GPSeq reveals the radial organization of chromatin in the cell nucleus

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Method Article

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Abstract

With the exception of lamina-associated domains, the radial organization of chromatin in mammalian cells remains largely unexplored. Here, we describe genomic loci positioning by sequencing (GPSeq), a genome-wide method for inferring distances to the nuclear lamina all along the nuclear radius that works by gradual enzymatic restriction of chromatin from the nuclear lamina towards the nucleus center, followed by sequencing of the generated cut sites. Using GPSeq, we mapped the radial organization of the human genome at 100 kb resolution, which revealed radial patterns of genomic and epigenomic features, gene expression, as well as A/B subcompartments. By combining radial information with chromosome contact frequencies measured by Hi-C, we substantially improved the accuracy of whole-genome structure modeling. Finally, we charted the radial topography of DNA double-strand breaks, germline variants and cancer mutations, and found that they have distinctive radial arrangements in A/B subcompartments. We conclude that GPSeq can reveal fundamental aspects of genome architecture.

Introduction

In eukaryotic cells, the genome is spatially organized and its three-dimentional (3D) architecture is vital to the proper execution of its functions[1]. One key feature of the 3D genome is that individual chromosomes are non-randomly positioned with respect to the nuclear periphery[2–9]. The nuclear lamina is thought to be the key organizer of the radial arrangement of chromatin in interphase nuclei[10], by creating a large nuclear compartment where the majority of inactive chromatin clusters in the form of lamina-associated domains (LADs)[11–13]. Specialized sub-chromosomal regions, such as centromeres and telomeres, as well as nucleolar organizing regions, are also non-randomly positioned in the nucleus[14–18]. The latter contain ribosomal RNA gene clusters that coalesce to form the core of the largest nuclear body, the nucleolus, and organize chromatin in and around them[19]. Furthermore, inter-chromosomal interactions around the nucleolus and nuclear speckles have also been implicated in shaping the 3D genome[20].

The preferential radial location of certain genomic loci in the nucleus has been variably attributed to gene density[3,5,6], GC-content[21–23], as well as chromosome size[4,7,8,24]. Additionally, transcriptional activity has also been implicated in radial nuclear organization, although it is still unclear whether transcription influences radiality or *vice versa*[25–35]. Overall, the role of genomic and epigenomic features in shaping radiality remains to be quantified, despite several attempts to model the contribution of various factors[35–37]. In particular, it is unclear whether the nucleus merely consists of a peripheral transcriptionally inactive compartment as opposed to a central transcriptionally active one, or whether a finer stratification exists. In this context, a major obstacle until now has been the lack of dedicated genome-wide methods to specifically tackle this aspect of chromatin organization at high resolution. To overcome this limitation, here we develop a method that allows inferring radial locations genome-wide, all along the nuclear radius, which we name genomic loci positioning by sequencing or GPSeq. Using GPSeq, we generate the first high-resolution map of radial chromatin organization in human cells, which reveals a clear tendency of individual genomic regions to occupy specific radial locations, as well as

gradients of chromatin modifications, transcriptional activity and replication timing, and a marked polar arrangement of chromosomes with respect to A/B compartments and subcompartments[38,39]. We develop a high-performance algorithm, *chromflock*, that dramatically improves the accuracy of whole-genome structure ensemble generation. Finally, we integrate GPSeq maps with DNA breaks and mutations data, revealing radial differences in DNA damage and mutational processes.

Reagents

· GPSeq adapter sequences are provided in **Supplementary Table 1**

· Poly-L-Lysine (PLL) solution (Sigma, cat. no. P8920-100 ml)

· Methanol-free paraformaldehyde (PFA) 16% (EMS, cat. no. 15710)

· Glycine (Sigma-Aldrich, cat. no. 50046-250G)

· Nuclease-free Phosphate-Buffered Saline (10X) pH 7.4 (Thermo Fisher Scientific, cat. no. AM9625)

· Nuclease-free water (Thermo Fisher Scientific, cat. no. 4387936) (for *in situ* reactions and library preparation)

· CutSmart® buffer (NEB, cat. no. B7204S)

· HindIII-HF® (NEB, cat. no. R3104)

· MboI (NEB, cat. no. R0147M)

· Calf intestinal alkaline phosphatase (Promega, cat. no. M1821)

· T4 DNA Ligase (NEB, cat. no. M0202M)

· UltraPure™ BSA (50 mg/ml) (Thermo Fisher Scientific, cat. no. AM2616)

· Triton® X-100 (Promega, cat.no. H5142)

· High-salt wash buffer (HSW): 10 mM Tris-HCl, 1M NaCl, 0.5% Triton X-100, pH 8 at 25 °C

· Dextran sulfate (Sigma, cat. no. D8906)

· Formamide (FA) (Ambion, cat. no. AM9342)

· *E.coli* tRNA (Sigma, cat. no. R1753)

· BSA (Ambion, cat. no. AM2616)

· YFISH wash buffer (YWB): 25% FA/2X SSC

· YFISH hybridization buffer (YHB): 10% Dextran sulfate/25% FA/1 mg/ml *E.coli* tRNA /0.02 % BSA/2X SSC

· Hoechst 33342 (Thermo Fisher Scientific, cat. no. H3570)

· DNA extraction buffer (DEB): 1% SDS, 100 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.5 at 25 °C

· Proteinase K, Molecular Biology Grade (NEB, cat. no. P8107S)

· Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM (Sigma-Aldrich, cat. no. P2069)

· Chloroform (Sigma-Aldrich, cat. no. C2432)

· EDTA (Sigma-Aldrich, cat. no. P2069)

· Glycogen (Sigma-Aldrich, cat. no. 10901393001)

· Nuclease-free TE buffer (Thermo Fisher Scientific, cat. no. AM9849)

· AMPure XP (Beckman Coulter, cat. no. A63881)

· MEGAscript® T7 Transcription Kit (Thermo Fisher Scientific, cat. no. AM1334)

· RiboSafe RNase Inhibitor (Bioline, cat. no. 65027)

· DNase I, RNase-free (Thermo Fisher Scientific, cat. no. AM2222)

· RA3 adaptor and RTP, RP1 and RPI primers are the same as in the TruSeq Small RNA Library Preparation kit (Illumina, cat. no. RS-200-0012), but are custom-synthesized and purified using standard desalting

· RNAClean XP (Beckman Coulter, cat. no. A63987)

· RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, cat. no. 10777-019)

· T4 RNA ligase 2, truncated (NEB, cat. no. M0242L)

· Deoxynucleotide (dNTP) Solution Set (NEB, cat. no. N0446S)

· SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific, cat. no. 18090050)

· NEBNext® High-Fidelity 2X PCR Master Mix (NEB, cat. no. M0541L)

Equipment

· Incubator (*e.g.*, Binder incubator, Model KB 53)

- Tabletop centrifuge (*e.g.*, Eppendorf® Microcentrifuge 5424)
- Thermoshaker (*e.g.*, Eppendorf® Thermomixer Compact)
- PCR cycler (*e.g.*, T3 Thermocycler, Biometra)
- Cell scrapers (Corning™ Cell Lifter, cat. no. 3008)
- Eppendorf® DNA LoBind microcentrifuge tubes (cat. no. 0030108035 and 0030108051)
- Sonication device (Bioruptor® Plus, Diagenode, cat. no. B01020001)
- DynaMag™-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)
- Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, cat. no. Q32866)
- Qubit™ dsDNA HS Assay (Thermo Fisher Scientific, cat. no. Q32851)
- Qubit™ Assay Tubes (Thermo Fisher Scientific, cat. no. Q32856)
- Bioanalyzer 2100 (Agilent, cat. no. G2943CA)
- Agilent RNA 6000 Pico Kit (Agilent, cat. no. 5067-1513)
- Agilent High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626)

Procedure

Cell preparation

Note: a separate set of samples (coverslips) should be prepared for visualization of gradual genome restriction by YFISH and for sequencing. The two sets of coverslips should be processed in parallel.

<u>For adherent cells</u>:

1. Wash the cells with 1X PBS containing Ca^{2+} and Mg^{2+} pre-warmed to 37 °C
2. Incubate for 2 min at room temperature (RT)
3. Aspirate the PBS solution from the dish
4. Add 0.4X PBS with Ca^{2+} and Mg^{2+} at RT
5. Incubate for 1 min at RT

6. Aspirate the PBS solution
7. Add 0.4X PBS/4% PFA at RT
8. Incubate for 10 min at RT
9. Aspirate the fixative solution from the dish
10. Quench the residual PFA by adding 1X PBS/125 mM glycine
11. Incubate for 5 min at RT
12. Briefly wash the cells in 1X PBS at RT
13. Wash the cells in 1X PBS/0.05% Triton X-100 three times, 5 min each at RT, while shaking
14. Aspirate the PBS solution and then add 1X PBS/0.5% Triton X-100 at RT
15. Incubate for 20 min at RT
16. Briefly wash the cells in 1X PBS at RT
17. Briefly wash the cells in 1X PBS/20% glycerol at RT
18. Incubate the samples in 1X PBS/20% glycerol for 18 h at RT
19. Fill a cryo-resistant Dewar flask with liquid N_2
20. **Remove the glycerol solution from the dish (leave a thin layer on top of the cells)**
21. **Place the dish on the surface of liquid N_2** for 20–30 sec
22. Place the dish at RT and let the samples thaw gradually, making sure that they do not dry
23. Once all the samples are thawed, fill the dish with fresh 1X PBS/20% glycerol at RT
24. Incubate for 3 min at RT
25. Repeat steps 20–23 four times
26. Wash the samples in 1X PBS/0.05% Triton X-100 two times, 5 min each at RT, while shaking
27. Briefly rinse the samples in 0.1 M HCl at RT
28. Incubate the samples in 0.1 M HCl for 5 min at RT

Note: the samples should be kept in HCl for a maximum of 5 min including the quick rinsing step below

29. Briefly rinse the samples in 1X PBS/0.05% Triton X-100 at RT

30. Wash the samples in 1X PBS/0.05% Triton X-100 two times, 5 min each at RT, while shaking

31. Wash the samples for 5 min in 2X SSC

32. Replace the solution to fresh 2X SSC and process the samples immediately or alternatively store them in 2X SSC/0.05% NaN_3 for up to 1 month at 4 °C

For suspension cells:

1. Place coverslips in a 10 cm dish, cover them with 5 ml of the PLL solution, and then gently shake the dishes for 15 min at RT AT WHICH TEMPERATURE?

Note: make sure that coverslips stay covered by liquid

2. Aspirate the solution and transfer it into a 15 ml tube

Note: the solution can be stored at 4 °C and used up to three times (STORED AT WHICH TEMPERATURE?)

3. Wash the coverslips three times with 1X PBS at RT and once with ethanol (EtOH) 70%

4. Air-dry the coverslips and proceed with cell spotting and fixation

Breakpoint: if not used immediately, coverslips can be stored in EtOH 70% at 4 °C and air-dried just before use.

5. Place the desired number of PLL-coated coverslips into a 6-well cell culture plate

6. In each well, dispense a cell suspension freshly prepared in 1X PBS with Ca^{2+} and Mg^{2+}

Note: we usually spot up to 5×10^5 cells onto a 22x22 mm coverslip

7. Let the cells sediment onto the coverslip for 10 min at RT

8. Slowly add 0.4X PBS with Ca^{2+} and Mg^{2+} at RT

9. Incubate for 1 min at RT

10. Aspirate the PBS solution

11. Slowly add 0.4X PBS/4% PFA at RT

12. Incubate for 10 min at RT

13. Aspirate the fixative solution from the dish

14. Quench the residual PFA by adding 1X PBS/125 mM glycine

15. Briefly wash the cells in 1X PBS at RT

16. Wash the cells in 1X PBS/0.05% Triton X-100 three times, 5 min each at RT, while shaking

17. Aspirate the PBS solution and then add 1X PBS/0.5% Triton X-100 at RT

18. Incubate for 20 min at RT

19. Briefly wash the cells in 1X PBS at RT

20. Briefly wash the cells in 1X PBS/20% glycerol at RT

21. Incubate the samples in 1X PBS/20% glycerol for 18 h at RT

22. Fill a cryo-resistant Dewar flask with liquid N_2

23. Remove the glycerol solution from the dish (leave a thin layer on top of the cells)

24. Place the dish on the surface of liquid N_2 for 20–30 sec

25. Place the dish at RT and let the samples thaw gradually, making sure that they do not dry

26. Once all the samples are thawed, fill the dish with fresh 1X PBS/20% glycerol at RT

27. Incubate for 3 min at RT

28. Repeat steps 20–23 four times

29. Wash the samples in 1X PBS/0.05% Triton X-100 two times, 5 min each at RT, while shaking

30. Briefly rinse the samples in 0.1 M HCl at RT

31. Incubate the samples in 0.1 M HCl for 5 min at RT

Note: the samples should be kept in HCl for a maximum of 5 min including the quick rinsing step below

32. Briefly rinse the samples in 1X PBS/0.05% Triton X-100 at RT

33. Wash the samples in 1X PBS/0.05% Triton X-100 two times, 5 min each at RT, while shaking

34. Wash the samples for 5 min in 2X SSC

35. Replace the solution to fresh 2X SSC and process the samples immediately or alternatively store them in 2X SSC/0.05% NaN_3 for up to 1 month at 4 °C

In situ digestion

1. Bring the 2X SSC/50 mM phosphate buffer/50% FA (FPS) solution to RT
2. Rinse the samples with FPS at RT
3. Exchange to fresh FPS and store at RT protected from light for 20 h
4. Wash the samples in 2X SSC for 5 min at RT
5. Wash the samples in 1X CutSmart, two times, 5 min each at RT, while gently rocking
6. Prepare the following digestion mix (volumes for one 22x22 mm coverslip):

· CutSmart (10X) 40 µl

· HindIII-HF (20,000 U/ml) 10 µl

· Nuclease-free water Up to 400 µl

Alternatively:

· CutSmart (10X) 40 µl

· MboI (25,000 U/ml) 8 µl

· Nuclease-free water Up to 400 µl

Note: The choice of enzyme depends on the desired resolution and affordable sequencing depth. As a rule of thumb, HindIII can be used to obtain highly reproducible radiality maps at 1 Mb resolution using five different digestion durations and one sequencing run on NextSeq 500. MboI is preferable when a higher resolution is needed.

7. Pre-warm the digestion mix for at least 10 min at 37 °C
8. Dispense the digestion mix on a piece of Parafilm placed inside a humidity chamber

Note: we make the chamber by covering the edges of a 15 cm dish with tissue paper pre-soaked in distilled water, and then laying a piece of parafilm on the dish bottom

9. Gently flip one sample at a time, onto a droplet of the digestion mix

10. Incubate each coverslip at 37 °C in the sealed humidity chamber, for a selected period of time

Note: each sample is incubated in the presence of the restriction enzyme for a different duration. HAP1 cells: for HindIII, we have used 10, 15, 30, 45 min, 1, 2, and 6 h, whereas, for MboI, we have used 1, 5, 10, 15, and 30 min, which were sufficient to produce reproducible radiality maps. GM06990 cells: for HindIII, we have used 10, 15, 30 min, and 2 h. For different cell types, it might be necessary to optimize the number and series of incubation times, by first monitoring the enzyme diffusion by YFISH (see below)

11. Stop the digestion by washing the samples with ice-cold 1X PBS/50 mM EDTA/0.01% Triton X-100 three times, 5 min each, on ice

12. Rinse the samples with 1X CutSmart/0.01 % Triton X-100 at RT

13. Wash with 1X CutSmart/0.01 % Triton X-100 for 5 min at RT

In situ dephosphorylation

1. **Wash the samples in 1X Alkaline phosphatase (AP) buffer for 5 min at RT**
2. **Prepare the following dephosphorylation mix (volumes for one 22x22 mm coverslip):**

· **AP buffer (10X)**	**40** µl
· AP (1 U/µl)	6 µl
· Nuclease-free water	354 µl

3. Dispense the dephosphorylation mix on a piece of Parafilm placed inside a humidity chamber
4. Gently flip one coverslip at a time, onto a droplet of the dephosphorylation mix
5. Incubate the samples for 2 h at 37 °C in the sealed humidity chamber
6. Transfer the samples back to a 6-well plate
7. Wash the samples with 1X CutSmart/0.01 % Triton X-100 two times, 5 min each at RT

In situ ligation

1. **Wash the samples in 1X T4 Ligase buffer two times, 5 min each at RT, while gently rocking**
2. **Prepare the following ligation mix I (volumes for one 22x22 mm coverslip):**

- **Ligase buffer (10X)** **21** µl
- ATP (10 mM) 24 µl
- BSA (5 mg/ml) 6 µl
- HindIII GPSeq adapter (10 µM) 6 µl
- Alternatively: MboI GPSeq adapter (10 µM) 18 µl
- Nuclease-free water Up to 210 µl

Note: thaw all reagents slowly on ice or at 4 °C

3. Dispense the ligation mix I on a piece of Parafilm placed inside a humidity chamber
4. Gently flip one sample at a time, onto the droplet of ligation mix I
5. Incubate the samples for 30 min at RT
6. Prepare the following ligation mix II (volumes for one 22x22 mm coverslip):

- Nuclease-free water 45 µl
- Ligase buffer (10X) 9 µl
- T4 Ligase enzyme 36 µl

7. Gently pipette the ligase mix II under each coverslip in and out 7–8 times, making sure not to make bubbles
8. Incubate the samples for 18 h at 16 °C in the sealed humidity chamber
9. In a new 6-well plate, wash the samples in 1X HSW five times, 1 hour each at 37 °C, while shaking
10. Wash the samples in 1X PBS three times, 5 min each at RT

Breakpoint: at this point, the samples for sequencing can be stored in 1X PBS at 4 °C for several days until the results of YFISH (next section) are available

YFISH

Note: we recommend to check that the genome has been gradually digested by the enzyme, from the nuclear periphery towards the nucleus interior, before proceeding to sequencing

1. Equilibrate the samples in 2X SSC/25% FA
2. Prepare the following hybridization mix (volumes for one 22x22 mm coverslip):

· YHB 297 µl

· Fluorescently labelled oligonucleotide (2 µM) 3 µl

3. Dispense the hybridization mix on a piece of Parafilm placed inside a humidity chamber
4. Gently flip one sample at a time, onto the droplet of hybridization mix
5. Incubate the samples for 18 h at 30 °C inside the sealed humidity chamber

Note: the samples are now light sensitive, therefore all the subsequent steps should be performed by protecting them from light

6. Transfer the samples into a 6-well plate pre-filled with 2 ml/well of YWB pre-warmed at RT
7. Exchange the YWB solution and incubate for 1 h at 30 °C, while gently shaking
8. Remove the YWB solution and add 2 ml/well of YWB/0.1 ng/ml Hoechst 33342
9. Incubate for 30 min at 30 °C, while gently shaking
10. Quickly rinse the samples with 2X SSC
11. Mount the samples and image them using wield-field microscopy or, if available, STED microscopy

Library preparation

Note: for each of the restriction times used, an individual sequencing library is prepared

DNA Extraction

1. **Wash the samples with nuclease-free water three times, 2 min each at RT**
2. **Scrape each coverslip in the following DNA extraction mix:**

· **DEB** **100** µl

· Proteinase K (0.8 U/µl) 10 µl

3. Transfer each sample into a 1.5 ml Eppendorf® DNA LoBind tube
4. Incubate the samples for 18 h at 56 °C, while shaking at 800 rpm

5. Inactivate the enzyme by incubating for 10 min at 96 °C

6. Place the samples on ice

DNA purification

1. To each sample, add an equal volume of phenol:chloroform:isoamyl alcohol 25:24:1, then shake the samples vigorously

2. Centrifuge the samples at 20,000 x g for 5 min at RT

3. Collect the upper phase and transfer it to a new 1.5 ml Eppendorf® DNA LoBind tube

4. Add an equal volume of chloroform to it, and shake vigorously

5. Centrifuge at 20,000 x g for 5 min at RT

6. Collect the upper phase and transfer it to a new 1.5 ml Eppendorf® DNA LoBind tube, then add sodium acetate to a final concentration of 0.3 M

7. Add 3.7 µl of glycogen per each 100 µl of the solution

8. Add 2.5X volumes of absolute ethanol at –20 °C and incubate the samples for 18 h at –80 °C

9. Centrifuge at 20,000 x g for 90 min at 4 °C

10. Discard the liquid and wash the pellet with 600 µl of ice-cold 70% ethanol

11. Centrifuge at 20,000 x g for 15 min at 4 °C

12. Discard the liquid and wash the pellet with 600 µl of ice-cold 70% ethanol

13. Centrifuge at 20,000 x g for 15 min at 4 °C

14. Discard the supernatant and dry the pellet

Note: do not over dry the pellet, as this might result in low DNA recovery

15. Dissolve the pellet in 100 µl of TE buffer

Breakpoint: at this point, the samples can be stored a –20 °C until sonication

DNA sonication

Sonicate the samples aiming to achieve a mean DNA fragment size of 300–500 base pairs (bp). We routinely use Bioruptor® Plus with the following settings: 30 sec ON, 90 sec OFF, high mode, 16 cycles. Alternatively, a Covaris S-series sonicator can be used with the following settings: duty 10%, intensity 4, time 30 sec, cycle/burst 200, 4 cycles in 50 µl TE/water.

DNA concentration using AMPure beads

1. **Pre-warm AMPure XP beads for 30 min at RT**
2. **To each sample, add 80 µl of AMPure XP bead suspension**
3. **Mix thoroughly by pipetting up-down 5–6 times**
4. **Incubate for 5 min at RT**
5. **Place the samples on a magnetic stand**
6. **Incubate for 5 min until all the beads have attached to the magnet and the liquid is completely transparent**
7. **Carefully aspirate and discard the supernatant**
8. **Add 200 µl per sample of ice-cold 80% ethanol, while the samples are attached to the magnetic stand**
9. **Aspirate the supernatant**
10. **Repeat the wash in ice-cold 80% ethanol once**
11. **Air-dry the beads for 5 min at RT**

Note: do not over-dry the beads, as this might result in low DNA recovery

12. Remove the samples from the magnetic stand
13. Resuspend each sample in 10 µl of nuclease-free water
14. Incubate for 5 min at RT
15. Place the samples on the magnetic stand
16. Incubate for 5 min until all the beads have attached to the magnet
17. Transfer 7.5 µl of the cleared solution into a 0.5 ml Eppendorf® DNA LoBind tube

18. Measure DNA concentration using the Qubit® dsDNA HS Assay Kit

Checkpoint: check the DNA fragment size distribution by loading 1 µl of sonicated DNA into an High Sensitivity DNA Kit, and run it on Bioanalyzer 2100.

Breakpoint: at this point, the samples can be stored at –20 °C until *in vitro* transcription

In vitro transcription (IVT)

1. **In a 0.5 ml Eppendorf® DNA LoBind tube prepare the following IVT mix:**

- **Sonicated DNA** **7.5 µl**
- **rNTPs mix*** **8 µl**
- **T7 polymerase buffer 10X*** **2 µl**
- **T7 polymerase*** **2 µl**
- **RiboSafe RNase Inhibitor** **0.5 µl**

Note: For HAP1 samples digested with HindIII, we typically use 300 ng of sonicated DNA as input to the IVT reaction, whereas for those digested with MboI we use 50 ng. For GM06990 samples digested with HindIII, we use 133 ng of sonicated DNA

Note: all the reagents marked by * are included in the MEGAscript® kit. The rNTPs mix is prepared by mixing equal volumes of each rNTP solution included in the MEGAscript® kit

2. Incubate the samples for 14 h at 37 °C in a thermocycler with the lid set at 70 °C

RNA cleanup

1. **To each sample, add 2U (1 µl) of DNase I**
2. **Incubate the samples for 15 min at 37 °C. Meanwhile, prewarm the Agencourt RNAClean XP beads for 30 min at RT**
3. **Transfer each IVT product into a 1.5 ml Eppendorf® DNA LoBind tube**
4. **Bring the volume of each sample up to 30 µl with nuclease-free water**
5. **Add a 1.8 vol./vol. ratio of RNAClean XP beads suspension to the IVT product**

6. **Mix thoroughly by pipetting up-down 5-6 times**

7. **Incubate for 10 min at RT**

8. **Place the samples on a magnetic stand**

9. **Incubate for 5 min until all the beads have attached to the magnet and the liquid is completely transparent**

10. **Aspirate and discard the supernatant**

11. **Add 200 µl per sample of ice-cold 80% ethanol, while the samples are attached to the magnetic stand**

12. **Incubate for 1–2 min at RT**

13. **Aspirate the supernatant**

14. **Repeat the wash with ethanol 80% two times more**

15. **Airdry the beads for 10 min at RT**

Note: do not over-dry the beads, as this might result in low DNA recovery

16. Remove the samples from the magnetic stand

17. Resuspend the beads in 7.5 µl of nuclease-free water

18. Incubate for 5 min at RT

19. Place the sample on the magnetic stand

20. Incubate for 5 min until all the beads have attached to the magnet

21. Transfer 6 µl of each sample into a separate 0.5 ml Eppendorf® DNA LoBind tube

Checkpoint: check the RNA fragment size distribution by loading 1 µl of purified IVT product into an RNA 6000 Pico Kit, and run it on a Bioanalyzer 2100

RA3 adapter ligation

Note: all the subsequent steps are done on ice, unless otherwise specified

1. To each sample, add 1 µl of RA3 adapter

2. In a thermocycler, incubate for 2 min at 70 °C with the lid set at 70 °C

3. Immediately place the samples on ice

4. To each sample, add 3 µl of the following RNA ligase mix:

· T4 RNA Ligase buffer 10X 1 µl

· T4 RNA Ligase 2, truncated (200 U/µl) 1 µl

· RNaseOUT™ 1 µl

5. Incubate the samples for 2 h at 25 °C in a thermocycler with the lid set at 30 °C

6. Transfer the samples onto ice

Reverse transcription

Note: all the subsequent steps are done on ice, unless otherwise specified

1. To each sample, add 3.5 µl of RTP (10 µM) primer

2. Incubate the samples for 2 min at 70 °C in a thermocycler with the lid set at 70 °C

3. Immediately place the samples on ice

4. Add 11.5 µl per sample of reverse transcription mix:

· 1st strand buffer* 5 µl

· dNTPs mix 0.5 µl

· DTT 100 mM* 2 µl

· SuperScript® IV* 2 µl

· RNaseOUT™ 2 µl

Note: all the reagents marked by * are included in the SuperScript® IV Reverse Transcriptase kit

5. Incubate the samples for 1 h at 50 °C in a thermocycler

Breakpoint: at this point, the samples can be stored at –20 °C

Library indexing and amplification

1. Transfer each sample into a 200 µl PCR tube
2. To each sample, add 4 µl of the desired RPI (10 µM) primer
3. To each sample, add 71 µl of PCR mix:

· NEBNext® 2X PCR Mix 50 µl

· RP1 primer 4 µl

· Nuclease-free water 17 µl

4. Perform the following cycles in a thermocycler:

A. 98 °C, 30 sec

B. 98 °C, 10 sec

C. 60 °C, 30 sec

D. 72 °C, 30 sec

E. Go to B and repeat for 8–14 cycles

F. 72 °C, 10 min

G. 4 °C, hold

Note: the number of PCR cycles should be adjusted based on the amount of genomic DNA used in the IVT reaction and the restriction enzyme used. For example, for a 22x22 mm coverslip fully covered with HAP1 cells digested with HindIII, we typically use 9 PCR cycles.

Library cleanup

1. Prewarm the AMPure XP beads for 30 min at RT
2. Transfer each PCR product into a 1.5 ml Eppendorf® DNA LoBind tube
3. Add a 0.8 vol./vol. ratio of AMPure XP bead suspension to each sample
4. Mix thoroughly by pipetting up-down 5-6 times
5. Incubate for 5 min at RT
6. Place the samples on a magnetic stand

7. Incubate for 5 min until all the beads have attached to the magnet and the liquid is completely transparent

8. Aspirate and discard the supernatant

9. Add 200 µl per sample of ice-cold 80% ethanol, while the samples are attached to the magnetic stand

10. Aspirate the supernatant

11. Repeat once the wash in ice-cold 80% ethanol

12. Airdry the beads for 5 min at RT

Note: do not over-dry the beads, as this might result in low DNA recovery

13. Remove the samples from the magnetic stand

14. Resuspend each sample in 20 µl of nuclease-free water

15. Incubate for 5 min at RT

16. Place the samples on the magnetic stand

17. Incubate for 5 min until all the beads have attached to the magnet

18. For each sample separately, transfer 17–18 µl of the cleared solution into a 1.5 ml Eppendorf® DNA LoBind tube

19. Measure DNA concentration using the Qubit® dsDNA HS Assay Kit

20. Store the libraries at −20 °C

Checkpoint: 1 µl of library can be checked on a Bioanalyzer 2100 using a High Sensitivity DNA Kit

Note: at this point, the libraries can be stored at −20 °C up to one year

Troubleshooting

Time Taken

Anticipated Results

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