RADseq Library Preparation

Updated 11 Aug 2020

【Measure DNA concentration with Qubit】

1. Qubit dsDNA HS standards are stored in fridge (Cusco) and taken out for 30 minutes before using. Dye (Qubit dsDNA HS Reagent *2000X) and HS Buffer are kept at room temperature.

2. Prepare enough Qubit buffer for the two standards + the number of samples to be measured: 199 µl HS buffer + 1 µl dye each. Vortex to mix.

3. On the Qubit machine, select DNA > dsDNA broad range. Choose “yes” to read new standards.

4. Beginning with standard #1, mix with Qubit buffer by gently shaking, and wait for 2 minutes.

    Prepare standards and samples for measurement as follows, in Qubit assay tubes:

    Standards: 190 µl Qubit buffer + 10 µl HS standard (both #1 and #2)

    Samples: 199 µl Qubit buffer + 1 µl DNA

5. After keeping dark for 15 minutes, immediately insert the tube into the Qubit, close the lid, and measure the DNA concentration.

6. Repeat with standard #2. The standards are used to calibrate the Qubit.

7. Continue with DNA samples and record the reading for each.

Note: Qubit tubes should be kept clean or results may be inaccurate.

【Genomic DNA preparation】

1. In new PCR tubes, dilute **160 ng** genomic DNA from each sample into a **12 µl** volume. For example, from a 40 ng/µl DNA extraction, add 160/40 = 4 µl DNA to 8 µl dH_2O, to make 12 µl total.

Note: don’t pool samples whose DNA concentrations are too different in the same library.

【Restriction digest】

This step digests genomic DNA into fragments with sticky-ended sites for adaptor ligation using restriction enzymes. Restriction enzymes used: EcoRI: GAATTC; MseI: TTAA

1. Put the below reagents (T4 stored in Kiev ddRAD library PCR box, enzymes in pouches in Kiev door, BSA + NaCl 50mL tubes in Cusco) on ice, and follow the table to make Master Mix I. Prepare enough for n + 1 samples.

Reagent	Each sample (µl)	Master Mix I (µl)
10 X T4 Buffer	2.3	
1M NaCl	1.2	
1 mg/ml BSA	1.2	
dH_2O	0.5	
MseI	0.24	
EcoRI	0.56	

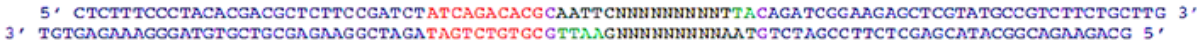
Total	**6**	

2. Add 6 µl master mix I into each sample (12 µl), for totals of **18 µl**.

3. Incubate at 37°C for 8 hours, then hold at 4ºC.

【Adaptor ligation】

Adaptors: EcoRI adaptor with barcode & MseI adaptor



Illumina PCR primer adaptor site, **barcode,** protector, **cut site**

1. Put the below reagents (NaCl + BSA stored in Cusco, others in Kiev) on ice and follow the table to prepare Master Mix II. Note that the EcoRI barcoded adaptor is added to each sample separately. Prepare enough for n + 2 samples.

	Reagent	Each sample (µl)	Master Mix II (µl)
mix together	MseI adaptor	2	
	dH_2O	0.065	
	10 X T4 Buffer	0.2	
	1M NaCl	0.1	
	1 mg/mL BSA	0.1	
	T4 DNA ligase	0.335	
	Total	**2.8**	
each sample separately	EcoRI with barcode	2	

Barcodes are located in lower door of Kiev, 101 different barcodes available.

2. Add 2.8 µl Master Mix II and 2 µl EcoRI unique barcode to each 18 µl digested DNA sample (total volume per reaction 22.8 µl). Record each sample's barcode number.

3. Vortex, spin down, and incubate at 16°C for 6 hours, then hold at 4ºC

【Gel Size Selection】

1. Make gel [how?] with comb with 2 large wells in the middle

2. Aliquot an equal amount of DNA (from 10-100 ng) from each sample using the measured DNA concentrations. Eg. for a 25ng/µl sample, if aiming for 100ng DNA from each sample, add 4µl. Combine aliquots from all samples into a new 1.5mL centrifuge tube.

3. Measure the volume of the combined samples, then add gel loading dye to the sample in a 1:4 ratio of dye:sample. Mix well by pipetting.

4. Load 4µl of 100bp DNA ladder into the first gel well. Load the entire sample into the large wells, using as many as necessary.

5. Run the gel for 25 minutes at [ ] voltage

6. Use NautiaZ Gel / PCR DNA Purification Mini Kit, following the included protocol

7. Place the gel on the Maestrogen LED transilluminator, and use the ladder as a reference to find the section containing 300-500bp DNA segments. Use a sterilized scalpel and forceps to cut a section (no more than 300 mg) from each well and place inside new 1.5mL centrifuge tubes.

8. Place the tubes in the sauna at 60ºC for 10 minutes to melt the gel.

9. Keep following the protocol: add buffer, transfer samples to EZ column filter tubes and centrifuge. DNA will bind to the filter. Wash. Elute. Combine. Measure.

【Bead cleanup】

1. Measure post-ligation DNA concentrations using Qubit.

2. Each sample needs 10-100 ng of DNA, depending on overall concentration [what if they don't?]. Mix all samples in a [1.5 mL] eppendorf tube.

3. Thoroughly resuspend beads. Add 1.8 µl beads per 1 µl of sample. Mix them by pipetting to be homogenized.

Note: don't vortex.

4. Incubate at room temperature for 5 minutes.

5. Put samples on a magnetic plate and wait for the solution to clear, which separates beads from the solution. DNA fragments above 100 bp will bind on beads.

6. Aspirate and discard upper cleared solution. It's better to leave a little solution (about 5 µl) because the beads are easily drawn out with the solution.

7. Wash the beads using 70% ethanol two times (200 µl, 200 µl for 30 seconds each time). Aspirate and discard all possible ethanol from the bottom after second washing.

Note: perform this step on a magnetic plate and don't disturb the beads when washing.

8. Keep the tube lid open, remove from the magnet, and wait until the beads look dry.

Note: don't overdry. The beads will appear cracked if overdried.

9. Add **40 µl** dH_2O and elute DNA by pipetting. Incubate for 2 minutes.

10. Put the sample on a magnetic plate to separate the beads from the DNA solution.

11 Transfer the DNA solution (supernatant) to a new eppendorf tube.

12. Measure DNA concentration using Qubit after ligation.

13. bluepippin size selection [?]

【PCR】

1. Add the below reagents (stored in ddRADseq box in Kiev) in order. DNA polymerase must be added last, because it will degrade the primers when dNTPs are absent (3'→5' exonuclease activity). 20 µl reaction is only used to test if adapter ligation is successful. 100 µl is used to amplify the library, and all reagents can be doubled to make a 200 µl reaction if desired.

Component	20 µl reaction (µl)	100 µl reaction (µl)
dH_2O	8.8 (add to 20)	add to 100
5X Phusion HF Buffer	4	20
10 mM dNTPs	0.4	2
Illumina PCR Primer 1 Forward	1	5
Illumina PCR Primer 2 Reverse	1	5
Template DNA	4	all (based on bluepippin output)
DMSO (optional for GC-rich)	0.6	3
Phusion HF DNA Polymerase	0.2	1

2. Vortex, spin down, and divide 100 µl reagent into 4 PCR tubes, **25 µl per tube.** (Or 8 tubes if 200 µl reaction.) Spin down again.

3. Measure DNA concentration from one tube with Qubit before PCR for later comparison.

4. Run the PCR with the following conditions:

Stage	Temp. & Time	Cycles
Initial denaturation	98°C 30s	1
Denaturation	98°C 10s	
Annealing	60°C 30s	10
Extension	72°C 30s	
Final Extension	72°C 10 minutes	1
Hold	4°C hold	1

5. Remeasure DNA concentration in the selected tube and compare it to previous measurement to ensure PCR was successful.

6. Recombine the PCR products into one tube. Clean up the product using magnetic beads, using the method described above.

7. Elute DNA in **40 µl** dH_2O.

8. Measure DNA concentration with Qubit.