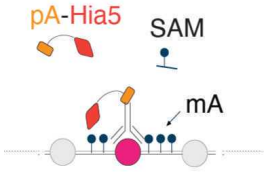
Dec 14, 2021 Version 2

DiMeLo-seq: Directed Methylation with Long-read sequencing V.2



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We use this protocol and it's working

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Abstract

Directed Methylation and Long-read sequencing (DiMeLo-seq) is a powerful method to map protein-DNA interactions at a single-molecule level across the genome (including repetitive regions). It can be multiplexed to analyze multiple base modifications at once (e.g. endogenous CpG methylation and directed pA-Hia5 adenine methylation). Additionally, PCR amplification is not necessary for this protocol, which means that sequencing readout is proportional to protein-DNA interaction frequency. Finally, DiMeLo-seq can be used to map multiple protein interactions across a long single molecule.

Materials

HEPES-KOH [M] 1 Molarity (M) pH 7.5 (Boston BioProducts BBH-75-K)

NaCl [M] 5 Molarity (M) (Sigma-Aldrich 59222C-500ML)

Spermidine [M] 6.4 Molarity (M) (Sigma-Aldrich S0266-5G)

Roche cOmplete™ EDTA-free Protease Inhibitor Tablet (Sigma-Aldrich 11873580001)
Bovine Serum Albumin (Sigma-Aldrich A6003-25G)
Digitonin (Sigma-Aldrich 300410-250MG)
Tween-20 (Sigma-Aldrich P7949-100ML)
KCl (Sigma-Aldrich PX1405-1)

EDTA [M] 0.5 Molarity (M) pH 8.0 (Invitrogen 15575-038)

EGTA [M] 0.5 Molarity (M) pH 8.0 (Fisher 50-255-956)

S-Adenosylmethionine [M] 32 millimolar (mM) (NEB B9003S)

PFA, 16% (if fixing) (EMS 15710)
Glycine (if fixing) (Fisher BP381-1)

Eppendorf DNA LoBind tubes 1.5 mL (Fisher 022431021)

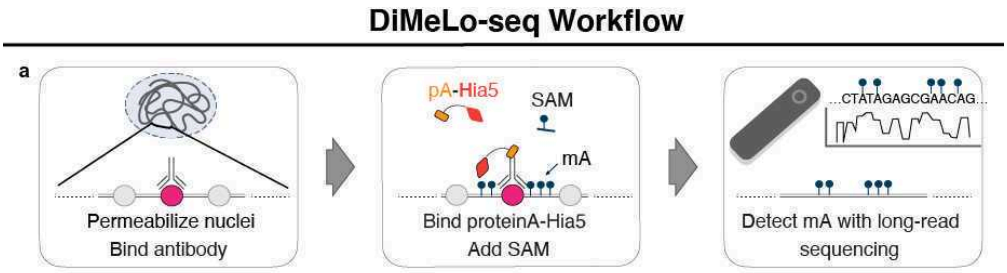
Wide bore 200 µl and 1000 µl tips (e.g. USA Scientific 1011-8810, VWR 89049-168)
pA-Hia5 (protocol for expression and purification available on protocols.io)
Primary antibody for protein target of interest, from species compatible with pA (e.g. Abcam ab16048)
Secondary antibody for immunofluorescence quality control (e.g. Abcam ab3554)
Trypan Blue (Fisher T10282)
Monarch® Genomic DNA Purification Kit (NEB T3010S)
Qubit dsDNA BR Assay Kit (Fisher Q32850)
Qubit Protein Assay Kit (Fisher Q33211)
Agencourt AMPure XP beads (Beckman Coulter A63881)
Blunt/TA Ligase Master Mix (NEB M0367S)
NEBNext quick ligation module (NEB E6056S)
NEBNext End Repair dA-tailing Module (NEB E7546S)
NEBNext FFPE DNA repair kit (NEB M6630S)
RNAse cocktail (Thermo AM2286)
Monarch Genomic DNA Purification Kit (NEB T3010S)
Monarch HMW DNA Extraction Kit (NEB T3050L)
Ligation Sequencing Kit (ON SQK-LSK109, ON SQK-LSK110)
Native Barcoding Expansion 1-12 (ON EXP-NBD104)
Native Barcoding Expansion 13-24 (ON EXP-NBD114)
Circulomics Short Read Eliminator Kit (SS-100-101-01)
Flow Cell Wash Kit (ON EXP-WSH004)
Flow cells (ON FLO-MIN106.1)

Troubleshooting

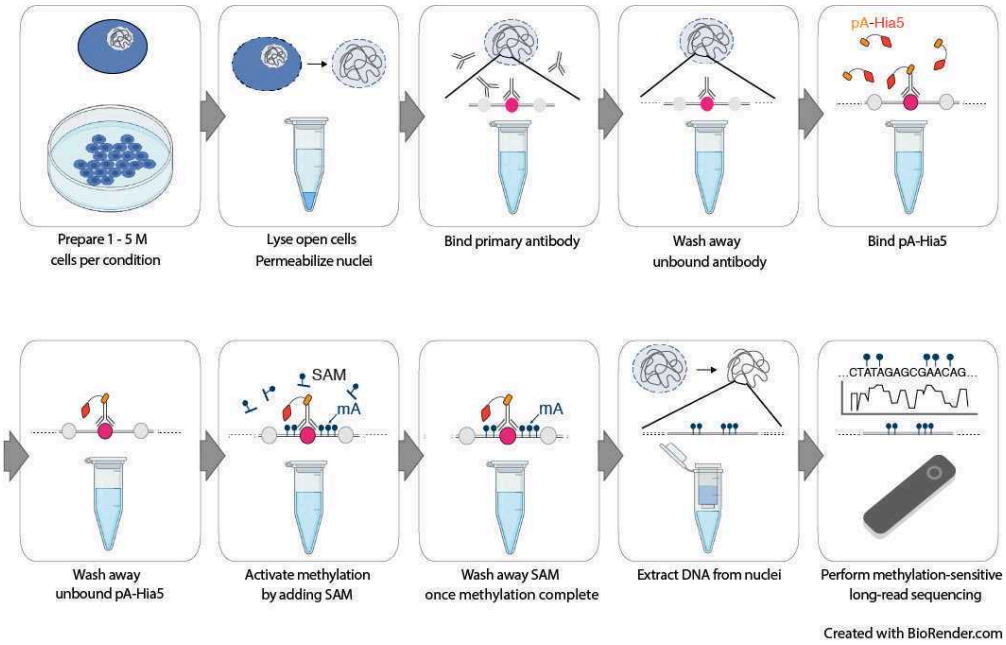
Introduction

1

This protocol is part of the broader Directed Methylation and Long-read sequencing (DiMeLo-seq) method. DiMeLo-seq targets a protein A-Hia5 methyltransferase fusion protein (pA-Hia5) to a protein of interest (using an antibody). Hia5 methylates adenines in the vicinity of the protein, the DNA is extracted, and long-read sequencing with modified base-calling is performed. Overall, this allows for the determination of protein location throughout the genome (including at repetitive regions) on a single-molecule level. This protocol is specifically used for the application of DiMeLo-seq *in situ*, on permeabilized nuclei.



General schematic of the DiMeLo-seq workflow for the mapping of DNA-protein interactions.



More detailed schematic of the DiMeLo-seq in situ methylation protocol, which involves a series of binding steps and washes followed by DNA extraction and sequencing.

Reagent Preparation

2 **General Notes**

- Prepare all reagents fresh
- Keep all reagents on ice
- Filter all buffers through a 0.2 µm filter

2.1 **Digitonin:**

Solubilize digitonin in preheated 95ºC Milli-Q water to create a 5% digitonin solution (e.g. 10 mg/200 µl).

2.2 **Wash Buffer:**

Prepare wash buffer with the following amounts/concentrations.

	Component	Amount	Final Concentration
	HEPES-KOH, 1M, pH 7.5	1 mL	20 mM

	Component	Amount	Final Concentration
	NaCl, 5M	1.5 mL	150 mM
	Spermidine 6.4M	3.91 uL	0.5 mM
	Roche cOmplete™ EDTA-free Protease Inhibitor Tablet	1 tablet	NA
	BSA	50 mg	0.1%
	H2O	fill to 50 mL	NA

2.3 **Dig-Wash Buffer:**

Add 0.02% digitonin to wash buffer. For example, add 20 µL of 5% digitonin solution to 5 mL wash buffer.

2.4 **Tween-Wash Buffer:**

Add 0.1% Tween-20 to wash buffer. For example, add 50 µL Tween-20 to 50 mL wash buffer.

2.5 **Activation Buffer:**

Create the activation buffer, but wait to add SAM until the activation step.

	Component	Amount	Final Concentration
	Tris, 1 M, pH 8.0	750 uL	15 mM
	NaCl, 5 M	150 uL	15 mM
	KCl, 1 M	3 mL	60 mM
	EDTA, 0.5 M, pH 8.0	100 uL	1 mM
	EGTA, 0.5 M, pH 8.0	50 uL	0.5 mM
	Spermidine, 6.4 M	0.391 uL	0.05 mM
	BSA	50 mg	0.1%
	H2O	fill to 50 mL	NA

	Component	Amount	Final Concentration
	SAM, 32 mM	(add at activation step)	800 uM

General Notes

3

- All spins are at 4ºC for 3 minutes at 500 x g.
- Spinning in a swinging bucket rotator can help pellet the nuclei.
- To prevent nuclei from lining the side of the tube, break all spins into two parts: 2 minutes with the tube hinge facing inward, followed by 1 minute with the tube hinge facing outward.
- Working with Eppendorf DNA LoBind tubes can reduce loss of material.
- Use wide bore tips when working with nuclei.
- Do not use NP-40 or Triton-X100, as they appear to dramatically reduce methylation activity.
- The best digitonin concentration may vary by cell type. For HEK293T, GM12878, and HG002 cells, 0.02% works well. You can test different concentrations of digitonin and verify permeabilization and nuclear integrity by Trypan blue staining. For example, you may try 0.02% to 0.1% digitonin.
- We use Tween to reduce hydrophilic non-specific interactions and BSA to reduce hydrophobic non-specific interactions. We've also found BSA at the activation step significantly increases methylation activity as well.
- The best primary antibody concentration may vary by protein target of interest. A 1:50 dilution works well for targeting LMNB1, CTCF, and CENP-A, but this may need to be varied depending on target abundance and antibody specificity.
- A secondary antibody binding step following primary antibody binding and before pA-Hia5 binding reduced total methylation and specificity. Including a secondary antibody binding step is not recommended.

Optional Fixation

4 **Fixation is optional - not necessary for completion of protocol.**

4.1 Resuspend cells in PBS.

4.2 Add PFA to 0.1% (e.g. 6.2 µl of 16% PFA to 1 ml cells) for 2 minutes while gently vortexing.

4.3 Add 1.25 M glycine (sterile; 0.938 g in 10 ml) to twice the molar concentration of PFA to stop the crosslinking (e.g. 60 µl of 1.25 M glycine to 1 ml).

4.4 Centrifuge 3 minutes at 500 x g at 4°C and remove the supernatant.

4.5 Resuspend the fixed cells in Dig-Wash buffer (Nuclear Isolation, step 5.3).

Nuclear Isolation

5 **Extraction and isolation of nuclei**

5.1 Prepare cells (1M-5M per condition).

5.2 Wash cells in PBS. Spin and remove supernatant.

5.3 Resuspend cells in 1 ml Dig-Wash buffer. Incubate for 5 minutes on ice.

5.4 Split nuclei suspension into separate tubes for each condition.

5.5 Spin and remove supernatant.

5.6 **Quality Control:**

Check permeabilization was successful by taking 1 µl of the nuclei following the 5-minute incubation on ice, diluting to 10 µl with PBS, and staining with Trypan Blue. Alternatively, fix with 1% PFA for 2 minutes at room temperature and wash with Tween-Wash. Spin and remove supernatant. Resuspend in fluoromount with 1:500 Hoechst.

Primary Antibody Binding

6 **Bind the primary antibody targeted to your protein of interest.**

6.1 Gently resolve each pellet in 200 µl Tween-Wash containing primary antibody at 1:50 or the optimal dilution for your antibody and target.

6.2 Place on rotator at 4°C for ~2 hr.

6.3 Spin and remove supernatant.

6.4 Wash twice with 0.95 ml Tween-Wash. For each wash, gently and completely resolve the pellet. This may take pipetting up and down ~10 times. Following resuspension, place on rotator at 4°C for 5 minutes before spinning down.

Quantify Protein Concentration

7 **Quantify concentration of pA-Hia5 for use in step 8 (pA-Hia5 binding).**

7.1 Thaw protein from -80°C at room temperature and then move to ice immediately.

7.2 Spin at 4°C for 10 minutes at 10,000 x g or higher.

7.3 Transfer the supernatant to a new tube and save it, discarding the previous tube .

7.4 Use Qubit with 2 µl sample volume to quantify protein concentration.

pA-Hia5 Binding

8 **Bind pA-Hia5 to antibody. Protocol to express and purify the pA-Hia5 protein can be found on protocols.io under the title "pA-Hia5 Protein Expression and Purification".**

8.1 Gently resolve pellet in 200 µl Tween-Wash containing 200 nM pA-Hia5. See protein quantification protocol above (Step 7).

8.2 Place on rotator at 4°C for 2 hrs.

8.3 Spin and remove supernatant.

8.4 Wash twice with 0.95 ml Tween-Wash. For each wash, gently and completely resolve the pellet. Following resuspension, place on rotator at 4°C for 5 minutes before spinning down.

Quality Control

9 **Quality control of steps completed so far, using a secondary antibody for immunofluorescence.**

Note: Can fix first or last (just before mounting)

9.1 Add 1.6 µl of 16% PFA to 25 µl of nuclei in Tween-Wash (taken from the 0.95 ml final wash) for 1% total PFA concentration.

9.2 Incubate at room temperature for 5 minutes.

9.3 Add 975 µl of Tween-Wash to stop the fixation by dilution.

9.4 Add 1 µl secondary antibody.

9.5 Put on rotator for 30 minutes at room temperature, protected from light.

9.6 Wash 2 times (or just once). Pellet likely won't be visible.

9.7 Resuspend in mounting media after last wash. Use as little as possible, ideally 5 µl.

9.8 Put 5 µl on a slide, make sure there are no bubbles, and put on a coverslip.

9.9 Seal with nailpolish along the edges.

9.10 Image or put at -20°C once the nail polish has dried.

Activation

10 **Activation of Hia5 MTase activity using S-adenosylmethionine (SAM).**

10.1 Gently resolve pellet in 100 µl of Activation Buffer per sample. Be sure to add SAM to 800 µM to the activation buffer at this step!

10.2 Incubate at 37°C for 2 hrs. Replenish SAM by adding 800 µM at 1 hr. Pipet mix every 30 minutes.

10.3 Spin and remove supernatant. Alternatively, add proteinase K and RNase A directly to the activation reaction before column extraction.

10.4 Resuspend in 100 µl cold PBS. Alternatively, add proteinase K and RNase A directly to the activation reaction before column extraction.

10.5 **Quality control:**
Check nuclei by Trypan blue staining to determine recovery and check integrity of nuclei if desired.

DNA Extraction for N50 of ~17kb

11 **Extract DNA from nuclei.**

This protocol will result in N50 ~17 kb. If longer fragments are desired (N50 ~50-70 kb), refer to step 13 for the DNA extraction protocol that better preserves longer fragments.

11.1 Use the Monarch Genomic DNA Purification Kit. Follow protocol for genomic DNA isolation using cell lysis buffer. Include RNase A. NB. If fixation was performed, be sure to do the 56°C incubation for lysis for 1 hour (not just 5 minutes) to reverse crosslinks.

11.2 Perform two elutions: 100 µl and then 35µl.

11.3 Quantify DNA yield by Qubit dsDNA BR Assay Kit.

11.4 Concentrate by speedvac if necessary for 3 µg DNA in 48 µl for input to library prep.

Library Preparation and Sequencing Run for N50 of ~17kb

12 **Perform library preparation on DNA from previous steps and start sequencing run.**

This protocol will result in N50 ~17 kb. If longer fragments are desired (N50 ~50-70 kb), refer to step 14 for library preparation protocol that better preserves longer fragments. If you are not multiplexing samples, then use LSK-110. LSK-109 is only used to barcode for multiple samples on a flowcell.

Follow Nanopore protocol for Native Barcoding Ligation Kit 1-12 and Native Barcoding Ligation Kit 13-24 with the following modifications:

12.1 Load ~3 µg DNA into end repair.

12.2 Incubate for 10 minutes at 20°C for end repair instead of 5 minutes.

12.3 Load ~ 1 µg of end repaired DNA into barcode ligation.

12.4 Double the ligation incubation time to at least 20 minutes.

12.5 Elute in 18 µl instead of 26 µl following barcode ligation reaction cleanup to allow for more material to be loaded into the final ligation.

12.6 Load ~3 µg of pooled barcoded material into the final ligation. If needed, concentrate using speedvac to be able to load 3 µg into the final ligation.

12.7 Double the ligation incubation time to at least 20 minutes.

12.8 Make sure to use LFB (NOT ethanol) for the final cleanup.

12.9 Perform final elution in 13 µl EB. Take out 1 µl to dilute 1:5 for quantification by Qubit (and size distribution analysis by TapeStation / Bioanalyzer if desired).

12.10 Load ~1 µg of DNA onto the sequencer.

12.11 Bubbles will absolutely destroy pores and ruin runs; mix and spin down all flush/wash solutions really well to eliminate bubbles.

12.12 The Flow Cell Wash Kit can increase the throughput per flowcell with <1% carryover of pre-wash barcodes.

12.13 Spiking in more library + SQB + LB during a run, without a wash step, can also increase pore occupancy if it's low.

DNA Extraction for N50 of ~50-70kb

13 **Extract DNA from nuclei and preserve long fragments with N50 of ~50-70kb**.

13.1 Use the NEB Monarch HMW DNA Extraction Kit. Follow protocol for genomic DNA isolation using cell lysis buffer. Include RNase A. Perform lysis with 2000 rpm agitation. We have validated 2000 rpm gives N50 ~50-70 kb but if longer reads are desired we expect 300 rpm would work.

Apart from using a different kit, all of the steps for the long fragment DNA extraction are the same as the general protocol. To reiterate, make the following changes to the protocol:

13.2 If fixation was performed, be sure to do the 56°C incubation for lysis for 1 hour (not just 10 minutes) to reverse crosslinks. Agitate for 10 minutes and then keep at 56ºC without agitation for 50 minutes.

13.3 Quantify DNA yield by Qubit dsDNA BR Assay Kit.

13.4 Concentrate by speedvac if necessary for 3 µg DNA in 48 µl for input to library prep.

Library Preparation and Sequencing Run for N50 of ~50-70kb

14 **Perform library preparation on DNA from previous steps and start sequencing run.**

This is a specialized library preparation and sequencing run that **preserves long fragments and results in N50 ~50-70 kb**. This protocol was adapted from "DNA extraction and Nanopore library prep from 15-30 whole flies" (Kim et al, dx.doi.org/10.17504/protocols.io.bdfqi3mw)

Follow Nanopore protocol for Ligation Sequencing Kit with the following modifications:

14.1 Increase end preparation time to 1 hour with a 30 minute deactivation.

14.2 Following end preparation, perform a cleanup by combining 60 µL SRE buffer from Circulomics (SS-100-101-01) with the 60 µL end prep reaction.

14.3 Centrifuge this reaction at 10,000 x g at room temperature for 30 minutes (or until DNA has pelleted).

14.4 Wash pelleted DNA with 150 µL of 70% ethanol two times, using a 2 minute spin at 10,000 x g between washes.

14.5 Resuspend the pellet in 31 µL EB.

14.6 Incubate at 50ºC for 1 hour. Incubate at 4ºC for at least 48 hours.

14.7 For the ligation step, reduce ligation volume by half (total of 30 µL DNA in a 50 µL reaction volume). Increase the ligation incubation to 1 hour.

14.8 Pellet DNA at 10,000 x g at room temperature for 30 minutes.

14.9 Wash the pellet twice with 100 µL LFB, using a 2 minute spin at 10,000 x g between washes.

14.10 Resuspend the pellet in 31 µL EB.

14.11 Incubate at least 48 hours at 4ºC.

14.12 Load 500 ng of DNA onto the sequencer.

14.13 Bubbles will absolutely destroy pores and ruin runs; mix and spin down all flush/wash solutions really well to eliminate bubbles.

14.14 Use the Flow Cell Wash Kit (it can increase the throughput per flowcell with <1% carryover of pre-wash barcodes).

14.15 Reload the sequencer every 24 hours.