Next-Generation Sequencing ddRad-seq Protocol
adapted for Ion Torrent PGM

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I. DNA extraction:

- Run a check gel on each extraction to verify that the DNA is of good quality.
- Quantify samples & make calculation to obtain 200 ng DNA from each sample.

***Preparation of adapters**

- All **AY,** to 10 µM	- Only **P1**, to 5 µM
10 µl of A stock (100 µM)	5 µl of P1-SdaI stock (100 µM)
10 µl of Y stock (100 µM)	5 µl of P1-RC stock (100 µM)
0.8 µl of 5M NaCl	0.8 µl of 5M NaCl
79.2 µl of TE 1X	89.2 µl of TE 1X
Total: 100 µl	**Total:** 100 µl

Thermocycler program: ***linkers***

95°C	3 min
65°C	2 min
55°C	2 min
45°C	2 min
35°C	2 min
25°C	2 min
15°C	1 min
4°C	∞

***Adapter dilution**

- Adapter **AY** (10 µM)	- Adapter **P1** (5 µM)
29.59 µl of AY stock (10 µM)	1.39 of P1 stock (5 µM)
20.41 µl of TE 1X	148.61 µl of TE 1X
Total: 50 µl	Total: Total: 150 µl

II. Digestion and ligation:

1.0 µl 200 ng* DNA **individual*
2.0 µl adapter AY, diluted*

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38.8 µl H_2O
5.0 µL TANGO buffer
2.0 µl P1 adapter, diluted
0.1 µl restriction enzyme SdaI
0.1 µl restriction enzyme Csp6I
0.5 µl T4 ligase
0.5 µl of ATP
Total: 50 µl

Thermocycler program: ***digest37***

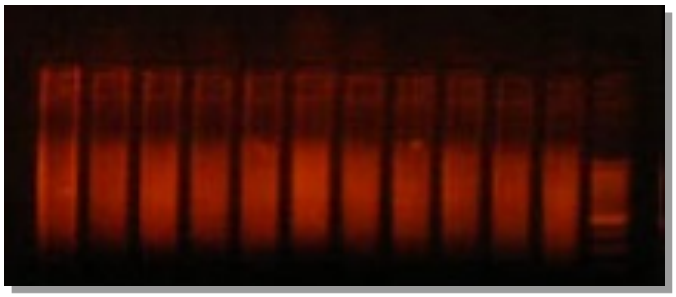
37°C	3 hr
68°C	15 min
4°C	∞

III. Digestion PCR (test):

5.25 µl H_2O
1.5 µL NH_4 buffer
1.5 µl primer P1
1.5 µl primer A-amp
1.5 µl BSA
1.2 µl dNTPs
1.2 µl $MgCl_2$ (reg., 25 mM)
0.35 µl Taq
Total: 15 µl (14 µl + 1 µl from digestion/ligation step II above)

Program: ***SNP Pre-amp*** (Biorad1 and 2)

94°C	2 min	
94°C	15 seg	35x
55°C	35 seg	
68°C	90 seg	
4°C	∞	



Gel showing results of digestion/ligation (step III).

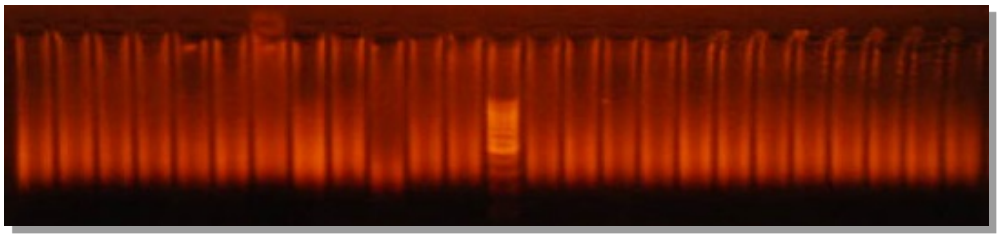
IV. Digestion PCR (4 replicates per SAMPLE):

11.44 µl H_20
2.0 µl $MgCl_2$
2.0 µl dNTPs
2.5 µl buffer NH_4
2.5 µl primer P1
2.5 µl primer A-amp
0.06 µl KlenTaq
Total: 25 µl (23 µl + 2 µl from digestion/ligation step II above)

Programa: ***PCR_digest_ion***

68°C	60 seg	
93°C	10 seg	18x
52°C	35 seg	
68°C	90 seg	
68°C	7 min	

- Combine the replicates for each sample into one tube, and then do a check gel to visualize the results.
- If you have some low molecular weight banding at the bottom of the check gel, you can do this PCR step for 20x instead of 18x.

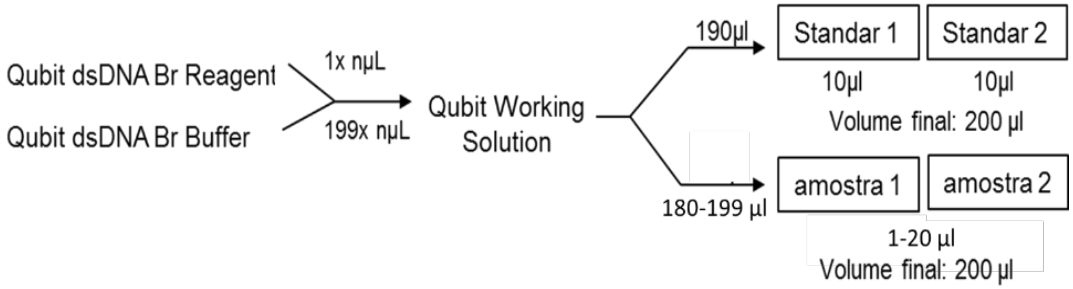


Gel showing results of the digestion/ligation amplification (step IV).

V. Sample quantification:

- Quantify DNA quantity and quality using the Qubit® 2.0 fluorometer (Thermo Fisher Scientific).

- Follow the manufacturer's protocol:



- Add 5 µl of each sample to 195 µl of the working solution.

- Vortex all tubes for 2-3 seconds.

- Incubate samples at room temperature for 2 minutes.

- Option: If the Qubit is not calibrated yet for DNA assays, do the following: **DNA → dsDNA HS** (double-stranded DNA High Sensitivity [10 pg/µl to 100 ng/µl] – if for that kit) → **Read New Standards** (place Standard 1 in Qubit) → **Read Sample;** next, read Standard 2

- Read the tube contents on the Qubit.

****Important:** the first value that appears in the Qubit read-out is the concentration of the tube in units of ng/mL. To calculate the original concentration of the sample go to the **Calculate Stock Conc.** option, and then adjust the volume of the sample used for the read. It is very important to choose the right units for subsequent calculations, which are in ***ng/µL***. *Concentrations in ng/µl must be saved for each of the samples*.

- Combine all of the individuals in one tube in an equimolar fashion (same quantity of DNA in each sample), taking the Qubit quantification results into account (maximum quantity of PCR fragment 4 ng = 4000 ng).

- Subsequently, **if necessary**, you may concentrate the library in the Speedvac (vacuum centrifuge). We [Justin and Sandra] did this by splitting the library equally among three 1.5 mL tubes, drying them down in the Speedvac for 1 h,

then resuspending the contents of each tube in 100 µl of autoclaved water. Prior to AMPure Bead Purification (next step), we mixed the solution in each tube by pipetting up and down a few times and then combining back into one tube.

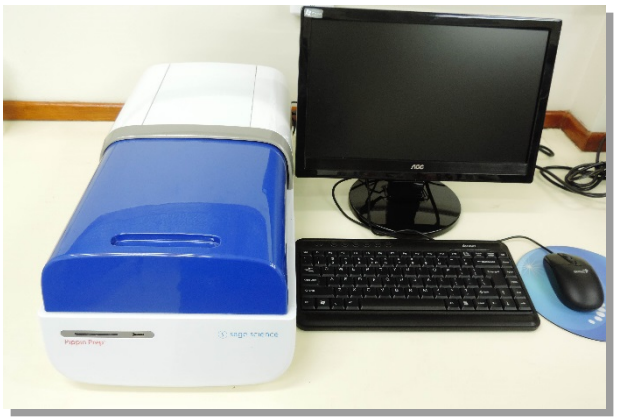
VI. Library purification – AmPure bead Purification

1. Add the correct quantity of AMPure Beads to the library – **0.8 µl** of beads for every 1 µl of product, and mix the tube by flicking with your finger.
2. Incubate at room temperature for 5 minutes.
3. Place the tube on the ThermoFisher magnetic rack (12 x 1.5 mL Magjet) and incubate at room temperature for 5 minutes.
4. Remove the supernatant.
5. Wash the beads by adding 500 µl of 80% alcohol.
6. Remove the tube from the rack and mix contents by flicking you're your finger.
7. Place the tube back on the rack and incubate for 5 minutes.
8. Remove the supernatant.
9. Repeat steps #5–8, then continue to step #10 below.
10. Leave the tube open to dry for 10–15 minutes. Do not dry excessively.
11. Resuspend in *X* µl (e.g. 61.5 µl for one tube) of water and mix.
12. Incubate away from the magnetic rack for 1 minute.
13. Place the tube on the magnetic rack and incubate for 2 minutes.
14. Remove (*X* − 1.5) µl (e.g. 60 µl for one tube) of the supernatant and transfer it to a new tube.

VII. Size selection:

Use SAGE Pippin Prep machine to conduct size selection. Only use 2% agarose dye-free (DF) cassettes, internal standard mix, marker type L.

- Sample of DNA → 30 µl of sample + 10 µl of marker solution.



- Program the protocol → Cassette 2% DF Folder L; Tight or Range (average = ± 400). We used Tight last.

- Calibrate the Pippin Prep by placing the calibration plate in the optical slot (LED target1: 0.80).

- Check the cassette → Visually check the buffer level in all wells (refill to “top off” where needed), the gel columns (e.g. make sure gels are not defective or broken), and for formation of any bubbles (try and pipet them out if possible).

- Prepare the cassette for a run → Remove bubbles, place the cassette in the optical slot, remove the adhesive (tape) covering, remove the buffer from the “elution” module and add 40 µl of electrophoresis buffer, recover the “elution” module (all of them) with tape, check the buffer level of the sample wells, and then start the “TEST” (if everything is OK, testing the cassette will give a result of “PASS”).

- Load the sample → Visually check the level of the buffer in the sample wells again, then remove 40 µl of buffer from the well and add 40 µl of the sample that you prepared above.

- Run! → Close the lid, go to the main menu and chose the appropriate program then click on “START.” The run will stop automatically when size-appropriate sample collection is complete.

- Collected fraction → Remove the sample (~40 µl) using a 100-200 µl pipette (the sample is in Tris-TAPS buffer; do not let it sit overnight in this buffer). Remove the cassette and dispose of it appropriately.

Target (bp)	Time to collect (min)
100	49
200	57
300	63
400	71
500	79

VIII. Library purification - AmPure bead Purification

1. Add the correct quantity of AMPure Beads to the library – **0.7 µl** of beads for every 1 µl of product, and mix the tube by flicking with your finger.
2. Incubate at room temperature for 5 minutes.
3. Place the tube on the ThermoFisher magnetic rack (12 x 1.5 mL Magjet) and incubate at room temperature for 5 minutes.
4. Remove the supernatant.
5. Wash the beads by adding 500 µl of 80% alcohol.
6. Remove the tube from the rack and mix contents by flicking you're your finger.
7. Place the tube back on the rack and incubate for 5 minutes.
8. Remove the supernatant.
9. Repeat steps #5–8, then continue to step #10 below.
10. Leave the tube open to dry for 10–15 minutes. Do not dry excessively.
11. Resuspend in *X* µl (e.g. 24.5 µl for one tube) of water and mix.

12. Incubate away from the magnetic rack for 1 minute.
13. Place the tube on the magnetic rack and incubate for 2 minutes.
14. Remove (X − 1.5) µl (e.g. 23 µl for one tube) of the supernatant and transfer it to a new tube.

IX. Calculation of library volume for *template* preparation

- Quantify the library on the Qubit by pipetting 3 µl of each sample into 197 µl of working solution.

- The original concentration of the sample given in ***ng/µL*** should be used to calculate the final volume of the library used for preparation of the *template*.

- Use the **nM Conversion Calculator** Excel spreadsheet (attached) to make the calculation.

Example: Library: 0.231 ng/µL

1st dilution (50 pM, 50 µL): 2.73 µL of library + 47.27 µL of H_2O

2nd dilution (15 pM, 25 µL): 7.50 µL of the 1st dilution + 17.5 µL of H_2O

X. *Template* preparation (see **User Guide *Ion PGM Hi-Q OT2 Kit***)

This step uses the Ion emulsion PCR machine.

XI. Enrichment (see **User Guide *Ion PGM Hi-Q OT2 Kit***)

This step uses the Ion enrichment machine.

XII. Sequencing - Ion 318 v2 chip (see **User Guide Ion PGM Hi-Q Sequencing Kit**)

Finally, the sample is run on the Ion Torrent Personal Genome Machine.