PAIR-END ILLUMINA LIBRARIES

Before you start:

- Prepare 1 μg of genomic DNA in appropriate volume of water/buffer (but see final notes regarding the starting amount of DNA). Depending on the Covaris tube you are going to use, the "appropriate volume" can be 50 μl or 130 μl. The smaller Covaris tubes are better, because you end up with a smaller volume, which makes the following purification step easier. I have been using the bigger Covaris tubes because I re-used some old ones, and I shear 2 μg of DNA and use half of the volume for library construction (see final notes about re-using Covaris tubes).

- Take the SPRI beads out of the fridge, mixed them well, let them go at room temperature for about 30 minutes. Shake them a bit again before using. I used home-made SPRI beads, following the protocol posted by Kristin on the Rieseberg Lab blog (based on Rohland *et al.* 2012). There is a fresh 50 ml batch in the freezer, in a Falcon tube wrapped in aluminum foil – ask me if you can't find it). I strongly recommend using these instead of the commercial AMPure beads, because they work exactly as well (see my blog post on the topic), and cost literally one tenth.

- Make ethanol 75% fresh.

Shearing

- Shear DNA in Covaris tube to ~350bp (well, that was easy…). I used the settings Kristen uses. For the 50 μl Screw Cap miniTUBEs that means Peak Incident Power 50, Duty Factor 20%, Cycle per Burst 200, Treatment time 65 s, Temperature 20°C.

Size selection

The volumes of beads used in this section are to select fragments of about 350 bp in size. You can modify the proportion of SPRI beads you use in order to obtain a different fragment size (again, my blog post of SPRI beads might be of help with that).

- add 0.875 volumes of beads to your DNA solution (so, if you started like me with 1 μg of DNA in 65 μl, add 56.9 μl of beads). Mix well by pipetting or vortexing, leave at room temperature for 5 minutes. I used 0.2 μl PCR tubes and the 96-well magnet throughout, so mixing by pipetting is the best option (the lids of our PCR tubes do not hold vortexing very well).
- Move the tubes to the magnet, leave them there for 5 minutes or until the solution is clear, then remove and discard the supernatant.

- Keeping the tubes on the magnet, add 200 μl of ethanol 75% to each tube. Wait at least 30 seconds, then remove the ethanol. Repeat the wash.
- Dry the tubes for 5-10 minutes at room temperature or at 37°C (I have used a thermal cycler with the lid open).
- Resuspend well by pipetting in 50.5 μl of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move 50 μl of the supernatant to a clean tube.

- Add 41.25 μl of SPRI beads (that's 0.825 volumes), mix well, leave at room temperature for 5 minutes
- Move the tubes to the magnet, leave them there for 5 minutes or until the solution is clear, then **move the supernatant to a clean tube**.

- Add 80 μl of SPRI beads to said supernatant, mix well, leave at room temperature for 5 minutes.
- Move the tubes to the magnet, leave them there for 5 minutes or until the solution is clear, then remove the supernatant.
- Keeping the tubes on the magnet, add 200 μl of ethanol 75% to each tube. Wait at least 30 seconds, then remove the ethanol. Repeat the wash.
- Dry the tubes for 5-10 minutes at room temperature or at 37°C.
- Resuspend well by pipetting in 42.5 μl of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move the supernatant to a clean tube.

End Repair

- Add: 5 μl of NEBNext End Repair 10X buffer
  2.5 μl of NEBNext End Repair enzyme mix.
- Incubate for 30 minutes at 20°C in a thermal cycler.

SPRI beads purification

- Add 80 μl of SPRI beads, mix well, leave at room temperature for 5 minutes.
- Move the tubes to the magnet, leave them there for 5 minutes or until the solution is clear, then remove the supernatant.
- Keeping the tubes on the magnet, add 200 μl of ethanol 75% to each tube. Wait at least 30 seconds, then remove the ethanol. Repeat the wash.
- Dry the tubes for 5-10 minutes at room temperature or at 37°C.
- Resuspend well in 34.5 μl of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move 34 μl of the supernatant to a clean tube.

A-tailing

- Add: 5 μl of NEB buffer 2
  10 μl of 1mM dATP
  1 μl of Klenow Fragments exo-
- Incubate for 30 minutes at 37°C in thermal cycler.

SPRI beads purification

- Add 80 μl of SPRI beads, proceed as before
- Resuspend in 11 μl of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move 11 μl of the supernatant to a clean tube.

Adapter ligation

- Add: 12.5 μl of NEB Quick Ligase buffer
  0.5 μl of 10 μM P1 adapter
  0.5 μl of 10 μM P2 adapter (eventually barcoded)
  0.5 μl of NEB Quick ligase
- Incubate for 15 minutes at 20°C, 5 minutes at 65°C, then cool down to 10°C (or put on ice).

SPRI beads purification

- Add 40 μl of SPRI beads, proceed as before
- Resuspend in 30 μl of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move the supernatant to a clean tube.

Enrichment (if you plan a depletion treatment, see alternative enrichment step at the and of the protocol)

- Set up the following PCR:

water	6.5 μl
KAPA HiFi HotStart 2X readyMix	12.5 μl
PE-PCR-F 10 μM	1 μl
PE-PCR-R 10 μM (indexed)	1 μl
Library	4 μl

Cycling conditions:

98°C	2 minutes	
98°C	10 seconds	X cycles
62°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	

In my experience 8 cycles are sufficient to get more than enough library for sequencing. If that is not the case you can repeat the PCR and add one or more cycles. It also depends on how much DNA you started with (see final notes). Since this PCR is necessary to obtain full-length Illumina adapters, you probably don't want to go much lower than 8 cycles – a higher proportion of your library won't have full length adapters.

SPRI beads purification

- Add 40 μl of SPRI beads, proceed as before
- Resuspend in 25 μl of of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move the supernatant to a clean tube.
- Add 40 μl of SPRI beads, proceed as before
- Resuspend in 32.5 μl of of Tris pH 8.0 10 mM. Leave at room temperature for 5 minutes.
- Put back on the magnet, wait for a couple of minutes or until the solution is clear. Move the supernatant to a clean tube.

A quick quality control is to check the concentration of your library on the Qubit, using the High Sensitivity kit. This will tell you how well the enrichment step worked. If you have about 5 ng/μl or more you should be good. Further quality control includes a run on a Bioanalyzer HS DNA chip to determine the size of your fragments and a qPCR assay to determine the molarity of your library. Remember that your fragments now have about 120 bp of adapters attached to them, so if you were aiming for 350 bp-long fragments, your main peak in a Bionalayzer chip analysis should be around 470 bp.

Enhanced enrichment for DSN treatment

A DSN treatment can be used in order to reduce the abundance of repetitive sequences (chloroplast DNA, transposons…) in your library. This treatment requires to start with substantially more library than you would get with a regular enrichment step (at least three μl at an ideal concentration of 160 ng/μl).

You can use any combination of increasing the amount of template in the enrichment PCR, increasing the number of cycles and/or making more than one PCR. What I did for the last batch of libraries I made was making for each library two enrichment reactions as follows:

	water		0 μl
	KAPA HiFi HotStart 2X readyMix		12.5 μl
	PE-PCR-F 10 μM		1.5 μl
	PE-PCR-R 10 μM (indexed)		1.5 μl
	Library		9.5 μl
Cycling conditions:	98°C	2 minutes	
	98°C	10 seconds	12 cycles
	60°C	30 seconds	
	72°C	30 seconds	
	72°C	5 minutes	

The annealing temperature is reduced to 60°C because I used shorter PCR primers, which are preferable when libraries are to be used for DSN treatments (see blog post about depletion treatments)

I pooled the two PCRs, bead purify them, re-susupend in 6 μl of Tris pH 8.0 10 mM, and quantified 0.5 μl on a qubit BR. That gave final concentrations of about 400-800 ng/μl, which is more than you need, so you could probably use one PCR cycle less. From that on you can follow the DSN depletion protocol I posted on the blog.

FINAL NOTES

Starting amount of DNA

There is actually no compelling reason why you should start with 1 μg of genomic DNA. You could start with more, and you will end up having a more concentrated library before the enrichment step. This means that you can shave off one or two cycles of PCR amplification. This is supposedly good, because PCR amplification introduces biases in your fragments pool. On the other hand, if you don't have 1 μg of DNA, you can start with less and compensate by increasing the number of PCR cycles in the enrichment step.

Unless you use much more DNA, the protocol should work well with different amounts of DNA. The only thing that is important to change is the amount of adapter you use. The ratio adapter:fragments is important to have an efficient ligation. So, if you half the amount of DNA input, remember to use half the amount of adapters. I used successfully the same protocol starting with 2 μg of DNA and doubling the amount of adapters, and the resulting library was roughly twice as concentrated (so I could have used one less PCR cycle). If you use more than 2 μg you might have to double all the reactions (end repairing, A tailing and ligation), so it might not be very convenient.

Re-using Covaris tubes

Covaris tubes are rather expensive (5$ each), and you are supposed to use them only once. I have used the same tube at least two or three times and there is no mechanical reason why you shouldn't re-use them (they don't break and the DNA is still properly sheared). The potential problem is to carry over some DNA from your previous experiment. I have been rinsing the tubes in water and then ethanol, left them a couple of hours in 20% bleach, rinsed them with water and then ethanol again. I doubt any DNA would be left after this, or so little as to have no influence on your final results.

Why 80 μl of beads for clean-ups?

That's 1.6 volumes. I found it to be a good concentration, in that you don't lose any of the larger fragments you care about, while it allows you to discard most of the fragments <100 bp (including adapters and primers).