**Phosphorylate the FORWARD as follow:**

Forward oligo (100uM)   10 ul

PNK 10x buff                  10 ul

ATP 10mM                      10ul

PNK enzym                     2 ul

Water (Nuclease free)     58 ul

\* We're using PNK from NEB.

Incubate @ 37, for 1 hour

Then add 10 ul of Reverse oligo (100uM) into the mix and anneal as follow in a thermocycler:

- In a PCR thermoblock, incubate all samples 5 min @ 95 °C, then ramp down to 25 °C by decreasing the temperature of 1.6 °C every minute (approx. 45 min will be needed to reach 25 °C)

- Store annealed oligos @ -20 °C

**If you do not want to phosphorylate, anneal the oligos as follow:**

Forward oligo (100uM)   10 ul

Reverse oligo (100uM)   10 ul

NEB buffer 2                  10 ul

Water (Nuclease free)     70 ul

- In a PCR thermoblock, incubate all samples 5 min @ 95 °C, then ramp down to 25 °C by decreasing the temperature of 1.6 °C every minute (approx. 45 min will be needed to reach 25 °C)

- Store annealed oligos @ -20 °C

**Cell lysis**

* 1. Exchange PBS to 1st Lysis buffer

Tris-HCl 10mM

NaCl 10mM

EDTA 1mM

Triton X-100 0.2%

pH 8 at 4°C

* 1. Incubate 1 h @ +4 °C
  2. Exchange to 1x PBS @ rt
  3. Incubate 5 min @ rt

Note: PBS wash- it is important that samples reach RT, otherwise SDS would precipitate

* 1. Exchange to 2nd Lysis buffer pre-warmed @ 37 °C

Tris-HCl 10mM,

NaCl 150mM

EDTA 1mM

SDS 0.3%

pH 8 at 25°C

* 1. Incubate 1 h in an incubator @ 37 °C
  2. Exchange to 1x PBS @ rt
  3. Incubate 1 min @ rt
  4. Repeat wash in 1x PBS

**DSB in situ blunting**

* 1. Exchange to 1x CutSmart buffer
  2. Incubate 2 min @ rt
  3. Repeat twice equilibration in 1x CutSmart buffer

* 1. Exchange to blunting mix:

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  | | --- | --- | |  | **1x** | | Nuclease-free water | 75 ul | | Blunting buffer 10x\* | 10 ul | | BSA 10 mg/ml\* | 1 ul | | dNTPs 1 mM\* | 10 ul | | Blunting enzyme mix\* | 4 ul | | **Total** | **100 ul** | |

\*Components included in Quick Blunting™ Kit

* 1. Incubate 1h@ rt

**DSB in situ ligation**

* 1. Exchange to 1x CutSmart buffer + 0.1% Tx100
  2. Incubate 2 min @ rt
  3. Repeat twice wash in 1x CutSmart buffer + 0.1% Tx100
  4. Exchange to 1x T4 Ligase buffer ….not needed
  5. Incubate 5 min @ rt
  6. Exchange to ligation mix:

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  | | --- | --- | | **Reganets** | **1x** | | Nuclease-free water | 74 ul | | T4 ligase buffer 10x (Thermo) | 10 ul | | ATP 10 mM (NEB) | 8 ul | | BSA 50 mg/ml  (Ambion) | 2 ul | | **BLISS linker @ 10uM** | 4 ul | | T4 ligase highly conc. 1000U/ul(Thermo) | 2 ul | | **Total** | **100** | |

Note: Make a mix without linkers, aliquoted to 96 ul and add 4 ul of corresponding linkers.

Incubate 16–18h in an incubator @ 16 °C

**Washing away the un-ligated linkers**

* 1. Wash 5x 1h @ 37°C in 1x W&B buffer

NaCl 2M

Tris-HCl 10mM

EDTA 2mM

Igepal 0.2%

pH 8

@ 25°C

**Scraping using cell scraper or razor/blade**

* 1. Wash 2x with 1xPBS
  2. Wash 3x in H2O
  3. Dry the cells
  4. Scrape the cells using scraper on a Parafilm and add 100 µl of TAIL Buffer and 10 µl Proteinase K on top of the coverslips

Ninsert to the 1,5ml DNA-low binding tube,

o Do

* 1. Incubate the tubes at 55°C for at least 5h or O/N, at 800rpm Overnight

* 1. Heat the tubes at 95°C-98°C (Use the thermo shaker) for 10 minutes then immediately put them on ice

Note: Proteinase K has to be inactivated! Make sure that the temperature is above 95°C. Incomplete inactivation of Proteinase K can cause problems in the PCR reaction

* 1. Vortex hard for 10-15 seconds. Spin down briefly. The DNA is ready.

* 1. Do not place on ice if you used TAIL buffer as the samples get frozen

**DNA Purification**

**Note: Genomic DNA purification by phenol-chloroform with 100% EthoH.**

There are two layers in the bottle, The **lower phase** is the phase that is to be used.

* 1. Add equal volume of phenol/chloroform from the lower layer of solution without mixing the two phases(here ul) and shake vigorously

* 1. Centrifuge at ~30000 x g, 10’, RT

Note: increase the centrifugation time until you see a good separation

* 1. Collect the upper phase (xx ul) and transfer to the new low bind tube and add an equal volume of chloroform to it and shake vigorously

* 1. Centrifuge at ~30000 x g, 10’, RT

Note: increase the centrifugation time until you see a good separation

* 1. Collect the upper phase (xx ul), transfer to the new low bind tube, add 1/10 of volume sodium acetate
  2. add xxul (normally around 3-4 ul) of Glycogen (20mg/ml stock) to 0.5 ug/ul final conc
  3. Add 2.5x volume of fresh 100% Ethanol
  4. and put at -20C for O/N

* 1. Centrifuge at ~30000 x g, **30 min to 1h**, 4C

Note: Turn on the Covaris sonicator, and start de-gassing for at least 30 before using it. If you're using Bioruptor to shear the DNA, turn on the bioruptor and its water coolant now.

* 1. Wash the pellet with 600ul of 70% EtOH (from -20C)

* 1. Centrifuge at ~30,000 x g, 10’, 4C
  2. Wash the pellet with 600ul of 70% EtOH (from -20C)

* 1. Centrifuge at ~30,00x g, 10’, 4C

* 1. Discard the supernatant and dry the pellet as below:

- 10 min upside-down on a Kimwipe in the petridish, not fully covered by the lid, just to protect from dust under the hood

* 1. Resuspend the dried pellet in 50ul of nuclease free water

* 1. Put on shaker @55 for 30 min.
  2. Keep on ice for 15 min before DNA fragmentation
  3. Shear the DNA with Covaris with setting named Reza\_350bp\_confirmed for 6 cycles.
  + If Bioruptor is used, turn on the instrument and water coolant 20-30 min before sonication, use the setting 30s ON/90s OFF, 30 cycles.

o Do

* + Qubit measurement

Diluted to 10ng /ul for BioA

If the profile looks good Continue to Beads purification

**DNA cleanup by AMPure beads**

* 1. To each sample add 0.8x of the volume of AMPure bead suspension (warmed to RT), then mix thoroughly by pipetting up-down 5-6x

* 1. Incubate 5 min @ RT

* 1. Place samples on a magnetic stand, until they are clarified (here 5min)

* 1. Manually aspirate supernatant

* 1. Wash beads with 200 ul fresh 70% ethanol for 30 second

* 1. Manually aspirate supernatant

* 1. Repeat wash 1x

* 1. Manually aspirate supernatant

* 1. Air-dry beads 5 min @ RT

* 1. Remove samples from magnetic stand, and resuspend beads in 10.5 ul nuclease-free water (from that 1ul for bioanalyzer , 1ul for Qubit)

* 1. Incubate 5 min @ RT
  2. Put back on the magnet rack
  3. Incubate 5 min @ RT

* 1. Transfer 9.5ul of samples into 0.5 ml DNA low-bind tubes
  2. Use 1ul for Qubit, and 1ul for BioA check up and 7.5ul goes to IVT
  3. Note: Adjust the IVT reaction volume if you use more DNA impute volume.

**Normalising DNA input**

Normalize the DNA amount before IVT if you plan to pool samples together

**IVT set up:**

* 1. ON ICE: to each sample, containing 7.5ul of DNA add the following reagents:

o Do

|  |  |
| --- | --- |
| Nuclease free water | 1.5 ul |
| A+U+G+C\* | 7.5 ul |
| 10x T7 polymerase buffer | 2 ul |
| T7 polymerase **10x** Megascript mix | 1 ul |
| RiboBlock RNase inhibitor 40U/ul | 0.5 ul |

\*Prepared from separate rNTP solutions provided with the MessageAmp II kit

* 1. Incubate 14 h @ 37 °C in a thermocycler with lid set @ 70°C

**Degradation of DNA Template in a Transcription Reaction** (adapted from Life Tech manual for Dnase I enzyme)

* 1. After transcription, add 2 U/ul (**1ul**) of DNase I (RNase free) to a 20 ul transcription reaction

Dnase I (Rnase-free)

Product No. AM2222, Lot No. 00299659, Life Technologies

* 1. Incubate at 37°C for 15min

**aRNA cleanup**

* 1. Transfer to 1.5ml low-bind tubes, bring volume up to 30ul or 50ul (depends on IVT volume), by Nuclease-free water

* 1. Prewarm RNAClean XP beads to room temperature

* 1. Gently pipette up and down/flick RNAClean XP beads until well dispersed, add to sample 1.8x of volume

68.Incubate at room temperature for 10 min

* 1. Place on magnetic stand for at least 5 min, until liquid appears clear

* 1. Remove and discard the supernatant

* 1. Add 200-1000 ul (depends on the reaction volume) of freshly prepared 70% EtOH

* 1. Incubate at least 30 seconds, then remove and discard supernatant without disturbing beads

* 1. Repeat wash two more times

* 1. Air dry beads for 10 min, or until completely dry

* 1. Resuspend in 8μl water. Pipette entire volume up and down ten times to mix thoroughly

* 1. Incubate at room temperature for 5 min

* 1. Place on magnetic stand for 5 min, until liquid appears clear

* 1. Transfer 7ul of supernatant to new 0.5ml DNA low-bind tube
  2. 1ul can be kept for Qubit/ BioA and
  3. **6ul** goes to ligation

**3’ Illumina adapter ligation**

Based on the Small RNA kit (Illumina) protocol

* 1. ON ICE: dilute RA3 adapter 5x dilution 1:5 in nuclease-free water

* 1. ON ICE: to each sample add **1ul** diluted RA3

* 1. In a thermocycler, incubate 2 min @ 70 °C then immediately place the samples on ice

* 1. Add **4ul** of nuclease free water

* 1. ON ICE: to each sample add 4 ul of the following mix

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  | | --- | --- | | RNA Ligase buffer (NEB) | 2 ul | | RNaseOUT (Invitrogen) | 1 ul | | T4 RNA Ligase truncated (NEB) | 1 ul | |

* 1. Incubate 1 h @ 28 °C in a thermocycler

* + Take out dNTPs (diluted and concentrated), RTP, DTT, 5x FS buffer, 30min before the incubation is done.

Breakpoint: if needed, at this point samples can be stored @ -20 °C

**Reverse transcription (1st strand synthesis)**

Based on the Small RNA kit (Illumina) protocol

* + ON ICE: dilute dNTPs @ 25 mM (Illumina) 2x dilution 1:2 in nuclease-free water

* 1. ON ICE: to each sample add 4 ul RTP

* 1. In a thermocycler, incubate 2 min @ 70 °C, then immediately place samples on ice
  2. ON ICE: to each sample add 11 ul of the following mix:

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  | | --- | --- | |  | 1x | | 1st strand buffer (Invitrogen) | 4 ul | | Diluted dNTPs | 1 ul | | 100 mM DTT (Invitrogen | 2 ul | | RNaseOUT (Invitrogen) | 2 ul | | SuperScriptIV(Thermo 200 U/µl) | 2 ul | |

* 1. Incubate in a thermocycler with lid set @ 50 °C:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| o Do | |  |  | | --- | --- | | 50 °C | 60 min | | 4 °C | Hold | |

**Library indexing and amplification**

Based on the Small RNA kit (Illumina) protocol

* 1. Transfer samples into 200 ul PCR tubes
  2. ON ICE: to each sample add **2 ul** of the desired indexed Illumina primer

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  |  | | --- | --- | --- | | Samples | indexes |  | | sample | # from idt |  | | sample | #from idt |  | |

* 1. ON ICE: to each sample add **68ul** of the following mix:

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  | | --- | --- | |  | **1x** | | Nuclease-free water | 16 ul | | NEBNext master mix 2x (NEB) | 50 ul | | RP1 primer | 2 ul | |

* + Note: RP1 is general for all of the reactions

* + In a thermocycler perform the following cycles:

Note: Number of PCR cycles depends on your DNA and IVT inputs

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| o Do | |  |  |  | | --- | --- | --- | | 1 | 98 °C | 30 sec | | 2 | 98 °C | 10 sec | | 3 | 60 °C | 30 sec | | 4 | 72 °C | 30 sec | | Cycles 12-16x |  |  | | 5 | 72 °C | 10 min | | 6 | 4 °C | Hold | |

Note: Take out AMPure beads suspension from +4°C at least 30 min before proceed, to reach RT, vortex

Note: Take out reagents for bioanalyzer

**Library purification**

* 1. Transfer samples into 1.5 ml DNA low-bind tubes

* 1. To each sample add 80ul AMPure bead (0.8x ratio) suspension (warm to RT), then mix thoroughly by pipetting up-down 5-6x

* 1. Incubate 5 min @ RT

* 1. Place samples on a magnetic stand, until they clarified

* 1. Manually aspirate supernatant

* 1. Wash beads with 200ul ice-cold 70% ethanol 30s

* 1. Manually aspirate supernatant

* 1. Repeat wash 1x

* 1. Manually aspirate supernatant

* 1. Air-dry beads 5 min @ RT

* 1. Remove samples from magnetic stand, and re-suspend

beads in 15ul nuclease-free water

* 1. Incubate 5 min @ RT

* 1. Put back on magnet for 5 min

* 1. Transfer 14 ul of supernatant into 1.5 ml DNA low-bind tubes

Qubit: