Protocol: Haplotagging

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1. *DNA dilution to 0.075ng/ul*
2. *Aliquot 1.25 ul of Hapltagging beads per sample*
3. *Mix 10 ul of DNA with tagmentation mix*
4. *Combine 2+3 and tagment the DNA*
5. *Stop reaction with SDS*
6. *Sub-sample and pool only portion of tagmented beads and DNA from each sample*
7. *Remove un-used barcoded transposons from beads with Exonucleases treatment*
8. *PCR amplify the library*
9. *Size-selection of library with magnetic beads*

1. *gDNA dilution into a 96-well plate to make all samples 0.075 ng/ul*

- Pipette 195 ul of 10mM Tris, pH=8 into each well of a 96-well plate.
- With very slow pipetting, transfer 15 ng of each DNA sample into the designated wells (now each well contains approx. 200 ul of 0.075 ng/ul DNA)
- Seal the plate and mix by gentle inverting, then spin down.
- Use 20 ul of each sample to check if it contains approx. 1.5 ng DNA, either with thermofisher's Qubit (Q33230, Qubit™ 1X dsDNA HS Assay-Kit) or in a 96-well format with PicoGreen (Quant-iT™ PicoGreen™ dsDNA Assay).

Pico-Green:

Check correctness of your DNA concentration/dilution using "Quant-iT™ PicoGreen™ dsDNA Assay" (96-well plate = 88 samples + 8 standards). I use Greiner, #655906: black plates with flat and transparent bottom, re-usable:

88-sample wells with DNA in 50 ul TE:

- *30 ul TE buffer into each sample well*
- *20 ul of 0.075 ng/ul DNA into each sample well*

8 calibration curve wells = standard DNA in 50 ul TE:

- *0 , 0.5, 2, 5 ng of Standard DNA, each 2-times, in 50 ul TE buffer each*

Prepare 50 ul of diluted Pico-green dye for every sample: 100 x 50ul = 5000 ul TE + 25 ul of PicoGreen dye

- *Pipette 50 ul of diluted Pico-Green dye into each well (samples and calibration)*

Read plate at Excitation 485nm, Emission 535nm (with our reader I use Auto GAIN and 20 pulses per sample)

2. *Haplotagging Beads: Aliquot the Haplotagging beads into PCR –strips*

2 seconds pulse-spin the stock haplo-beads strips, mix gently with multichannel pipette and 200ul tips till completely re-suspended. Adjust the pipette volume and transfer the desired volume of beads (1.25 ul worth of haplotagging beads) into a 8-well-pcr-strip. Keep on ice.

Alternative aliqouting: put the stock haplo-beads strip on the magnet for only 1-4 seconds so the beads move up if stuck on the bottom. Then keep inverting gently the stock-strip till the beads are completely re-suspended. Then shake down, or 1-second pulse spin in a small table-top centrifuge (beads must not sediment at all), and transfer the desired amount of beads into the prepared labeled pcr-strips. Make sure no liquid/beads are left in the tips.

3. *Tagmentation Mix:*

Prepare Tagmentation MIX in a 96-well plate

Per sample: 38 ul **WASH buff**er + 12ul **5xTAPS-Mg-DMF** buffer + 10 ul **DNA** (0.075ng/ul)

for 100 samples: **4 ml WASH buffer** (100 samples x 38 ul) + **1.2 ml 5xTAPS-Mg-DMF** buffer (100 sample x 12ul)

- Aliquot 50 ul of the into each well
- With wide-orifice pipette transfer 10 ul of DNA (0.075 ng/ul) into each well

Pre-heat cycler to 55°C

4. DNA-tagmentation

- **Put strips on magnet and remove the dialysis buffer** from the beads (I usually just do several strips at a time, to keep the incubation time constant and to not get overwhelmed).

Take the beads off of the magnet and:

- **Transfer all the tagmentation MIX** with wide-orifice tips onto the haplotagging beads in strips (set the pipette to > 75ul to ensure transferring all the mix onto the beads), and pipette thoroughly but gently! up-and-down to fully re-suspend the beads with DNA (5-6 times). Close with lids and mix gently by inverting several more times.
- **Incubate the tagmentation reaction at 55°C for 10 minutes**.
- Mix by inverting the strips after 5 min incubation to re-suspend the beads during tagmentation one more time.

5. STOP the tagmentation and strip the Tn5 from DNA

- 1-second pulse spin the strips after tagmentation to collect all liquid stuck at the lids
- **add 30 ul of STRIPPING buffer** (WASH buffer with 0.6% SDS) in each well with multichannel pipette
- Mix by inverting the strips 5-times or until fully re-suspended
- **Incubate at 55°C for 10 minutes**
- 3-seconds pulse spin the strips and put on magnet
- Aspirate/remove all the stripping buffer from the wells
- Add 100 ul of WASH buffer into each well, mix by gently inverting the tubes 5-times. Pulse spin down.

Beads are now ready for sub-sampling before the PCR. Possible stopping point. Keep samples at 4C.

I consider 1-plex scenario being 5 ul haplotagging beads and 3 ng DNA input.

*1-plex : **5 ul beads + 3 ng DNA** => 3 ng DNA = 1000 human genomes (=> max approx. 1000 sequencing coverage)*
(HiSeq3000 output per lane = 100 GB => approx. 30 sequencing coverage of human 1-plex => only every 30th out of 1000 reads/molecules covering any position is sequenced)

To make 50-plex look like 1-plex: take 1/50th of each samples' beads after tagmentation into PCR:
Then 50-plex will again be 5 ul beads + 3 ng DNA = 1000 human genomes => 1000/50 = 20 human genomes per sample in a human 50-plex (ore equals to approx. 120 butterfly genomes)

This protocol uses 1/4th of beads and 1/4th the amount of DNA already (so the 50-plex is already a 12.5-plex by using less beads and DNA):
1.25 ul beads + 0.75 ng DNA = already equals to 1/4th -plex = 250 human genomes per sample
if 50 samples to look like 1-plex : take 1/12th of each samples beads => 20 human genomes per sample of a 50-plex (or 120 butterfly genomes)

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6. Sub-sampling of beads:

SUB-SAMPLING of beads for the PCR

After tagmentation, each samples has 1.25 ul of beads that carry 0.75 ng of tagmented and barcoded DNA in 100 ul of WASH buffer.

TIP: If beads settled to the bottom, put on magnet for 5 seconds, then mix by inverting till re-suspended.

- Mix one strip at a time, to fully re-suspend the beads and immediately transfer with multichannel pipette the calculated proportion of beads from each strip (e.g. 1/12th of 100 ul = 8.3 ul) **into a single 8-well-pcr-strip**.

(One can also do another pooling afterwards, and pool 4 + 4 of the single 8 wells-strip to make 4 final bead pools instead of 8. By pooling 1/12th of beads from each row of the 96 samples into one well, you end up with one 8-well-pcr-strip, where each well has total of 1.25 ul beads pooled from 12 wells, and thus 8 x 1.25 ul beads = 10 ul haplo-beads => ***96 samples converted into a 2-plex****.)*

7. ***Exonuclease treatment*** *of the beads to remove un-integrated barcoded oligos/transposons from the bead surface.*

Prepare exonuclease mix for 9 samples:

Per 1 sample add: 25 ul H2O + 3 ul 10x Lambda Exonuclease buffer + 1 ul Lambda Exonuclease (NEB, M0262) + 0.8 ul Exonuclease 1 (NEB, M0293)

- On magnet, remove WASH buffer from the 8-well-PCR strip
- Pipette 30 ul of mix in each well
- Incubate 20 min at 37°C
- Add 15 ul Stripping buffer (WASH buffer+0.6% SDS) and gently mix by inverting, incubate 2 min at RT
- Spin down 3 seconds, and on magnet remove all liquid and add 150 ul WASH buffer, mix by inverting, incubate 2 min at RT
- Keep in WASH buffer until PCR mix is ready

8. ***PCR amplification of libraries***

50 ul PCR mix per sample: 25 ul NEBNext® High-Fidelity 2X PCR Master Mix (NEB, M0541)
23 ul H2O
2 ul TruSeq F+R primer mix (20 uM each)

NOTE: can use other High-Fidelity PCR mixes/Polymerases, but ***never use*** *a hot-start polymerase!*

1.	**10 min**	**72 °C**	*crucial step!*
	Mix by inverting the tube at minute 5 of incubation and at the very end to re-suspend the beads before the 10 PCR cycles		
2.	30 sec	98 °C	
3.	20 sec	98 °C	
4.	30 sec	65 °C	
5.	1 min	72 °C	
	Repeat 3-5 another 9-times => 10 PCR cycles total		
6.	3 min	72 °C	
7.	Forever	4 °C	

After PCR keep on ice, transfer 10 ul of PCR into new strip to run 1.5-2% agarose gel electrophoresis.

NOTE: Use narrow wells (e.g. ~3mm width) and very little amount of ladder, e.g. max of 50 ng of ladder/well (GeneRuler DNA Ladder Mix), less if using ladder that has all bands shorter than 3kb.

Run for 15 minutes at 120V (20 cm electrode distance) and image the gel. It is best not to run for too long for initial assessment, as the intensity of library fades as it stretches over longer distance if run for long.

If successful and comparable amounts of library are visible among the 8(4) samples on gel, place PCR reaction on magnet and pool all wells into **one single 1.5 ml eppi tube.**

9. Size-selection of library pool

Do 300-800 bp size selection of the library *(use 0.45x -> 0.85x PCR volume of size selection buffer/beads)*

- *Measure the volume of the PCR and use 0.45x the volume of size selection beads (binds ~800+ bp DNA only), mix well and incubate at RT for 5 min. (If the volume is 320 ul, add 320x0.45=144 ul of size selection beads)*
- *Spin down, put on magnet and **transfer the supernatant** (no beads) **into a new tube**, which either already has fresh 128 ul of size-selection beads in it to bind the final size-selected library; or the 128 ul of size-selection beads are added afterwards.*
  (the bottom size selection to bind 300+ bp library, is done with 0.85x PCR volume of size-selection buffer/beads, so 320 ul PCR x 0.85 = 272 ul beads buffer, but 144 is already there from the upper size selection, so 272 - 144 = 128 ul size selection beads)
- *Incubate at RT for 10-15 minutes, with occasional mixing of beads*
- *Pulse spin down 3 seconds and put on magnet*
- *Remove supernatant*
- *Add 150 ul 80% EtOH, buffered with 10 mM Tris, pH=8, incubate 1 min RT*
- *Remove EtOH wash and repeat the EtOH wash 1 more time*
- *Remove all the EtOH wash and let evaporate open on magnet for 2 minutes*
- *Add 52 ul of 10mM Tris, pH=8 to the bead, mix well and incubate at RT for 5 min*
- *Measure concentration in 2 ul with QuBit. (ideally , it should be more than 3 ng/ul if a 2-plex done => 10ul haplotag beads into PCR in total)*
- *Do final size-selection bead clean-up to remove any leftover primers, with 0.85x size selection*

if 50 ul total library, then add 42.5 ul of size selection beads

- *Mix well and incubate at RT for 10 min, with occasional mixing*
- *Spin and put on magnet and do 2-times 80% EtOH wash as before.*
- *Elute in volume of 10mM Tris that would give at least 2 ng/ul, when expecting 10-20% loss during final clean-up compared to the previous Qubit measurement.*
- *Adjust all or part of library to 1.3 ng/ul for HiSeq sequencing (2.5 nM)*

BUFFERS and REAGENTS

Size-selection Magnetic-BEADS:

Stock Beads = *15ml, SpeedBeads™(GE65152105050250 SIGMA) from Sigma or GElifesciences Sera-Mag SpeedBead (65152105050250)*

on magnetic stand, add 1 ml of the Stock Beads in an 1.5 ml Eppendorf tube

after 1 min remove the solution and wash beads with 1 ml of 10mM Tris-HCl, pH=8

on magnet, remove Tris buffer and use some of the Bead Buffer to transfer and re-suspend the beads into the rest 50 ml of Bead Buffer.

Beads are ready to use, store at 4C, use at RT.

Magnetic-BEAD BUFFER:

for 50 ml of Bead buffer, dissolve in 35 ml of mol.biol. grade H_2O:

9 g PEG8000 (Promega, V3011)
7.3 g NaCl
500 µl 1M Tris-HCl, pH=8.0
100 µl 0.5M EDTA, pH=8.0

When all PEG/NaCl dissolves, *add mol.biol. grade H_2O to 50 m*

Note: (warm bath helps with dissolving; also you will probably end up with lots of tiny bubbles that will clear up in some time)

Tagmentation buffer, 5x TAPS-Mg-DMF (contains: 50mM TAPS, 25mM MgCl2, 50% DMF),

DMF = N,N-Dimethylformamide

TAPS = *T5130 SIGMA*

1M $MgCl_2$ = e.q. SERVA ,1 M Magnesium chloride, 38772.01

1M TAPS STOCK (50 ml):

1M TAPS in H_2O, adjust to pH:8.5 with 10M NaOH (for 25 ml of 1M TAPS you will need ca 5 ml of 3M NaOH)

⇨ 0.2/0.45 um Filter sterilize the 1M TAPS

To prepare 10 ml of 5 x 5x TAPS-Mg-DMF buffer mix on ice in same order:

4.25 ml H_2O + 500 µl 1M TAPS (pH=8.5) + 250 µl 1M $MgCl_2$ + 5 ml DMF

Note: Buffer becomes warm after adding DMF (keep in ice)

Aliquot in 1.5 ml tubes, store at 4C (for short time, 1-2 months), for longer storage at -20C (buffer doesn't freeze)

Dialysis / Tn5 storage buffer

50 mM HEPES-KOH pH 7.2
0.2 M NaCl
0.2 mM EDTA
0.2 % Triton X-100
20 % glycerol

WASH buffer:

10 mM Tris,pH=8,
30 mM NaCl,
0.1 % Triton X-100

STRIPPING buffer (WASH+0.6% SDS)

WASH buffer + 0.6% SDS (from stock 20% SDS in H2O)