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Assay for Transposase Accessible Chromatin using Sequencing (ATAC-Seq)

Protocol modified from:

- Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. (Buenrostro et al., Nat Methods. 2013 Dec;10(12):1213-8).

- Cell freezing protocol suitable for ATAC-Seq on motor neurons derived from human induced pluripotent stem cells. (Milani et al., Scientific Reports 6, Article number: 25474 (2016)).

Overview of the protocol and QC

ATAC-Seq detects open-chromatin regions and maps transcription factor binding events genome-wide by means of direct *in vitro* transposition of native chromatin. Specifically, hyperactive Tn5 transposase is used to interrogate chromatin accessibility by inserting high-throughput DNA sequencing adapters into open genomic regions, which allows for the preferential amplification of DNA fragments located at sites of active chromatin.

The ATAC-Seq protocol was adapted from Buenrostro et al. (2013), with some modifications. Given that a successful ATAC-Seq experiment begins with the isolation of high-quality intact nuclei, we have first introduced a quality control checkpoint consisting of the **morphological evaluation of nuclei with either Trypan Blue or DAPI staining**, followed by the accurate quantification of those nuclei using an automated cell counter. Precise counting of nuclei is important to ensure optimal tagmentation (the simultaneous fragmenting of the DNA and insertion of adapter sequences) and to limit the technical variability across samples. **From a qualitative perspective, individual intact nuclei with a round or oval shape should be observed with no visible clumping.** To exclude samples with severe degradation or over-tagmentation, we assess the quality of the treated chromatin samples by gel electrophoresis, as described in Buenrostro et al. (2013); if the chromatin was intact and the transposase reaction was optimal, **a**

DNA laddering pattern with a periodicity of about 200bp should be observed, corresponding to fragments of DNA that were originally protected by an integer number of nucleosomes (nucleosome phasing). Furthermore, we measure the enrichment of DNA accessible regions by performing real-time qPCR analysis using a known open-chromatin site as a positive control and a Tn5-insensitive site as a negative control. **When assayed by real-time qPCR, high-quality ATAC-Seq samples should show at least a 10-fold enrichment of positive control sites compared to Tn5-insensitive sites.** Finally, as we are principally interested in open-chromatin profiling and not in nucleosome positioning, we have introduced a size-selection step to enrich for nucleosome-free fragments. This step increases the signal-to-noise ratio and improves the sensitivity of the methodology. After size-selection, libraries are PCR-amplified and submitted for single-end sequencing. Figure 1 shows the outline of ATAC-Seq procedure.
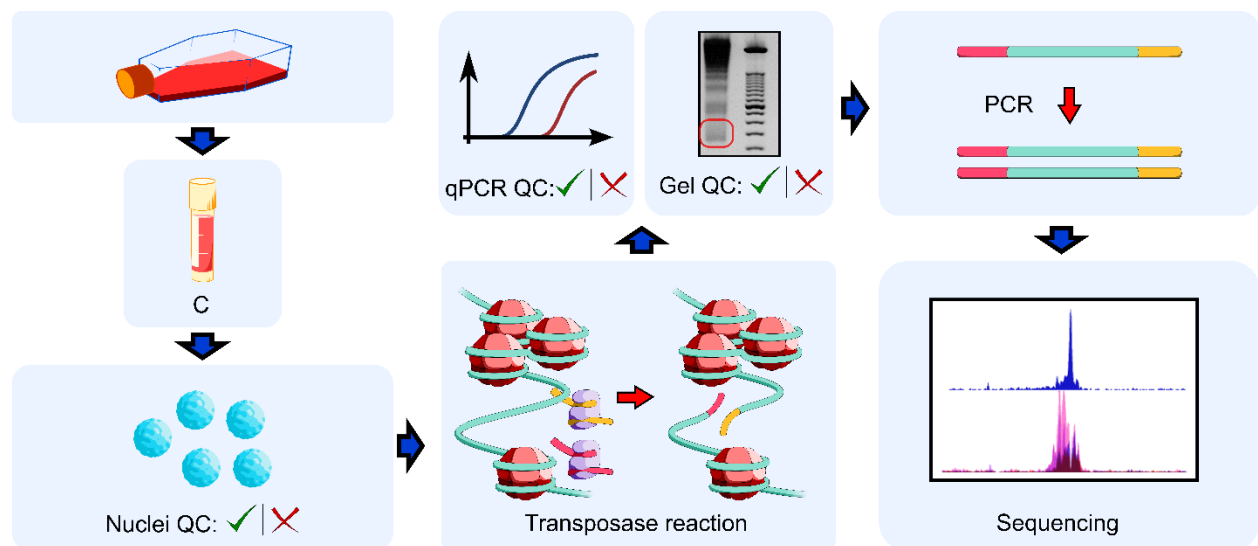

Figure 1. Outline of ATAC-Seq key experimental steps and quality control (QC) checkpoints.

Materials and equipment:

Agencourt AMPure XP (5 mL), Beckman Coulter, cat #: A63880

cOmplete Protease Inhibitor Cocktail Tablets EDTA-free, Sigma-Aldrich, cat #: 11873580001

Countess Cell Counting Chamber Slides, Thermo Fisher Scientific, cat #: C10228

Countess II FL Automated Cell Counter, Thermo Fisher Scientific, cat #: AMQAF1000

CryoStor® CS10, Stemcell Technologies, cat #: 07930

DNA Clean & Concentrator – 5 Kit, Zymo Research, cat #: D4013

DR88M transilluminator package, Clare Chemical Research

E-Gel® EX Agarose Gels, 2%, Thermo Fisher Scientific, cat #: G4020-02

E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit, Thermo Fisher Scientific, cat #: G6465

E-Gel®50 bp DNA Ladder, Thermo Fisher Scientific, cat #: 10488-099

Elution Buffer (EB) (10 mM Tris·Cl, pH 8.5), Qiagen, cat #: 19086

Ethanol, Pure, 200 Proof (100%), KOPTEC, VWR, cat #: 71001-862

KAPA SYBR FAST qPCR Master Mix, (5 mL), Kapa Biosystems, cat #: KK4601

Magnetic stand for PCR tubes

Mr. Frosty™ Freezing Container, Thermo Fisher Scientific, cat #: 5100-0001

NEBNext® High-Fidelity 2X PCR Master Mix, New England Biolabs, cat #: M0541S

Nextera DNA Sample Preparation Kit (24 samples), Illumina, cat #: FC-121-1030

Nextera Index Kit (24 indexes, 96 samples), Illumina, cat #: FC-121-1011

Nuclease-Free Water, Integrated DNA Technologies, cat #: 11-05-01-14

Single-edge industrial razor blades, VWR, cat #: 55411-050

Thermal Cycler

Zymoclean™ Gel DNA Recovery Kit, Zymo Research, cat #: D4007

Procedure

I.   Cell Preparation and Nuclei Isolation

1. Wash the cells once or twice with 1X PBS, isolate them via cell scraper in 1X PBS, and centrifuge at 250 rcf for 5 min at 4°C.
2. Cryopreservation: aspirate the supernatant, resuspend the pellets in Cryostor media, transfer the cell suspension to cryovials and freeze slowly in a Mr. Frosty Freezing Container (Thermo Fisher Scientific), filled with isopropyl alcohol, at -80°C.

3. <u>Thawing:</u> remove the cryovials from -80°C and quickly warm them for 2 min in a 37°C water bath. Transfer the samples to 12 ml of warm 1X PBS supplemented with 1X protease inhibitor cocktail. Gently mix each tube by inversion and centrifuge at 250 rcf for 5 min at 4°C. Carefully aspirate the supernatant and proceed with nuclei isolation.

4. <u>Nuclei isolation:</u> gently resuspend the cell pellet in 100 µL of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% IGEPAL CA-630*, 1X protease inhibitor cocktail). Transfer the cell suspension to 0.2 ml tubes and incubate on ice for 5 min. Spin down at 250 rcf for 5 min at 4°C.

5. Carefully discard the supernatant and resuspend the nuclear pellet in 20 µL of ice-cold 1X TD buffer (diluted from 2X TD buffer (Illumina, FC-121-1011) with ultrapure nuclease-free water)

6. Take 2 µL of nuclear suspension and dilute 1:5 by adding lysis buffer (without IGEPAL). Add 10 µL of Trypan Blue Solution (Thermo Fisher Scientific) and count the nuclei with the Countess II FL Automated Cell Counter (Thermo Fisher Scientific), according to manufacturer's instruction. Calculate the number of nuclei/µL.

7. <u>QC checkpoint</u>: inspect the nuclei by light microscopy; high-quality nuclei should be intact with round or oval shape and no clumping should be observed, as shown in Figure 2 (sample on the left).
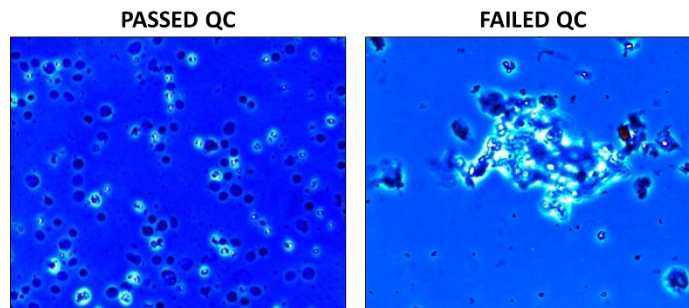

Figure 2. Nuclear morphological evaluation: the nuclei on the left are of high quality, while excessive clumping is observed for the nuclei on the right.

*Note: the optimal concentration of IGEPAL CA-630 to achieve maximal cell lysis without nuclear damage must be determined empirically for each specific cell type.

II.   Transposition Reaction and Purification

1. Ensure that the nuclei are set on ice and prepare the transposition reaction mix as indicated below (final volume = 50 µL):

> − **25 – x/2 µL** 2X TD buffer (Illumina, FC-121-1011)
>
> − **22.5 – x/2 µL** ultrapure water
>
> − **x µL nuclei** (previously resuspended in 1X TD buffer – total number = 50,000)
>
> − **2.5 µL** TDE1 (Tagment DNA Enzyme – Illumina, FC-121-1011)

4. Gently pipette to resuspend the nuclei in the transposition reaction mix.

5. Incubate the transposition reaction mix at 37°C for 30 min (in the thermal cycler).

6. Immediately following transposition, purify using a DNA Clean & Concentrator – 5 Kit (Zymo Research) according to the manufacturer's protocol. Elute the DNA with 21 µL of Elution Buffer (Qiagen).

7. Store 10 µL of purified transposed DNA at -20°C for possible future usage.

8. Use the remaining 10 µL for PCR reaction ("PCR amplification (8 cycles)" step).

III.   PCR amplification (8 cycles)

1. To amplify transposed DNA fragments, combine the following in a PCR tube (final volume = 50 µL):

> − **15 µL** Nextera PCR Master Mix (NPM – Illumina, FC-121-1011)
>
> − **5 µL** PCR Primer Cocktail (PPC – Illumina, FC-121-1011)
>
> − **5 µL** Index primer 1 (i7 – Illumina, FC-121-1011)
>
> − **5 µL** Index primer 2 (i5 – Illumina, FC-121-1011)
>
> − **10 µL** Ultrapure nuclease-free water
>
> − **10 µL** Transposed DNA

2. Perform PCR using the following program on a thermal cycler (Note: ensure that the thermal cycler lid is heated during the incubation):

- 72°C for 3 minutes

- 98°C for 30 seconds

- **8 cycles** of:

   — 98°C for 10 seconds

   — 63°C for 30 seconds

   — 72°C for 3 minutes

- Hold at 4°C

3. Purify using DNA Clean & Concentrator – 5 Kit (Zymo Research) according to the manufacturer's protocol. Elute the DNA with 21 µL Elution Buffer (Qiagen).

IV.   Gel-based qualitative evaluation of libraries and size selection

1. Load 20 µL of purified PCR reaction from step III.3 on E-Gel® EX Agarose Gels, 2% (Thermo Fisher Scientific). Load also 4 µL of E-Gel®50 bp DNA Ladder (Thermo Fisher Scientific) diluted with ultrapure water to a final volume of 20 µL.

2. Run for 10 min using the pre-set E-Gel® EX program on the E-Gel® iBase™ Power System (Thermo Fisher Scientific).

3. QC checkpoint: high-quality ATAC-Seq libraries should display clear nucleosome phasing, as shown in Figure 3 (sample on the left).
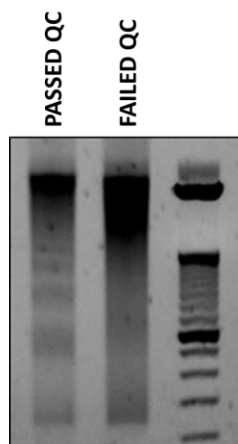

Figure 3. The sample on the left passed the QC checkpoint, while the sample on the right shows DNA smearing on the gel, likely caused by sample degradation.

4. Using the DR88M transilluminator and a clean razor blade, size select **175 - 250 bp** (fraction "A", corresponding to a <u>nucleosome-free fragment size</u>) and **250 - 625 bp** (fraction "B").

5. Purify the DNA from both gel fractions using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research), following the manufacturer's recommendation, and elute the DNA with 22 µl of Elution Buffer (Qiagen).

6. <u>QC checkpoint</u>: use the DNA from fraction "B" for qPCR-based qualitative analysis of libraries with primers mapping to open-chromatin regions as positive control sites and gene desert regions as negative control sites (Figure 4). Perform the qPCR assay also using non-transposed genomic DNA as a template to correct for any difference in primer efficiency. Prepare the amplification reaction with KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) and forward and reverse primers (500 nM final concentration). Calculate the fold enrichment of the open-chromatin site (OC) over the Tn5-insensitive site (INS) with the following formula:

$$\text{Fold Enrichment (FE)} = 2^{[(OCg-OCa) - (INSg-INSa)]}$$

where OCg is the qPCR threshold cycle number obtained for the OC qPCR primer pair using non-transposed genomic DNA as template, and INSa is the qPCR threshold cycle number obtained for the INS qPCR primer pair using ATAC-Seq library as template.
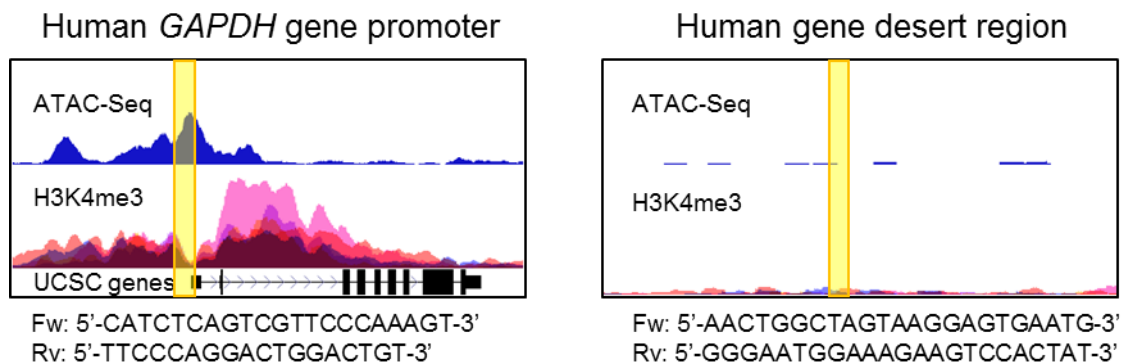

Figure 4. Sequences and genomic locations, displayed on the UCSC Genome Browser, of the primers used to amplify positive (human *GAPDH* gene promoter) and negative (human gene desert region) control sites.

V.   Final amplification

1. Combine the following reagents in a PCR tube (final volume = 50 µL):

- **20 µL** size-selected DNA (fraction A = nucleosome-free fragments)
- **3 µL** Nuclease Free water
- **1 µL** 10 µM PCR Primer IS5 (PAGE or HPLC purified)
- **1 µL** 10 µM PCR Primer IS6 (PAGE or HPLC purified)
- **25 µL** NEBNext High-Fidelity 2x PCR Master Mix

IS5 (reamp.P5) 5'-A*ATGATACGGCGACCACCG*A

IS6 (reamp.P7) 5'-C*AAGCAGAAGACGGCATACG*A

2. Perform PCR using the following program on a thermal cycler (Note: make sure that the thermal cycler lid is heated during the incubation):

- 98°C for 30 seconds
- **5-7* cycles** of
    — 98°C for 10 seconds
    — 65°C for 30 seconds
    — 72°C for 30 seconds
- 72°C for 5 minute
- Hold at 4°C

* Note: optimize the number of cycles to avoid saturation in order to reduce GC and size bias.

3. Use AMPure XP beads (50 µl) to purify the DNA following the manufacturer's instruction.

4. Store 10 µL at -20°C and submit 10 µL for quality control (BioAnalyzer and qPCR) and sequencing (Illumina HiSeq2000).