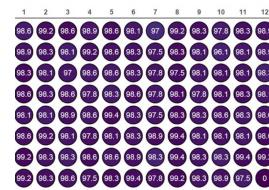


Jul 12, 2024

## GT-seq protocol EFGL

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**Protocol status:** Working

**We use this protocol and it's working**

**Created:** December 28, 2023

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**Protocol Integer ID:** 92797

**Keywords:** PCR, barcoding, SequalPrep, Normalization, Ampure, Qubit, NextSeq 2000, Eagle Fish Genetics Lab,

## Abstract

The purpose of this protocol is to prepare extracted samples of DNA to be read on a NextSeq 2000. This will be achieved through SNP-PCR, barcoding, SequalPrep Normalization, Ampure bead size selection, and Qubit quantification. This will result in a single pooled vial of DNA ready for the NextSeq 2000 EFGL Loading Protocol.

## Materials

### SNP-PCR

- Print out Library Prep Sheet from project checklist excel file
- Qiagen Master mix ( aliquots in box in refrigerator)
- Panel specific GT-seq Primers ( aliquots in box in refrigerator)
- Low profile non-skirted PCR tray
- 0.1mL combi-tip
- 1000ul tips for larger master mixes
- DNA tray
- p10 multi-channel pipette and box of tips
- Heat seal
- Non-Lo Bind 1.5ml or 1.7ml tube
- Gloves

### Barcoding

- SNP-PCR tray
- 25ml reservoir
- multi-channel pipette and box of tips
- lab grade H2O
- 3 heat seals
- i7 tube(s)
- i5 barcode plate(s)
- HotStart master mix
- low profile non-skirted PCR tray
- 1.5ml non-Lo Bind tube
- 2 boxes of 0.1-10ul pipette tips
- 0.1 mL combi-tip
- Gloves

### SequelPrep Normalization

- SequelPrep Normalization tray
- SequelPrep binding buffer - Red
- SequelPrep wash buffer – Blue
- SequelPrep elution buffer –Yellow
- 2 1.0 mL combi-tip
- 5mL combi-tip
- Multichannel pipette and one box of tips
- 2 heat seals
- Paper towels
- 1.5mL Lo-Bind tube
- Divided reservoir (25mL)
- Gloves

## Bead Size Selection

- Ampure beads
- Desktop cooler
- 1 1.5ml tube
- 3 1.5ml Lo-Bind tubes
- Magnet Stand
- p100 pipette and tips
- p1000 and tips
- 80% ethanol, made fresh daily
- Low TE
- Gloves

## BSS quantification with Qubit

- BSS tube at room temperature
- Qubit dsDNA high sensitivity kit (Buffer and fluorescent reagent)
- Kimwipes
- Tube/vial for Qubit working solution
- Qubit assay tube
- 1 1.5ml Lo-Bind tube
- Gloves

## Protocol materials

-  QIAGEN Multiplex PCR Kit **Qiagen Catalog #206145** Step 1
-  Ampure XP beads **Beckman Coulter Catalog #A63881** Step 11
-  low TE **Fisher Scientific Catalog #AAJ75793AP** Step 11
-  SequalPrep Normalization Plate Kit 96-well **Thermo Fisher Scientific Catalog #A1051001** Step 7
-  Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854** Step 12
-  TE pH 8.0 (1X TE Solution) **IDT Technologies Catalog #11-01-02-05** Step 12
-  Invitrogen Nuclease-Free water **Fisher Scientific Catalog #43-879-36** In 2 steps
-  Hot Start Taq 2X Master Mix - 500 rxns **New England Biolabs Catalog #M0496L** Step 4

## SNP-PCR

19m 30s

- 1 Prepare SNP-PCR master mix using species specific primer panel.

## Note

**Equipment and supplies needed for this step:**

Thermalcyclers (we have several thermalcyclers. Here's an example of one we use often)

## Equipment

**Mastercycler Pro**

NAME

thermalcycler

TYPE

Eppendorf

BRAND

No Longer Manufactured

SKU



## Equipment

**EPPENDORF SCIENTIFIC CENTRIFUGE 5804**

NAME

plate centrifuge

TYPE

Eppendorf

BRAND

02-262-8153PM; discontinued

SKU



## Equipment

Repeater-M4	NAME
pipette	TYPE
Eppendorf	BRAND
14-287-150	SKU

## Equipment

ALPS 25 V	NAME
manual heated plate sealer	TYPE
Thermo Scientific	BRAND
AB-0384/110; discontinued	SKU

 FILE

p10 multi-channel pipette and tips  
p1000 pipette and tips  
1.5 mL microcentrifuge tube  
benchtop centrifuge  
0.1 mL Eppendorf combi-tips (Fisher Scientific; 13-683-703)  
Vortex  
Heat seals (Fisher Scientific; AB-0745)  
Unskirted 96 well PCR trays (Fisher Scientific; AB-0700)

**Reagents needed:**

DNA tray(s)

 QIAGEN Multiplex PCR Kit **Qiagen Catalog #206145**

Panel specific GT-seq primers

- 1.1 Vortex and spin down aliquots of Qiagen Multiplex Master Mix and panel specific GT-seq primers, keep them in benchtop coolers. The table has been formulated to account for pipette errors.

	Per sample	1 tray	2 trays	4 trays
<b>Qiagen Multiplex MM</b>	3.5	371µL	742µL	1,484µL
<b>Panel specific GT-seq Primers</b>	1.5	159µL	318µL	636µL
<b>Total:</b>		530µL	1,060µL	2,120µL

Combine the ingredients in a non- Lo-bind tube. Vortex and spin down tube and keep in benchtop cooler till ready to dispense.

- 1.2 Label unskirted PCR plate(s): SNP-PCR, date, tray#, project initials, and your initials.
  
- 2 Remove the DNA tray(s) from fridge. Briefly vortex and spin down  DNA trays.
  
- 2.1 Using a 0.1mL combi-tip, dispense  5 µL of PCR master mix into each well.
  
- 2.2 Using a p10 multichannel pipette, add  2 µL of DNA into corresponding well. Discard tips after each use.
  
- 2.3 Heat seal the SNP-PCR plate(s). Vortex and briefly spin down  SNP-PCR plate(s).
  
- 3 Run the SNP-PCR plate(s) on a thermalcyler using the following program. (In our lab, the program can be found using the menu options GTseq -> **gs-fxtd+10c-65**) 19m 30s

1.  95 °C for  00:15:00
2.  95 °C for  00:00:30
3.  57 °C for  00:00:30
4.  72 °C for  00:02:00

Repeat 2-4 for 5 cycles, with a 5% ramp

5.  95 °C for  00:00:30
6.  65 °C for  00:00:30
7.  72 °C for  00:00:30

Repeat 5-7 for 10 cycles

8.  4 °C indefinitely

- 3.1 Heat seal the DNA tray(s), and stored them back in the fridge.

## Barcoding

21m 40s

- 4 Barcoding amplicons with i5s and i7s.

### Note

**Equipment and supplies needed for this step:**

thermal cycler  
manual heated plate sealer  
plate seals  
p10 multi-channel pipette and tips  
p200 multi-channel pipette and tips  
p1000 pipette and tips  
Repeater-M4 pipette  
0.1mL combi-tip (Fisher Scientific; 13-683-700)  
unskirted 96 well PCR trays (Fisher Scientific; AB-0700)  
1.5mL microcentrifuge tubes  
50mL reservoir  
vortex  
Plate centrifuge

**Reagents needed:**

SNP-PCR plate(s)

 Invitrogen Nuclease-Free water Fisher Scientific Catalog #43-879-36

 Hot Start Taq 2X Master Mix - 500 rxns New England Biolabs Catalog #M0496L

aliquoted i5 tray from IDT

diluted unique i7s

- 4.1 After SNP-PCR thermalcycler program has completed, remove SNP-PCR plate(s). Briefly vortex and spin down .

- 4.2 Pour lab-grade water into a 50mL reservoir. Using a p200 multichannel pipette, dispense 133ul of lab grade water into each well.

### Note

To prevent contamination, discard tips after each use if you don't feel comfortable pipetting over the wells.

4.3 Heat seal diluted SNP-PCR tray(s). Vortex well, and centrifuge  3000 rpm, 00:00:30

30s

5 Make barcoding master mix.

**IMPORTANT! Each DNA tray needs to have an unique i7, if they will be loaded on the same library.**

5.1 Check that i7s have been diluted to working stock of 11 $\mu$ M, and proceed with preparing barcoding mix. If not, add  961  $\mu$ L of lab-grade water to  119  $\mu$ L of 100 $\mu$ M i7 to make working stock.

Vortex Hotstart MM and i7(s), spin down and store in benchtop cooler.

Barcode cocktail/tray	1 plate
Desired i7 Index [11 $\mu$ M]	106 $\mu$ L
HotStart MM	530 $\mu$ L
Total:	636 $\mu$ L

Combine ingredients in a 1.5mL non- Lo-bind tube(s). Vortex, spin down and store in benchtop cooler.

5.2 Label unskirted PCR plate: BARCODE, Date, Tray#, project initials and your initials.

5.3 Remove the pre-aliquot i5 trays. Briefly vortex, and spin down .

5.4 Using a 0.1mL combi-tip, dispense  6  $\mu$ L of barcoding mix into each well of the Barcode plate(s).

5.5 Using a p10 multi-channel pipette, add  2  $\mu$ L of i5 barcode into each corresponding well of the Barcode plate(s). Discard tips after each use.

- 5.6 Once the i5s have been added, add  3 µL of the diluted SNP-PCR into each corresponding well of the Barcode plate(s). Discard tips after each use.
- 5.7 Heat seal the Barcode plate(s). Gently vortex, and spin down .

- 6 Run the Barcode plate(s) on a thermalcycler using the following program. (In our lab, the program can be found using the menu options GTseq --> **RAD-amp**) 21m 10s

1.  95 °C for  00:15:00
2.  95 °C for  00:00:10
3.  65 °C for  00:00:30
4.  72 °C for  00:00:30

Repeat 2-4 for 10 cycles

5.  97 °C for  00:05:00
6.  4 °C indefinitely

- 6.1 Heat seal the i5 tray, and diluted SNP-PCR plate(s). Store them in fridge.

## Sequalprep Normalization

1h 5m

- 7 Normalize the barcoded samples with SequalPrep kit.

## Note

**Equipment and supplies needed for this step:**

Applied Biosystems SequalPrep Normalization plates  
manual heated plate sealer  
plate seals  
p10 multi-channel pipette and tips  
Repeater-M4 pipette  
1mL combi-tip  
5mL combi-tip  
1.5mL Lo-bind microcentrifuge tube (Fisher Scientific; 13-698-791)  
25mL reservoir with divider  
vortex  
Plate centrifuge

**Reagents needed:**

Barcode plate(s)

 SequalPrep Normalization Plate Kit 96-well Thermo Fisher  
Scientific Catalog #A1051001

- 7.1 After Barcode thermalcycler program has completed, remove Barcode plate(s). Briefly vortex and spin down .
- 7.2 Label a new SequalPrep Normalization plate: Date, Tray#, project initials and your initials.
- 7.3 Using a 1mL combi-tip, dispense  10 µL of Binding Buffer into each well of the SequalPrep plate(s). If you have a partial tray, only dispense binding buffer that contains barcoded samples.
- 7.4 Using a p10 multi-channel pipette, transfer all  11 µL of barcoded samples into corresponding well of the SequalPrep plate(s). Discard tips after each use.
- 7.5 Heat seal SequalPrep plate(s). Vortex, and briefly spin down .
- 7.6 Incubate SequalPrep plate(s) at room temperature for  01:00:00. (OK to leave plate(s) at room temperature overnight if necessary) 1h
- 8 After incubation, empty out all contents from the SequalPrep plate(s) into sink.

- 8.1 Tap plate(s) on paper towels to remove any residual binding buffer/barcoded samples.
  - 8.2 Using a 5mL combi-tip, dispense 50ul of Wash Buffer into each well of the SequalPrep plate(s). Avoid touching the side of the wells, change tip if you think there might be contamination.
  - 8.3 Empty out all wash buffer from SequalPrep plate(s) into sink.
  - 8.4 Tap plate(s) on paper towels to remove any residual wash buffer in wells.
- 
- 9 Using a 1mL combi-tip, dispense  20  $\mu\text{L}$  of Elution Buffer into each well of the SequalPrep plate(s). Avoid touching the side of the wells, change tip if you think there might be contamination.
  - 9.1 Heat seal SequalPrep plate(s). Vortex, and spin down  .
  - 9.2 Incubate SequalPrep plate(s) at room temperature for  00:05:00 . (OK to store plate(s) in fridge overnight before pooling if necessary) 5m
  - 10 Using a p10 multi-channel pipette, pool  10  $\mu\text{L}$  of each sample into a 25mL reservoir. OK to reuse tips.
- 
- 10.1 Transfer all pooled samples from reservoir into a 1.5mL Lo-bind tube. Label tube(s): NP# (tray#), project initials. Store in fridge.
  - 10.2 Heat seal SequalPrep plate(s), and store in fridge.

## Beads Size Selection

32m 30s

- 11 **Invitrogen™ DynaMag™- Spin Magnet**

## Note

### Equipment and supplies needed for this step:

p10 pipette and tips  
p200 pipette and tips  
p1000 pipette and tips  
1.5mL microcentrifuge tube  
1.5mL Lo-bind microcentrifuge tube (Fisher Scientific; 13-698-791)  
vortex  
Invitrogen DynaMag Spin Magnet (taped in place so it does not spin)

### Reagents needed:

NP tube(s)

 Ampure XP beads **Beckman Coulter Catalog #A63881**

100% ethanol (laboratory grade, non-denatured)

 Invitrogen Nuclease-Free water **Fisher Scientific Catalog #43-879-36**

 low TE **Fisher Scientific Catalog #AAJ75793AP**

- 11.1 Vortex Ampure XP beads well, and briefly spin down to avoid beads getting stuck on lid. Keep in benchtop cooler.

## Note

Ampure XP beads have been aliquoted into 2 mL tubes to prevent contamination of the stock. In general, whenever they are used in the protocol, they should be vortexed thoroughly to ensure mixing

- 11.2 Vortex NP tube(s), and spin down. Keep in benchtop cooler.
- 11.3 Label 1.5mL Lo-bind tube(s) with the corresponding NP tube number, if performing multiple bead size selection simultaneously.
- 11.4 Add  25 µL of Ampure XP beads into each labeled 1.5mL Lo-Bind tube.
- 11.5 Add  50 µL of NP tube into the corresponding labeled 1.5mL Lo-bind tube containing Ampure XP beads.

11.6 Gently vortex, and quick spin down. Incubate tube(s) at room temperature for  00:05:00 . 5m

Meanwhile, store the NP tube(s) back into fridge.

11.7 Place tube(s) on magnetic stand for  00:05:00 . 5m

11.8 Carefully transfer supernatant (~  75  $\mu\text{L}$ ) into a new 1.5mL Lo-bind tube. Try to avoid disturbing the bead pellet, and be sure to label the new tube(s).

11.9 To the supernatant, add  35  $\mu\text{L}$  of Ampure XP beads. Repeat step if having multiple tube(s).

11.10 Gently vortex, and quick spin down. Incubate tube(s) at room temperature for  00:05:00 . 5m

In the meantime, prep the 80% ethanol wash solution. Each tube will need a total of  400  $\mu\text{L}$  80% ethanol wash solution.

Add  400  $\mu\text{L}$  of 100% ethanol with  100  $\mu\text{L}$  of lab-grade water, multiply volume according to the total number of tubes you'll be doing. Vortex, spin down and set aside on benchtop.

11.11 Place tube(s) on magnetic stand for  00:05:00 . 5m

If time allows, store Ampure XP beads back in fridge.

11.12 Transfer and discard all supernatant (~  110  $\mu\text{L}$ ) without disturbing the bead pellet.

11.13 While on magnetic stand, gently add  200  $\mu\text{L}$  of 80% ethanol wash solution to each tube(s).

11.14 Incubate for  00:00:30 , and discard supernatant. Repeat wash step one more time. 30s

11.15 While on magnetic stand, air dry beads for  00:05:00 . Avoid overdrying beads. 5m

11.16 Remove tube(s) from magnetic stand, and add  17  $\mu\text{L}$  low TE. Resuspend beads by pipetting or gently vortex.

11.17 Incubate tube(s) at room temperature for  00:02:00 . 2m

11.18 Place tube(s) on magnetic stand for  00:05:00 . 5m

11.19 Carefully transfer all  17  $\mu\text{L}$  supernatant into a new 1.5mL Lo-bind tube(s), labeled BSS#.

This is now your final, undiluted GT-seq library(s).

## Quantify library

### 12 Qubit GT-seq library

## Note

**Equipment and supplies needed for this step:****Equipment****Qubit Fluorometer**

Fluorometer to quantify DNA

NAME

Invitrogen

TYPE

Q33226

BRAND

SKU



Qubit assay tubes (Fisher Scientific, Q32856)

2.0mL microcentrifuge tube

1.5mL Lo-bind microcentrifuge tube (Fisher Scientific; 13-698-791)

p10 pipette and tips

p200 pipette and tips

vortex

benchtop centrifuge

**Reagents needed:**

BSS tube(s)

 Qubit dsDNA HS Assay kit Thermo Fisher Scientific Catalog #Q32854 TE pH 8.0 (1X TE Solution) IDT Technologies Catalog #11-01-02-05 (not sure if the right one)

12.1 If BSS tube(s) were stored in fridge, allow tube(s) to come to room temperature prior to qubit.

12.2 Make 1:200 diluted dye mix in a 2.0mL microcentrifuge tube.

	# BSS tubes				
	x1	x2	x3	x4	x5
Qubit dsDNA HS Buffer (µL)	199	398	597	796	995
Qubit dsDNA HS Reagent (µL)	1	2	3	4	5
Total	200	400	600	800	1000

Label each qubit assay tube(s). Only label on the lid, never the side of the tube(s).

- 12.3 Pipette  198 µL of diluted dye mix into each qubit assay tube(s). Add  2 µL of corresponding BSS to labeled qubit assay tube(s).

Vortex thoroughly, quick spin.

- 12.4 Incubate tube(s) at room temperature for  00:03:00 .

3m

- 12.5 Put qubit assay tube in Qubit Fluorometer and take 2 readings per sample and enter them into Library Prep sheet in PROJECT CHECKLIST\_v2.4 spreadsheet. The spreadsheet has a column that will take the average, and make the calculations in the Tray-Quant tab to dilute each library down to 1nM.

- 12.6 In a new 1.5mL Lo-bind tube, transfer  10 µL of BSS and dilute it with the calculated amount with 1X TE. Label tube as D#, include your initials and project initials.

- 12.7 Store diluted BSS tube(s) in GT-seq run box in fridge. Discard the undiluted BSS tube(s). Print out the Library Prep sheet when all tube(s) are ready for the run.

## Protocol references

Steele, C. A., Hess, M., Narum, S., & Campbell, M. (2019). Parentage-based tagging: Reviewing the implementation of a new tool for an old problem. *Fisheries*, 44(9), 412–422. <https://doi.org/10.1002/fsh.10260>