Pyromark Sequencing Protocol

Step1: Isolate genomic DNA from GA muscle. (small piece about half of slo.)

1. Cut small piece GA muscle place into 1.5 ml tube.
2. Add 700ul of Lysis buffer and 10ul of proteinase K (20mg/ml).
3. Incubate at 55C overnight.
4. Next day add 700ul of phenol, shake tube for 30". Spin full speed for 5' at room temp.
5. Transfer top layer to clean tube, Add 600ul of Chloroform , shake tube for 30", Spin for 5'.
6. Transfer top layer to clean tube, Add 1 ml of ETOH, Mix by shake the tube, Spin 5'
7. Wash pellet with 500ul 70% ETOH, Spin 5'. Pour off the supernatant. Quick spin to collect all the liquid and remove by using pipette.
8. Air dry the pellet. Resuspend the pellet in 20 – 50ul of TE.
9. Read OD260.

Step2: Methlcode Bisulfite Conversion.

Using Conversion kit from Invitrogen. Cat# MECOV-50. Dilute 200ng of genomic DNA with Water to bring the volume up to 20ul in the PCR tube. (0.1 ml)

Making CT conversion reagent. (Each tube only do 10 samples at a time).

Add: 900ul of ddh2O

50ul of Resuspension buffer

300ul of dilution buffer

Shaking at 360C rocker for 10'

Add 130ul of CT conversion reagent to the PCR tube with DNA. Mix well. Run program: BIOCOM

PCR: BIOCOM

94C 10’

64C 2.5 hours

12C hold

Place spin column in a collection tube. Add 600ul of binding buffer to the column. (Do 12 samples at a time)

Add 150ul of PCR conversion sample to the binding buffer. Mix tube by inverting several times.

Spin full speed for 30”, Discard the flow – through.

Add 100ul of wash buffer , Spin 30”,’

Add 200ul of Desulphonation buffer, incubate at RT for 15’ – 20’, Spin 30”

Add 200ul of wash buffer, Spin 30”, repeat one more time wash.

Place column to a new tube.

Add 10ul elution buffer to the center of the column, spin 30”. Sample ready for PCR.

Run 3ul of PCR products on 1% agarose gel to confirm has products.

Step3: Pyoseq:

Go to Rhianna’s computer. Open window Virtura box, go to Ptromark Q24, go to New run, Save to shared folder. Go to Tool, Get pre run information. Set up plate. Print out. Transfer information to the USB.

Before start pipetting. Turn on the machine and set up heat block at 80C.

Prepare all the solution:

1. Clean water 1L (Autoclaved)
2. Wash buffer (Dilute from 10X , Qiagen) store in cold room
3. Deanature solution (Qiagen)

4. 70% ETOH
5. Fill the solution up to the marker in the container.

Do all the pipetting on ice. Use filter tips. Cut PCR plate with 424 wells. This plate for binding reaction. Use Q24 plate for annealing reaction. Each plate have one well for control oligo. (Order from Qiangen)

Binding Cocktail (making for all the samples)

	1X
Binding buffer	40ul
Beads	2ul
ddH2O	18ul

Vortex, Mix well before pipetting.

Annealing Cocktail (for each primmer)

	1X
h-260 seq. primer	0.75ul
Annealing Buffer	24.25ul

Control Oligo:

Binding reaction:	1X	Annealing Buffer	
Binding buffer	40ul		1X
Beads	2ul	AB	5ul
H2O	13ul		
Oligo	25ul		

Pipet 60.ul of binding beads to the binding plate. Add 20ul of each PCR products. Mix by pipet up and done.

Add 25ul of annealing buffer to the Q24 plate. Works at RT. Tap plate to remove all the bubbles.

Add 80ul of oligo control in the binding plate. Marker the well. Cover top with sealed plastic cover.

Shaking the binding plate at 8 speed setting for 10 min.

Place Q24 annealing plate on the rack of the machine, cover the top with a piece of paper.

After 10' shaking, quickly move the plate to rack. Remove top cover. Gently put probes in the well and suck out the liquid for 20". No shaking. Move the probe into 70% ETOH. Wait sucking all done. Move probe into Deanaturation wait all done. Lift probe up and move probe around to get out all the liquid. Turn off the sucking switch. Put probe into annealing plate. Shaking about 1'. Move the probe to the 50ml water tray.

Transfer annealing plate on to heat rack (Pre-heat at 80C) FOR 2'.

Pipet Enzyme and Nucletides to the Chamber in other

E A S

T C

G

E-Enzyme

S-Substrates

A-dATP, C-dCTP, G-dGTP

Put Chamber into Machine. Also put annealing plate into the machine. Cool for 5'

Set up run. Put USB in the computer. Select program then start run.

* Enzyme and Substrates have to be fresh.