Protocol for **Assay for Transposase-Accessible Chromatin (ATAC-seq)**

Adapted from


i. **Summary**

The Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) is a powerful technique that enables the genome-wide analysis of chromatin accessibility. At the level of individual loci this allows an understanding of the positional relationship between chromatin, transcriptional regulators and gene expression whilst on a global level can provide an understanding of how large areas of the genome are regulated.

ATAC-seq relies upon the engineered activity of the hyperactive transposase Tn5, which both cleaves accessible DNA and anneals specific adaptors to either end of the resulting fragments. These DNA fragments can be isolated, amplified and analysed by NGS. ATAC-seq requires a clean nuclear extraction that has limited contamination from, in particular, chloroplast DNA.

This article outlines a consensus methodology for ATAC-seq that includes descriptions of a crude nuclear extraction, cleavage by Tn5 and amplification of DNA fragments in preparation for NGS.

ii. **Keywords**

Chromatin, Transposase, Chromatin accessibility, Genome, Nucleus
1. Introduction

Chromatin that is packed into the plant nucleus exhibits varying levels of accessibility. Heterochromatin contains tightly packed nucleosomes that allow poor accessibility, which result in low levels of gene expression. In contrast, within open euchromatin the nucleosomes are dispersed, allowing increased accessibility for transcription factors and other regulators of gene expression.

Over the past decade next generation sequencing (NGS) has enabled the analysis of chromatin accessibility on a whole-genome level. Initially the analysis of chromatin accessibility was facilitated by genomic digestion by DNaseI, which cleaves DNA that is unprotected because it is not wrapped in a nucleosome. This cleavage generates DNA that can then be amplified and sequenced to reveal the accessible sequences. Unfortunately using DNaseI requires a large amount of starting tissue so has practical limitations when attempting to analyse chromatin extracted from rarer cell types.

The more recently developed method of Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) has been widely used in many experimental systems. This technique takes advantage of an engineered hyperactive Tn5 transposase that is preloaded with DNA adapters thus removing the need for a separate annealing step. Purified nuclei are treated with the transposase complex, the enzyme freely enters nuclei where it cleaves accessible DNA, after which the transposase attaches sequencing adapters. Regions of higher accessibility will be cleaved more frequently by the transposase and therefore generate more DNA fragments—and ultimately more reads—once the sample is sequenced. Conversely, less accessible regions will yield both fewer fragments and subsequent reads. Importantly the integration of cleavage and adaptor-annealing steps allows for a shorter protocol that requires less starting material and therefore can be used to analyse chromatin accessibility from nuclei extracted from rarer tissue types.

The activity of Tn5 is agnostic with regard the source of the chromatin so this can be utilised on samples extracted from any plant species. The high efficacy of the enzymatic reaction means that ensuring the purity of nuclear extraction is arguably the most important part of this protocol. This reflects that the risk of contamination from organelle genomes is a particular challenge when working with plant tissues. Tn5 will cut both mitochondrial and chloroplast DNA with high frequency due to the absence of nucleosomes thus generating a lot of contaminating fragments. As such care must be taken to reduce (especially) chloroplast contamination, either by extracting from a non-green tissue or by using more precise nuclear extraction techniques.

ATAC-Seq has been successfully paired with both INTACT (isolation of nuclei tagged in specific cell types; Deal and Henikoff, 2011) and FANS (fluorescent activated nuclear sorting) nuclear extraction methods (Bajic et al, 2018; Maher et al, 2018; Karaaslan et al, 2020). Of course these techniques require plants that express a particular transgene so are not applicable for use with many species.

Fortunately Maher et al (2018) and Bakic et al (2018) successfully used ATAC-seq on a crude nuclear preparation extracted by sucrose sedimentation. However as cautioned in the previous paragraph, Maher et al (2018) found that analysis of samples from a crude extract resulted in 50% of sequencing reads mapping to an organellar genome whilst when using the INTACT method this was reduced to 10%. The former case necessitates a higher number of these reads to be discarded prior to downstream processing. Fortunately comparison of datasets compiled from ‘crude’ or INTACT extracted samples was highly
similar (Figure 1). Another benefit of using INTACT or FANS is that analysis can be restricted to specific cell types, which is not possible with the same level of precision in samples obtained by a crude extraction method.

![Figure 1: Comparison of peaks obtained by ATAC-Seq from nuclei samples isolated using the INTACT or crude method extraction methods. Adapted from Maher et al (2018).](https://www.brookes.ac.uk/indepth/indepth-academy/)

In this article we provide a consensus protocol for ATAC-seq for samples extracted by 'conventional methods' and is therefore applicable for, in theory, any plant species. For use of INTACT nuclear extraction please consult Bajic et al (2018), Maher et al (2018), and Tannenbaum et al (2018). For use of FANS please consult Karaaslan et al, (2020) or the general FANs protocol published as part of the INDEPTH Academy, [https://doi.org/10.24384/fvmf-km57](https://doi.org/10.24384/fvmf-km57).
2. Materials

- Liquid nitrogen (Extraction #1)
- Mortar and pestle (Extraction #1)
- Blotting paper (Extraction #1)
- Razor blades (double edged blades are often sharper) (Extraction #2)
- Plastic Petri-dish (Extraction #2)
- 30um non-sterile filters (Extraction #1 and #2) (available from CellTrics or similar
- 15ml Falcon tubes (sterile)
- 1.5ml Eppendorf tubes (sterile)
- DAPI stock solution: 1 mg/mL of stock solution. Dilute 2000× for DAPI staining (0.5ug/ml)
- 37% paraformaldehyde stock solution
- Wash solution: 10 mM Tris–HCl pH 7.5, 10 mM Na EDTA pH 8, 100 mM NaCl.
- Nuclei Extraction Buffer ([NEB1](#)): 20 mM MOPS pH 7, 40 mM NaCl, 90 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, and a broad action protease inhibitor (such as cOmplete™ Protease Inhibitor, [https://www.sigmaaldrich.com/catalog/product/roche/04693159001?](https://www.sigmaaldrich.com/catalog/product/roche/04693159001?)). Prepare by adding spermidine, spermine, and protease inhibitor just prior to starting the experiment. Use within 1hr of preparation.
- Nuclei Extraction Buffer 2 ([NEB2](#)): 0.25 M sucrose, 10 mM Tris, pH 8, 10 mM MgCl$_2$, 1% Triton X-100, and a broad action protease inhibitor. Use within 1hr of preparation.
- Nuclei Extraction Buffer 3 ([NEB3](#)): 1.7 M sucrose, 10 mM Tris, pH 8, 2 mM MgCl$_2$, 0.15% Triton X-100 and a and a broad action protease inhibitor. Use within 1hr of preparation.
- Tagmentation-based library preparation kit (such as Nextera reagents from Illumina; [https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-xt-dna.html](https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-xt-dna.html))
- Elution Buffer (EB). Usually provided with PCR purification column: If not 10 mM Tris-Cl, pH 8.5
- High Fidelity PCR polymerase (available from many suppliers)
- Specific ATAC primers (100uM)
- qPCR machine and appropriate reagents
- PCR Purification magnetic beads (such as from AMPure XP, [https://www.beckman.com/reagents/genomic/cleanup-and-size-selection/pcr](https://www.beckman.com/reagents/genomic/cleanup-and-size-selection/pcr))
- Magnetic rack
- 80% ethanol
- 10mM Tris pH8
- Bioanalyser or similar (optional)
3. Methods

3.1 Plant Growth

Plants are grown according to experimental needs. The reference protocols have used Arabidopsis root tips, leaves or dark-grown hypocotyl tissues for crude nuclei extraction. Root tips and etiolated hypocotyls will have less chloroplast contamination but will require a larger amount of starting tissue. For reference Maher et al (2018) harvest up to 1gram of tissue from 1cm sections of root tips from seven-day old Arabidopsis, medicago, rice or tomato plants.

Unless mentioned all steps of the workflow should be conducted at 4°C. Whilst speed of activity is important, for optimal reproducibility it is critical that where possible samples are treated identically.

Two tissue extraction methods are outlined that use different techniques for tissue extraction, either using grinding of frozen tissue in liquid nitrogen (#1) or by chopping fresh tissue (#2).

3.2 Tissue Extraction #1

1- Collect plant tissue into a falcon tube with 10ml 4% paraformaldehyde in Wash solution and fix for 15minutes [NOTE 1].

2- Tissue is washed twice for 10minutes in Wash solution.

3- Tissue is blotted as well as possible before being frozen in liquid nitrogen in a mortar and pestle [NOTE 2].

4- Tissue is ground under liquid nitrogen into a fine powder [NOTE 3]

5- Transfer frozen tissue to 10ml cold NEB1 and vortex well.

6- Proceed to Step 14

3.3 Tissue Extraction #2

7- Collect plant tissue into a falcon tube with 10ml 4% paraformaldehyde in Wash solution and fix for 15minutes [NOTE 1].

8- Tissue is washed twice for 10minutes in 10ml Wash solution.

9- Tissue is transferred to 10ml NEB1.

10- Remove a maximum amount of 0.5g tissue and add to a 30ml petri-dish.

11- Chop sample with a new razor blade for up to 10minutes/sample before transferring tissue to a new falcon tube [NOTE 4].

12- Repeat until all samples have been chopped. After chopping final sample rinse petri dish with at least 500ul NEB1 to ensure all nuclei have removed.

13- Proceed to Step 14
3.4 Nuclear Isolation

14- Strain nuclear extraction through a pre-wet 30um filter [NOTE 5].
15- Centrifuge the flow-through at 1200xg for 10minutes at 4°C
16- Remove as much supernatant as possible without disturbing the pellet [NOTE 6]
17- Gently resuspend pellet in 1ml cold NEB2 in a cooled 1.5ml eppendorf tube.
18- Centrifuge the resuspended nuclei at 12,000x g for 10 min at 4°C.
19- Remove supernatant and resuspend in 300ul NEB3.
20- Add 300ul cold NEB3 (without nuclear sample) to a fresh 1.5ml eppendorf tube.
21- Layer 300ul pellet from Step 19 onto 300ul NEB3 from Step 20
22- Centrifuge the layers at 16000xg for 10mins at 4°C [NOTE 7]
23- Remove supernatant and resuspend nuclei in 1ml NEB1
24- Remove 25ul of the nuclear extraction and quantify with a haemocytometer using standard protocols [NOTE 8]
25- Remove a volume of liquid from Step 23 that includes 50,000 nuclei and add to a new cooled 1.5ml eppendorf tube [NOTE 9]
26- Centrifuge at 1500xg for 10minutes at 4°C
27- Resuspend in 45ul in ice-cold Tn Reaction Mix (Step 28) and proceed immediately with Step 28.

Figure 2: Examples of DAPI-stained nuclei extracted by sucrose sedimentation (labelled with arrows). Adapted from Bajic et al (2018)

3.5 Tagmentation Reaction

This portion of the protocol uses Tn5 to fragment chromatin and add adaptor DNA sequences used for downstream sequencing [NOTE 10]. This protocol must be planned alongside local technical experts who have knowledge of the NGS platform that will be used. Each NGS platform will require a different procedure but this article outlines use of the Nextera kit for preparing samples for Illumina NGS (Nextera DNA library Prep Reference Guide, 2016). Alternatively Picelli et al (2014) outline a protocol to generate a ‘home-made’ Tn5. This procedure recommends use of 50000 nuclei per reaction as isolated in Step 27.
28- Add 50000 nuclei extracted in Step 25 to the Tn Reaction Mix (22.5ul nuclease-free water, 25ul TD buffer, 2.5ul TDE1 transposase) and incubate at 37°C for 30 minutes [NOTE 11].

29- Purify DNA sample using a sample column-based PCR purification kit.

30- Elute DNA in 11ul of standard Elution buffer (EB). Digested genomic DNA can now be stored at -20°C until needed.

![Figure 3: Diagram of Tagmentation reaction and limited cycle PCR. Taken from Nextera DNA Library Prep Reference Guide.](https://www.brookes.ac.uk/indepth/indepth-academy/)

### 3.6 Amplification of DNA library

Genomic DNA is present at low concentration but the standardised adaptor sequences allow unbiased amplification in an initial 5-cycle PCR reaction.

31- Set up the following PCR reaction
- Genomic DNA from Step 30 10ul
- 25uM ATAC primer 1 2.5ul
- 25uM ATAC primer 2 [NOTE 10] 2.5ul
- High Fidelity PCR Mix [NOTE 12] Xul
- Nuclease-free water to 50ul

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<td>4</td>
<td>Hold</td>
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*Table 1: PCR reaction for initial amplification of samples*
32- To determine the optimal number of PCR cycles prior to final amplification, take 5ul from the PCR reaction from Step 31 and amplify by qPCR reaction using the available machine 

[NOTE 13].

33- Determine number of cycles to generate 1/3 total DNA from the qPCR. This cycle number is adequate for final amplification (Step 34). Using any more cycles can cause biased amplification of the input DNA.

34- Run the remaining 45ul in a standard PCR reaction with chosen High Fidelity polymerase.

35- Purifiy amplified DNA library using PCR Purification magnetic beads with a ratio of PCR products:Beads of 1:1.5 [NOTE 14]

36- Incubate at room temperature for 5 minutes

37- Capture beads on magnetic rack for 1 minute and discard supernatant

38- Wash beads 2x 30seconds with 200ul 80% ethanol

39- Air-dry beads for 5 minutes to remove all traces of ethanol

40- Resuspend beads in 20ul 10mM Tris pH8 and incubate for 2 minutes at room temperature.

41- Capture beads and transfer supernatant to fresh tube.

42- Run 1ul small aliquot on Bioanalyser to test the size and quality of DNA. Alternatively run sample on an agarose gel stained with ethidium bromide or other visualisation aid [NOTE 15].

43- NGS and downstream bioinformatic analysis can then proceed according to the researcher preferences.
4. Notes

NOTE 1: Fixation can ensure that the native state of the tissue is maintained during extraction. When tissue is extracted using a mortar and pestle under liquid nitrogen samples may not be treated with paraformaldehyde. However, this treatment will not diminish the quality of the extraction and will better preserve the native state of chromatin-TF interactions. During nuclear extraction by FANS, Karaaslan et al. (2020) show that use of 4% rather than 1% paraformaldehyde allowed the extraction of a better quality nuclear GFP signal, which yielded clearer ATAC-seq peaks (Figure 4).

![Figure 4. ATAC-seq reads obtained from nuclei fixed with 4% and 1% Formaldehyde (FA). Adapted from Karaaslan et al. (2020)](https://www.brookes.ac.uk/indepth/indepth-academy/)

NOTE 2: After blotting the samples can be wrapped in foil, frozen in liquid nitrogen and kept at -80°C until needed.

NOTE 3: Grinding tissue under liquid nitrogen is most effective when tissue is initially ground up, scraped to the bottom of the pestle using a cold spatula before carefully adding new liquid nitrogen to grind up the tissue again. Ensure the metal spatula is cooled in liquid nitrogen prior to scraping tissue as otherwise tissue can melt before transfer to NEB1.

NOTE 4: Ensure samples are coated with NEB1 but not in excess liquid as this will decrease the effectiveness of the chopping.

NOTE 5: If filter gets clogged up with debris then wash in Wash buffer or use a fresh filter (depending on supplies).

NOTE 6: It may be tempting to pour off some of the supernatant but this may partially dislodge the lightly attached pellet. Take time to use increasingly smaller pipettes to remove the maximum amount of the supernatant. Better to leave some supernatant than to lose some of the pellet.

NOTE 7: Proper separation of nuclei from other cellular debris requires the nuclei to pass through the sucrose cushion during centrifugation. After centrifugation, the contaminating organelles and debris may be visible at the top of the tube and will be green if leaf tissue was used.

NOTE 8: Add DAPI from stock solution to a final concentration of 0.5ug/ul. Mix well and place on ice in the dark for 5minutes. Count nuclei on haemocytometer using standard protocols.

NOTE 9: Bajic et al. (2018) suggest using 50000 nuclei from either an INTACT or crude nuclear extraction for the tagmentation reaction. Karaasian et al. (2020) use 10000 nuclei from their FANS extraction for Tagmentation. Therefore a recommended amount of between...
10-50K nuclei reaction should be used. Of course more starting tissue is needed if INTACT or FANS protocols are used to extract nuclei from rare tissue types.

**NOTE 10**: The adaptor/ primer sequences will vary depending on the needs of the experiment and the possible intention to multiplex samples for NGS. In this case the adaptor/primer sequences will be unique to each reaction. This strategy should be discussed in advance with an expert in the NGS platform that the researcher will be using. For reference see Nextera Index Adapter Pooling Guide.

**NOTE 11**: Keep the Tn Reaction Mix on ice prior to being used to resuspend nuclear extract in Step 27. Mix well by pipetting before transferring to reaction temperature of 37°C.

**NOTE 12**: Researchers should choose their own High Fidelity Polymerase for this reaction. Usually the reaction mix is supplied as a 2x solution but this may vary.

**NOTE 13**: The optimal number of PCR cycles for the final reaction must be determined empirically by qPCR and will depend on the quality of nuclear extraction, the accuracy of nuclear counting and therefore the starting DNA content. Bajic et al (2018) report that typically when samples are amplified to 1/3 of the maximum fluorescence obtained by qPCR the quantified molarities are between 50nM to 300nM.

**NOTE 14**: When using PCR purification beads the ratio of Beads to Product determines the size of the DNA that is retained by the beads. A Bead:DNA ratio of 1.5:1 will gather DNA >150bp whilst removing contaminating smaller fragments.

**NOTE 15**: The size of the amplified DNA fragments is expected to be between 180bp -> 500bp. All smaller primer dimer or adaptor dimer fragment ~100bp should have been removed.
5. References


Nextera DNA Library Prep Reference Guide
https://support.illumina.com/sequencing/sequencing_kits/nextera_dna_kit.html

Nextera Index Adapter Pooling Guide
