87.2 Redband dloop region: dloop\_Brunelli\_F and 28RIBa\_DL\_R

87.3

Reagents	Stock Conc. (µM)	Final Conc. (µM)	Per rxn (µl)
10X LA PCR Buffer II	10	1	2
MgCl <sub>2</sub>	25	2.5	2
dNTPs	10	1.6	3.2
Forward Primer	10	1	2
Reverse Primer	10	1	2
TaKaRa LA Taq	5	0.05	0.2
Water			6.6
DNA template	raw	raw	2

88 **Thermo Scientific PCR Master Mix (2X)** (Cat# K0171) -  50 µL reactions

88.1 Whitefish CytB region: whitefish2\_cytb\_765F and whitefish2\_cytb\_765R

88.2

Reagents	Stock Conc. (μM)	Final Conc. (μM)	Per rxn (μl)
PCR Master Mix	2X	1X	25
Forward Primer	10	0.6	3
Reverse Primer	10	0.6	3
Water			18
DNA template	raw	raw	1

89 Thermo Scientific Phusion Hot Start II HF DNA Polymerase (Cat# F-549S) -  20 μL reactions

89.1 Redband mitogenome: EmsermtDNA\_F and EmsermtDNA\_R (work in progress)

89.2

Reagents	Stock Conc. (μM)	Final Conc. (μM)	Per rxn (μl)
5X Phusion HF Buffer	5X	1	4
dNTPs	10mM	200	0.4
Forward Primer	10	0.5	1
Reverse Primer	10	0.5	1
Phusion Hot Start II DNA Polymerase	2	0.04	0.4
Water			9.2
DNA template	raw	10ng/μl	4

## Appendix – Successful PCR cycling programs

90 ND1\_ND2 and dloop

90.1 Run ND12NEW (General → Sanger → ND12NEW)  
Program located on machines #10-14

90.2

	Temperature (°C)	Time	Cycles
Initial denature	95	3 mins	36
Denature	94	30 secs	
Annealing	65	1 min	
Extension	72	5 mins	
Final Extension	72	10 mins	
Hold	4	∞	

## 91 Whitefish Cytb

91.1 Run pstar60 (General → Sanger → pstar60)

Program located on machines #1-5

91.2

	Temperature (°C)	Time	Cycles
Initial denature	98	30 secs	35
Denature	98	10 secs	
Annealing	67	25 secs	
Extension	72	8 mins 20 secs	
Final Extension	72	5 mins	
Hold	4	∞	

## 92 Mitogenome

92.1 Run Emser\_THERMO (General → Sanger → Emser\_THERMO) (work in progress)

Program located on machines #10-14

92.2

	Temperature (°C)	Time	Cycles
Initial denature	98	30 secs	35
Denature	98	10 secs	
Annealing	67	25 secs	
Extension	72	8 mins 20 secs	
Final Extension	72	5 mins	
Hold	4	∞	