Simplified GBS protocol (Marco's approach)

Including notes

DNA

1. Check DNA for Quality/quantity
    a. Nanodrop to check purity. 260/280 should be ~1.8; 260/230 should be ideally 1.8-2, but lower values (down to 1.4) should be acceptable as well.

    b. Use Qubit BR kit to assess quantity (of all samples). Ideally it should be at least 20 ng/µl, to make the whole set up of the plate easier.

    c. Normalize DNA samples to 20 ng/µl with 10 mM Tris-HCL, pH 8. DNA normalization should be done using Qubit concentration estimates, not Nanodrop (they are not too far off, but far off enough to noticeably increase variability among samples).

I Digestion

2. Create a digestion/ligation master mix and aliquot 10 µl of it into each well in a 96-well plate

Reagent	**Cat. Num**	**x1 sample**	**x102 (master mix)**
MilliQ H_2O		7.2 µl	734.4 µl
NEB 10X Cutsmart buffer	B7204	2.0 µl	204 µl
NEB PstI HF	R3140	0.4 µl	40.8 µl
NEB MspI	R0106	0.4 µl	40.8 µl
		10 µl	1020 µl

3. Add 10 µl of DNA (20ng/µl) to each well. Each well will receive a different barcode, so it is important to keep track of where samples are located. Make sure to always check that the orientation of the new plate matches the old when transferring samples. In the 1-96 set of adapters, F12 consistently fail, so keep that well empty. D4 has sometimes given troubles in the 97-192 set of adapters.

4. Digest for 5 hrs at 37°C, followed by 20 min at 80°C to (partially) heat inactivate the enzymes, then a hold at 4°C. **Don't freeze at this stage, you can leave the samples at 4°C, but it is best to proceed to ligation as soon as possible.**

II Ligation

5. Anneal adapters before first use (using protocol from Marco). The adapters are ordered as normal oligos (standard desalting; 25µM amount). For each adapter two oligos are ordered in complementary pairs and must be annealed to form the double stranded adapter prior to use of the protocol. They are very stable after annealing and can be stored at -20ºC indefinitely.

6. The working dilution for the barcoded adapters is 0.4 ng/µl. Since preparing accurate dilutions for all the 96/192 adapters is a pain, it is convenient to prepare a larger volume of dilutions, aliquot 4.5 µl of them (the amount needed for each library) in several 96-well plates and store them at -20ºC. This minimizes the number of times that the adapters need to be thawed, and even if there is some evaporation you are sure that you have the right amount of adapter in the well (this has been a problem for us when storing larger volumes of adapters at the working concentration).

7. Add entire volume of digested samples (18 – 20 µl depending on evaporation) to plate with barcoded adaptors (~ 24.5 total volume).
8. Add 15.5ul ligation mix (see below) to each sample (total volume of ~ 40ul per tube).

Reagent	Cat. Num	x1 sample	x102 (master mix)
MilliQ H_2O		8.4 µl	856.8 µl
NEB 10X Cutsmart buffer	B7204	2.0 µl	204 µl
10 mM ATP	P0756	4.0 µl	408 µl
T4 Ligase (2,000 U/µl)	M0202T	0.1 µl	10.2 µl
Common Adapter (10 µM)		1.0 µl	102 µl
	Total	15.5 µl	1581 µl

9. Incubate in the PCR machine to ligate. Program below.

Temperature	Time
22°C	2 hours
65°C	20 minutes
4°C	forever

III Cleaning and Concentration

When using the SPRI beads, make sure you allow them time to come to room temperature (~30 min.) and MIX VERY WELL EACH TIME before using (but do NOT vortex!). In general, a clean-up with 1.6 volumes of bead will recover almost all of your DNA except the very small stuff (e.g. adapters, primers).

10. All steps use multichannel pipettes:
    a. Add 64 µl of SPRI (or AMPure XP) beads, mix well by pipetting, leave at room temperature for ~10-15 minutes
    b. Move the tubes to the magnet, leave them there for 5 minutes or until the solution is clear, then remove the supernatant. While waiting for the beads to separate, you can poke larger bubbles if there are any, since they might interfere with beads separation or get in the way when removing the supernatant - each well with a new tip.
    c. Keeping the tubes on the magnet, add 200 µl of 75% EtOH to each tube. Wait a few seconds (or until clear), then remove the ethanol.
    d. Repeat wash.
    e. Dry the tubes for 5-10 minutes at room temp or in a PCR machine with the lid open at 37ºC.
    f. Resuspend well in 12 µl of 10 mM Tris-HCl pH 8.0. You need to pipette the solution up and down on the sides of the wells to get all the beads into the solution. Leave at room temperature for 10 minutes.
    g. Put back on the magnet, wait 5 minutes or until the solution is clear. Move 10 µl of the supernatant to a clean plate, being careful not to carry over any beads. If it looks like there are still beads, move the plate onto the magnet before taking 4 µl for the amplification step (residual beads might affect the PCR).

IV Amplification (PCR)

11. After cleaning, do individual PCRs in a 10 µl volume as follows:

Reagent	**x 1 well**	**x 105 (master mix)**
MilliQ H_2O	0.2 µl	21 µl
Kapa HIFI HotStart MasterMix 2X	5.0 µl	525 µl
IlluminaFor_PE (10µM)	0.4 µl	42 µl
IlluminaRev_PE (10µM)	0.4 µl	42 µl
Total	**6 µl**	**735 µl**
DNA Template from step #12	4 µl	
Total	**10 µl**	

Step	**Temperature**	**Time**		**Cycles**
1	98°C	30 sec		
2	98°C	30 sec		
3	62°C	20 sec		
4	72°C	30 sec	Return to step 2	14 x
5	72°C	10 min		
6	4°C	forever		

12. Quantify 1 µl of each PCR sample with the Qubit BR kit. With 13-14 cycles you should get DNA concentrations of between 30 and 150 ng/µl. If you pool 96 samples, that translates to 30+ µg of library before size selection, which is plenty (after gel size selection you'll end up with about 10% of the starting amount of library). While for some plates all samples are relatively similar in concentration (up to 2-fold differences), other plates can have up to 4-5-fold difference between the most and least concentrated samples. Normalize and pool the samples accordingly (**you'll have to normalize the quantities to the least abundant sample).**
13. Pool one plate into one 1.5 ml tube. If the volume exceeds 400-500 µl, split in two tubes before concentration (the beads on top won't be attracted efficiently by the magnet if the volume is too high).
14. Concentrate:
    a. Add 1.6X beads and mix well by pipetting.
    b. Leave at room temperature 10-15 minutes.
    c. Move the tubes to the magnet, leave them there for 5 minutes or until the solution is clear, then remove the supernatant.
    d. Keeping the tubes on the magnet, add 1 ml of 75% EtOH. Wait a few seconds (or until clear), then remove the ethanol.
    e. Repeat
    f. Dry the tubes for 5 -10minutes at room temp or at 37ºC in a heating block, until it looks like the ethanol is gone.
    g. Resuspend in 40 µl of 10 mM Tris-HCl, measure concentration.

V Size Selection

15. Pour 150 ml of a 1.5% agarose gel in a small square gel rig, with EtBr.
16. To make sure you'll have enough library for the depletion step, it is best to start size selection with 10-20 µg of library. Divide it into two aliquots containing 5-10 µg of DNA each. If you load too much DNA on a single lane, separation will be poor. On the other hand, if you divide your library between many lanes, you'll have several gel slices to deal with.
17. Add gel dye to each sample and load the gel, using a 50 bp ladder as size standard. Leave at least 1 lane between each sample and other occupied wells (e.g. ladder, other samples). It is preferable to use a separate gel for each library. My preferred organization is: empty, ladder, empty, sample, empty, sample, empty, ladder, empty.
18. Add the same volume of loading dye and Tris to empty wells (makes the gel run better).
19. Add 5 µl EtBr to the bottom reservoir of the gel rig. Since EtBr is attracted to the negative pole (opposite to DNA), this helps ensure that the lower portion of the gel is well-stained.
20. Run for ~20 minutes at a lower voltage, e.g. 80 V. Then increase to a higher voltage (100 V) and run until the desired size range seems well enough separated (about half way in the tray). You can also run gel constantly on 80V, the separation is better with lower V.
21. To excise bands:
    a. Label and pre-weigh a number of 2ml tubes (depending on the width of your selected band and the number of wells).
    b. Clean blue light viewer before placing gel there, and cover with saran wrap to protect the glass. You will need to wear the orange glasses OR place the (cleaned) orange plate over the gel to visualize the stained DNA, and will likely need to turn off the light.
    c. Use new, sterile blades—a new one for each library. Cut carefully and as precisely as possible—you want to have the lowest possible gel volume that contains all of your desired fragments. Size range should be 400 - 600 bp (this includes ~120 bp of adapters, so the actual fragments selected will be ~300-500 bp long). If you are preparing several plates of GBS that need to be compared, it is important that the size selection is as reproducible as possible. Unfortunately, since you are loading a lot of DNA, there can be quite some variation between different gels, and the ladder is not always the best way to compare them. I find it better to use the thicker chloroplast bands as internal standards; when you cut out the first library, record where you cut relative to the banding pattern in the samples, and keep that constant for the following libraries
    d. Carefully transfer your excised gel fragments into the pre-weighed tubes.
    e. Weigh the tubes containing the gel fragments to get the weight of the excised fragment. If a fragment weighs more than 400 mg (the declared maximum that can be processed on a Qiagen column), divide it into two tubes.
    f. Proceed to follow the Qiagen Gel Extraction kit protocol. Note that the 3X (step 2) and 1X (step 4) volumes both refer to the original weight of gel in each tube. Pre-warm Buffer QG to 50ºC. Wait 2–5 minutes after applying the wash buffer (step 7), as suggested by the note. For maximum yield, pre-warm your elution buffer (10 mM Tris-HCl recommended) to 50 °C and allow to sit on column for a few minutes before centrifugation. Elute in 30 µl Tris-HCl, and pool samples belonging to the same set of 96 samples.

g. Measure concentration (QuBit BR). The ideal concentration for the depletion step in 160 ng/µl, so you will likely need to concentrate your sample (with SPRI beads). Elute into an appropriate volume of 10 mM Tris, pH 8. I had good recovery with as little as 6 µl of Tris.
h. Measure concentration using 0.5 µl of solution and the QuBit BR kit. If concentration is higher than 160 ng/µl, dilute accordingly. Lower concentrations (down to ~80 ng/µl) do not seem to affect the result of the depletion much.

Perform depletion according to the protocol on the lab blog.