MinION PROTOCOL

Adapted from Janneke Wit by Robyn Tanny May 2016

Company	Kit/Item	Catalog Number
Fisher	Eppendorf™ DNA/RNA LoBind Tubes	13-698-791
Fisher	Covaris g-TUBE	NC0380758
NEB	NEBNext FFPE Repair Mix	M6630S
NEB	NEBNext Ultra II End Repair/ dA-Tailing Module	E7546S
NEB	NEB Blunt / TA Ligase Master Mix	M0367S
Fisher	Ampure XP 5ml kit	NC9959336
Fisher	Dynabeads® MyOne™ Streptavidin C1	65001
Invitrogen (via Fisher)	Qubit® dsDNA HS Assay Kit, 500 Assays	Q32854
Oxford NanoPore	MinION R7 chemistry set	-

Prior to starting the assay, make sure you have all incubators set to appropriate temperatures and that you have a thermocycler available.

A. Shear 1*µ*g genomic DNA in g-TUBE in a total volume of <u>46</u> *µ*l

1. Make up total volume with nuclease free water, mix thoroughly (by inversion), and spin down. Transfer this to the Covaris g-TUBE. Use an Eppendorf 5424 centrifuge and spin at 4,200 rpm for 1 minute **twice.**
    - About 1 mm fluid remains in the filter. Tweaking no result, we find that spinning twice is the optimal compromise.
    - We started using 1.5 *µ*g starting material, as the protocol recommends starting with more if you're doing the end repair steps. After the two AMPure bead cleanup steps that puts us at about the right concentration

2. To retrieve DNA, invert Covaris g-TUBE according to Covaris directions. Use an Eppendorf 5424 centrifuge and spin at 4,200 rpm for 1 minute twice.

B. DNA Repair step (optional, but recommended for gDNA)

1.

Reagent	Volume (µl)
>1 µg Fragmented DNA (1.5 µg)*	45
Nuclease-free H_2O	8.5
FFPE Repair Buffer	6.5
FFPE Repair Enzyme Mix	2
Total volume:	62

Fragmentation is not necessary, can be performed directly on high mol. weight samples

2. Mix gently, spin down, and incubate the reaction at **20˚C** (or room temp if heating block not available) for 15 min.
3. Clean up the reaction with Agencourt AMPure XP beads (sample:beads, 1:1):
   - Resuspend beads by vortexing, add 62 µl of the resuspended beads to the FFPE-repair reaction and mix by inversion. Allow DNA to bind to beads by rotating for 5 minutes on a nutator.
   - Spin down and pellet on magnet. Once clear and colorless, aspirate off supernatant.
   - Wash beads with 200 µl of fresh 70% ethanol. Remove the 70% ethanol using a pipette.
   - Repeat above wash step.
   - (Optional) Briefly spin tube to collect residual liquid at bottom of tube. Return the tube to the magnet and aspirate residual wash solution.
   - Let the pellet air dry, avoid over drying (indicated by big cracks in the pellet)
   - Resuspend the pelleted beads in 48 µl nuclease-free water. Incubate for 2 minutes at RT.
   - Pellet beads on a magnet, and remove eluate (~46 μl) once it is clear and colourless.
   - Quantify 1 µl of eluted sample using the Qubit (>800 ng of material expected).
     - When I start with 1.5 μg of DNA, I often get close to 1.5 μg back at this point.
   - Take at least 1 μg of FFPE repaired DNA in 45 μl into End-Prep.

C. End-Prep:

1. In a 0.2 ml thin walled PCR tube, setup the end-prep reaction using NEBNext Ultra II End Repair/dA-tailing module and ~ 1 μg DNA in 45 μl.

Reagent	General (µl)	cDNA only (µl)
DNA (1 µg)	45	45
Ultra II End-Prep buffer	7	7
Ultra II End-Prep enzyme mix	3	3
DNA CS	5	-
Nuclease-free water	-	5
Total volume:	60	60

2. Mix gently by inversion, spin down and using a Thermal cycler incubate at **20˚C** for 5 mins and 65 ˚C for 5 mins.
3. Clean with AMPure beads (sample:beads, 1:1):
   - Resuspend beads by vortexing, add 60 µl beads to the End-prep reaction and mix by pipetting. Transfer sample to LoBind DNA 1.5 ml Eppendorf tube. Incubate 5 minutes.
   - Spin down solution and pellet on magnet. Once clear and colorless aspirate off supernatant.
   - Wash beads with 200 µl of fresh 70% ethanol. Remove the 70% ethanol using a pipette.
   - Repeat above wash step.
   - (Optional) Briefly spin tube to collect residual liquid at bottom of tube. Return the tube to the magnet and aspirate residual wash solution.
   - Let the pellet air dry, avoid over drying (indicated by big cracks in the pellet)
   - Resuspend the pelleted beads in 33 µl nuclease-free water. Incubate for 2 minutes at RT.
   - Pellet beads on a magnet, and remove eluate (~31μl) once it is clear and colorless (used in step 5).
   - Quantify 1 µl of eluted sample using the Qubit fluorimeter. (>700 ng of material expected).
     - At this point, I usually get close to 1 μg of DNA.

4. Thaw and prepare the kit reagents as follows, mix by inverting and spin down briefly before use:

Reagent	On ice	At room temperature
Bead binding Buffer		X
Elution Buffer		X
HP Tether		X
HP Adapter	X	
Adapter Mix	X	
Fuel Mix	X	
Running Buffer		X

Ligation

If fragment sizes are generally smaller than 3 kb, adjustments should be made to use 0.2 pmoles of DNA in the adapter ligation step.

5. Ensure that the Blunt/TA master mix is mixed thoroughly before use. Assemble the following reagents in the order given below, in a DNA LoBind 1.5 ml Eppendorf.

Reagent	Volume (µl)
Nuclease-free water	8
End-prepped DNA	30
Adapter Mix	10
HP Adapter	2
Blunt/TA Ligase Master Mix	50
Total volume:	**100**

6. Mix by pipetting between **each sequential addition (**briefly spin down in a microfuge if needed)
7. Incubate for 10 min at room temperature Prepare MyOne Beads.
8. **Add 1 µl HP Tether**, mix by pipetting, (briefly spin down), and incubate for 10 mins at room temp.

MyOne C1-bead preparation

9. Resuspend MyOne C1-beads by vortexing until homogeneous.

10. Transfer 50 µL of homogenized beads to a 1.5 ml DNA LoBind tube. Pellet beads on a magnet and aspirate off supernatant.
11. Add 100 µl Bead binding Buffer to the pelleted beads. Resuspend beads by pipetting. Place tube on magnet, pellet and aspirate.
12. Repeat step 11
13. Add 100 µl Bead Binding Buffer to the pelleted beads. Resuspend pellets by pipetting.
14. These "washed beads" are required for the subsequent purification.
15. Go back to add the HP tether to the ligation reaction.

MyOne C1-bead binding

Do not invert or vortex, carefully use pipet for mixing during MyOne C1 bead step. Otherwise most beads stick to the walls, regardless of the type of tube used.

16. To the adapter-ligated, tether-bound DNA add 100 µl "washed beads", carefully mix by pipetting and incubate at room temperature for 5 min (make sure beads don't sink to the bottom).
17. Place on a magnetic rack, allow beads to pellet and aspirate supernatant.
18. Resuspend the pelleted beads in 150 µl Bead Binding Buffer. Place on magnet, aspirate off Bead Binding Buffer.
19. Repeat step 17

Elution of library from MyOne C1-beads

20. Resuspend the pelleted beads in 25 µl of Elution Buffer. Incubate for 10 min at **37 ˚C**.
21. Place on magnet, pellet and retain eluate (contains library).
22. Quantify 1 µl of eluted sample on the Qubit. One should expect to retain more than 200 ng of material.
    - At this point, I get a large loss of sample. The most I have ever gotten back is 130 ng.
    - Have tried the following: Increase MyOne elution volume, increase MyOne elution time, and adding ligation mix to the beads rather than beads to ligation mix (this last step prevents unnecessary handling of the sticky beads). None of these made a difference.
    - Janneke Wit has also tried these adaptations and she did see an increase. She did a

side-by-side prep and eluted one sample in 25 µl and the other in 35 µl. Both samples gave ~250 ng, so the elution volume did not have an affect. As of 8/16/2016, Janneke is not sure what made the difference. It might have been the starting material as she noted it was one of cleanest preps she ever had and had some fairly long fragments (avg fragment size was 16.24 kb).

23. The library is called the **Pre-sequencing Mix.**

For loading, mix (in order) 75 µl 2x running buffer, 65 µl nuclease-free water, 4 µl Fuel Mix and 6 µl Pre-sequencing Mix in DNA LoBind tube. ☒ Check *Preparing the library for loading* for details.