

Feb 29, 2024

Version 2

Protocol SAM-Seq A.Thaliana V.2

DOI

<https://dx.doi.org/10.17504/protocols.io.8epv5x1xdg1b/v2>

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Protocol Citation: basile.leduque , Quadrana Leandro 2024. Protocol SAM-Seq A.Thaliana. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.8epv5x1xdg1b/v2> Version created by [basile.leduque leduque](#)

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Protocol status: Working

We use this protocol and it's working

Created: February 29, 2024

Last Modified: February 29, 2024

Protocol Integer ID: 95953

Keywords: dna methylation sequencing, dna methylation genome, characterising epigenetic pattern, resolution methylation profiles over nucleosome, dna methylation, epigenetic patterns along repetitive genomic region, resolution profiling of cytosine methylation, dna methylation within single molecule, chromatin accessibility, chromatin profiling, including chromatin accessibility, utilising bacterial adenine methyltransferase, use in chromatin profiling, bacterial adenine methyltransferase, somatic epigenetic variation at bivalent chromatin domain, tagged chromatin accessibility, somatic epigenetic variation, resolution methylation profile, cytosine methylation, chromatin accessibility along individual chromatin fibre, epigenetic modification, repetitive genomic region, epigenomic information, chromatin, naked genomic dna, investigation of chromatin, significant sequence preferences of m6a, using naked genomic dna, read sequencing, genome function, shaping genome function, individual chromatin fibre, limited genomic, in

Funders Acknowledgements:

European Research Council

Grant ID: 948674

Abstract

Background: Epigenetic modifications, including chromatin accessibility, nucleosome positioning, and DNA methylation (5mC), are pivotal in shaping genome function. However, current short read sequencing approaches present challenges in characterising epigenetic patterns along repetitive genomic regions.

Results: We developed Simultaneous Accessibility and DNA Methylation Sequencing (SAM-seq), a robust method utilising bacterial adenine methyltransferases (m6A-MTases) to mark accessible regions in purified plant nuclei. Coupled with Oxford Nanopore Technology sequencing, SAM-seq enables high-resolution profiling of cytosine methylation and m6A-tagged chromatin accessibility along individual chromatin fibres in *A. thaliana* and maize. Importantly, using naked genomic DNA we uncovered significant sequence preferences of m6A-MTases, which we show must be taken into account in order to obtain reliable accessibility profiles, particularly for the analysis of highly repetitive sequences such as centromeric repeats. Using this method we found intriguing antagonism between accessibility and DNA methylation within single molecules, somatic epigenetic variation at bivalent chromatin domains, periodicity of nucleosomal strings over centromeric repeats, and high-resolution methylation profiles over nucleosomes.

Conclusions: Our study highlights the importance of considering intrinsic substrate preferences of DNA modifying enzymes for their use in chromatin profiling. Hence, SAM-seq is a robust and cross-species method to chart high-resolution accessibility and DNA methylation genome-wide. This method and findings will enable the investigation of chromatin-based regulation across plant species, with implications for the study of non-model plant species with limited genomic and epigenomic information.



Reagent Preparation

1 Extraction Buffer (EB) 1

To prepare 50mlm of fresh EB1 buffer

	Component	Amount	Final Concentration
	2M Sucrose	10 ml	0.4M
	1M Tris-HCl pH 8	0.5 ml	10mM
	1M MgCl ₂	0.5ml	10mM
	14.3M 2-Mercaptoet hanol	17.5 µl	5mM
	+ Pls (1 tablet per 50mls)		

2 Extraction Buffer (EB) 2

To prepare 4ml of fresh EB2 :

	Component	Amount	Final Concentration
	14.3M 2-Mercaptoet hanol	1,4 µL	5mM
	20% Triton X-100	200 µL	1%
	H ₂ O	2818,6 µL	
	1M MgCl ₂	40 µL	10mM
	1M Tris-HCl pH 8	40 µL	10mM
	Pls (1 mini- tablet diluted in 1 ml)	400 µL	
	2M Sucrose	500 µl	0.25M



3 Extraction Buffer (EB) 3

To prepare 3ml of fresh EB 3 :

	Component	Amount	Final Concentration
	1M Tris-HCl pH 8	30 μ L	10mM
	1M MgCl ₂	6 μ L	2mM
	20% Triton X-100	25 μ L	0.15%
	+ Pis (1 mini-tablet diluted in 1 ml)	300 μ L	
	2M sucrose	2550 μ L	1.7M
	14.3M 2-Mercaptoethanol	1,05 μ L	5mM
	H ₂ O to volume	90,3 μ L	

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Nuclear Preparation Buffer (NPB)

To prepare 10ml of fresh NPB:

	Component	Amount	Final Concentration
	0.5M MOPS pH7	400 μ l	20 mM
	5M NaCl	80 μ l	40 mM
	1M KCl	900 μ l	90 mM
	0.5 M EDTA	40 μ l	2 mM
	250mM EGTA	20 μ l	0.5 mM
	3.5M spermidine	1.2 μ l	0.5 mM



	Component	Amount	Final Concentration
	70 mM spermine	28.5µl	0.2 mM
	1 mini-tablet diluted in 1ml	1ml	1X

5 **Plant-Tween-Wash-Buffer (PTWB)**

To prepare 3ml of fresh PTWB:

	Component	Amount	Final Concentration
	20% Tween-20	30 µl	0.2%
	1M pH7,5 HEPES-KOH	60 µl	20 mM
	5M NaCl	90 µl	150 mM
	PI Mini	300 µl	
	spermidine 3.5M	0,4 µl	0,5 mM
	H2O	2530 µl	

6 **Reagents:**

for m6A reaction

EcoGII/ rCutSmart/ SAM (M0603S New England Biolabs)

for library preparation:

NEBNext Companion Module for Oxford Nanopore Ligation Seq (E7180S New England Biolabs)

SQK-LSK110 (Oxford Nanopore Technologies)

for nanopore sequencing:

FLO-MIN106.1 or FLO-PRO002 (Oxford Nanopore Technologies)

Plant nuclei purification and permeabilization

7 Starting material: 1g of powder (Aerial part of ~17 days seedling)



- Add the powder to 25 ml of Extraction Buffer (EB) 1 in a 50 ml falcon tube. Let sit on ice for 5 min.
- 8 Add 1% Formaldehyde for crosslinking (i.e. 675 μ l Formaldehyde 37% in 25ml of EB1). Incubate 5 minutes
 - 9 Add 1.25 M glycine to stop the crosslinking (i.e. 1.7ml of Glycine 2M per 675 μ l Formaldehyde 37%)
 - 10 Filter the solution through Miracloth into a new 50 ml falcon tube. Repeat once.
 - 11 Filter the solution through a 0.4 μ m filter into a new 50 ml falcon tube.
 - 12 Centrifuge the filtered solution for 20 minutes at 4,000 rpm at 4°C.
 - 13 Gently remove supernatant and resuspend the pellet in 2 ml of Extraction Buffer (EB) 2. Transfer the solution into four 1.5ml Eppendorf tubes.
 - 14 Centrifuge at 12,000 g for 10 minutes at 4°C. Repeat step 7 & 8 once
 - 15 Remove supernatant and resuspend each pellet in 300 μ l of Extraction Buffer (EB) 3.
 - 16 In a clean Eppendorf tube, add 300 μ l of EB3. Take the 300 μ l solution (resuspended pellet) from step 7 and carefully layer it on top of the clean 300 μ l of EB3.
 - 17 Centrifuge for 45 min at 12,000 at 4 degrees.
 - 18 Resuspend each chromatin pellet in 300 μ l Nuclear Preparation Buffer (NPB) and incubate on ice for 5 min

m6A-MTases reaction

- 19 Spin down nuclei at 1000g for 5 min at 4°C and resuspend in Plant-Tween-Wash-Buffer (PTWB).



- 20 Resuspend each pellet in 193.5 μ l 1X rCutSmart, containing 5 μ l SAM and 1.5 μ l EcoGII (i.e. 3.75U for 100 μ l). Incubate 30 minutes at 30°C
- 21 Add 200 μ l rSmartCut 1X + 20 μ l NaCl (5M) and incubate overnight at 65 °C (or 1h30min if not crosslinked).
- 22 Add 5 μ l of RNase A (20mg/ml) and 2 μ l Proteinase K. Incubate 30 minutes at 42°C.

Phenol/Chloroform extraction of genomic DNA

- 23 Add 1 volume of 1:1 phenol:chloroform (i.e. 400 μ l)
- 24 Centrifuge at 12,000 g for 10 minutes at 4°C
- 25 Recover the aqueous phase (upper) in a new LoBind Eppendorf tube
- 26 Add 1 volume of chloroform (i.e. 400 μ l)
- 27 Centrifuge at 12,000 g for 10 minutes at 4°C.
- 28 Recover aqueous phase (upper) in a new LoBind Eppendorf tube
- 29 Precipitate DNA by adding 1/10 volume of NaAcétate 3M pH5,2 + 2,5 volume of EtOH 100%
- 30 Incubate at -20°C from 45 minutes to overnight.
- 31 Centrifuge at 12000g for 30 minutes at 4°C to precipitate DNA.
- 32 Wash the pellet with 300 μ EtOH 70%.



33 Remove EtOH and dry pellets.

34 Elute in 20µl TE pH 8.0

Library preparation

35

Follow the protocol provided by Oxford Nanopore technology (Genomic_DNA_by_Ligation_SQK-LSK110) with the following modification:

- Use 0.3 to 1 µg of DNA per library
- For a better library preparation increase the End-Prep step up to 20 minutes at 20°C and Ligation step incubation to at least 1h
- Elute in Tris-HCl 10 mM.
- LFB was used for final size selection step
- Quantify DNA using the Qubit dsDNA HS Assay Kit (Q33230) to check yield.

Sequencing

36 Sequence during 72h on v9.4.1 flow cell FLO-MIN106.1 or FLO-PRO002 in MinION or PromethION sequencer, respectively.