Aug 06, 2024 Version 3

Protocol SAM-Seq Zea Mays V.3

DOI
dx.doi.org/10.17504/protocols.io.kqdg3x25zg25/v3

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DOI: dx.doi.org/10.17504/protocols.io.kqdg3x25zg25/v3

Protocol Citation: basile.leduque leduque, s Quadrana Leandro 2024. Protocol SAM-Seq Zea Mays. **protocols.io https://dx.doi.org/10.17504/protocols.io.kqdg3x25zg25/v3**Version created by **basile.leduque leduque**

Protocol status: Working
We use this protocol and it's working

Created: August 28, 2023

Last Modified: August 06, 2024

Protocol Integer ID: 104133

Funders Acknowledgement:
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.

Materials

Reagents:

1.5 ml Eppendorf DNA LoBind tubes (022431021 Fisher)
Pierce, protease inhibitor without EDTA (A32965)
Pierce, mini-tablets protease inhibitor without EDTA (A32955)

for m6A reaction

EcoGII/ rCutSmart/ SAM (M0603S New England Biolabs)
Proteinase K (25530049 Thermofisher)
RNAse A (T3018L 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)

Reagent Preparation

1 **Extraction Buffer (EB) 1**

To prepare 50 ml of fresh EB1 buffer

	Component	Amount	Final Concentration
	2M Sucrose	10 ml	0.4M
	1M MgCl2	0,5 ml	10mM
	14.3M 2-mercaptoethanol	17,5 µl	5mM
	protease inhibitor (PI)	1 tablet	1X
	1M Tris-HCl pH 8	0,5 ml	10mM

Fill to 50 ml with distilled water

2 **Extraction Buffer (EB) 2**

To prepare 4 ml of fresh EB2 :

3 **Extraction Buffer (EB) 3**

To prepare 3 ml of fresh EB 3 :

	Component	Amount	Final Concentration
	1M Tris-HCl pH 8	30 µl	10mM
	1M MgCl2	6 µl	2mM
	20% Triton X-100	25 µl	0.15%
	PI (from 1 mini-tablet resuspended in 1 ml)	300 µl	
	2M sucrose	2550 µl	1.7M
	14.3M 2-mercaptoethanol	1,05 µl	5mM
	Distilled water	90,3 µl	

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Nuclear Preparation Buffer (NPB)

To prepare 10 ml of fresh NPB:

	Component	Amount	Final Concentration
	0.5M MOPS pH7	400 µl	20 mM
	5M NaCl	80 µl	40 mM
	1M KCl	900 µl	90 mM
	0.5 M EDTA	40 µl	2 mM
	250mM EGTA	20 µl	0.5 mM
	3.5M spermidine	1,2 µl	0.5 mM
	70 mM spermine	28,5 µl	0.2 mM
	PI (from 1 mini-tablet resuspended in 1 ml)	1 ml	

Fill to 10 ml with distilled water

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 (from 1 mini-tablet resuspended in 1 ml)	300 µl	
	Spermidine 3.5M	0,4 µl	0,5 mM
	Distilled water	2530 µl	

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Activation Buffer (AB)

To prepare 3ml of freash AB:

	Component	Amount	Final Concentration
	1M Tris-HCl pH 8	750 µl	15 mM
	5M NaCl	150 µl	15 mM
	1M KCl	3 ml	60 mM
	0.5 M EDTA	100 µl	1 mM
	250mM EGTA	100 µl	0.5 mM
	3.5M spermidine	0,715 µl	0.05 mM
	BSA	50 mg	0.1%
	SAM 32 mM	(add before reaction)	800µM

Add enough distilled water to raise the volume to 3 mL.

7 **Reagents:**
1.5 ml Eppendorf DNA LoBind tubes (022431021 Fisher)
Pierce, protease inhibitor without EDTA (A32965 Thermofisher)
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for m6A reaction
EcoGII/ rCutSmart/ SAM (M0603S New England Biolabs)
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Plant nuclei purification and permeabilization

8 Starting material: 0.75 g of grinded powder (7 days old leaves)

9 Add the powder to 12.5 ml of Extraction Buffer (EB) 1 in a 50 ml falcon tube. Homogenise and let sit on ice for 5 min.

10 Add 1% Formaldehyde for crosslinking (i.e. 338 µl Formaldehyde 37% in 12.5 ml of EB1). Incubate 5 minutes on ice.

11 Add 1.25M glycine to stop the crosslinking (i.e. 850 ml of Glycine 2M per 338 µl Formaldehyde 37%), homogenise well before going throught the next step.

12 Filter the solution through Miracloth into a new 50 ml falcon tube. Repeat once.

13 Filter the solution through a 0.4µm filter into a new 50 ml falcon tube.

14 Centrifuge the filtered solution for 20 minutes at 4,000g at 4°C.

15 Gently remove the supernatant and resuspend the pellet in 2 ml of Extraction Buffer (EB) 2. Transfer the solution into two 1.5ml LoBind Eppendorf tubes.

16 Centrifuge at 11,000 g for 10 minutes at 4°C. Repeat step 15 & 16 once

17 Remove the supernatant and resuspend each pellet in 300 µl of Extraction Buffer (EB) 3.

18 In two clean 1.5 ml LoBind Eppendorf tubes, add 300 µl of EB3. Take the 300 µl solution (resuspended pellet) from step 17 and carefully layer it on top of the clean 300 µl of EB3.

19 Centrifuge for 45 min at 11,000g at 4 degrees.

20 Resuspend each chromatin pellet in 300 µl of Nuclear Preparation Buffer (NPB) and incubate on ice for 5 min.
Only one 1.5 ml LoBind Eppendorf tube of chromatin is used for the m6A-MTase reaction and is sufficient to produce enough material for one promethION run; the second tube can be stored at -80°C.

m6A-MTases reaction

21 Spin down nuclei at 1000g for 5 min at 4°C and resuspend in 300 µl of Plant-Tween-Wash-Buffer (PTWB).

22 Spin down nuclei at 1000g for 5 min at 4°C and resuspend pellet in 380 µl 1X rCutSmart, add 10 µl SAM (32mM) and 6 µl EcoGII (i.e. 7.5U for 100 µl). Incubate 30 minutes at 30°C

23 Add 20 µl NaCl (5M) and incubate overnight at 65 °C (or 1h30 if the chromatin has not been cross-linked).

24 Add 5 µl of RNAse A (20mg/ml) and 2 µl Proteinase K (20mg/ml). Incubate 30 minutes at 42°C.

Phenol/Chloroform extraction of genomic DNA

25 Add 1 volume of 1:1 phenol:chloroform (i.e. 400 µl)

26 Centrifuge at 12,000 g for 10 minutes at 4°C

27 Recover the aqueous phase (upper) in a new 1.5 ml LoBind Eppendorf tube

28 Add 1 volume of chloroform (i.e. 400 µl)

29 Centrifuge at 12,000 g for 10 minutes at 4°C.

30 Recover aqueous phase (upper) in a new 1.5 ml LoBind Eppendorf tube

31 Precipitate DNA by adding 1/10 volume of NaAcétate 3M pH5,2 (ie 40 µl) and 2,5 volume of EtOH 100% (ie 1 ml)

32 Incubate at −20°C from 45 minutes to overnight.

33 Centrifuge at 12000g for 30 minutes at 4°C to precipitate DNA.

34 Wash the pellet with 300 µl EtOH 70%.

35 Remove EtOH and dry pellets.

36 Elute in 20 µl Tris-HCl 10mM pH 8.0

Library preparation

37 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 10mM pH 8.0.
- LFB was used for final size selection step
- Quantify DNA using the Qubit dsDNA HS Assay Kit (Q33230) to check yield.

Sequencing

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