Chapter 12

Genome-Wide Mapping and Microscopy Visualization of Protein–DNA Interactions by pA-DamID

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Abstract

Several methods have been developed to map protein–DNA interactions genome-wide in the last decades. Protein A-DamID (pA-DamID) is a recent addition to this list with distinct advantages. pA-DamID relies on antibody-based targeting of the bacterial Dam enzyme, resulting in adenine methylation of DNA in contact with the protein of interest. This ^{m6}A can then be visualized by microscopy, or mapped genome-wide. The main advantages of pA-DamID are an easy and direct visualization of DNA that is in contact with the protein of interest, unbiased mapping of protein–DNA interactions, and the possibility to select specific subpopulations of cells by flow cytometry before further sample processing. pA-DamID is particularly suited to study proteins that form large chromatin domains or that are part of distinct nuclear structures such as the nuclear lamina. This chapter describes the pA-DamID procedure from cell harvesting to the preparation of microscopy slides and high-throughput sequencing libraries.

Key words Protein A-DamID (pA-DamID), Protein–DNA interactions, Genome-wide mapping, Fluorescence microscopy, ^{m6}A-tracer, High-throughput sequencing, Chromatin, Nuclear lamina

1 Introduction

Mapping of protein binding sites in the genome is an essential tool in the fields of chromatin biology and gene regulation. Chromatin immunoprecipitation followed by high-throughput sequencing has been the most widely used method for many years, but various alternatives have been developed, each one with its own strengths and weaknesses (reviewed in [1–3]). Protein A-DamID (pA-DamID) is a newly developed alternative, which combines the versatility of antibody-based detection with the principles of the DamID technology [4, 5].

pA-DamID is an implementation of a protein A-based profiling method, following the principles of ChIC and CUT&RUN [6, 7]. In these methods, permeabilized cells are incubated with an antibody against a protein of interest, which in turn is tagged with a fusion of protein A and micrococcal nuclease. Subsequent

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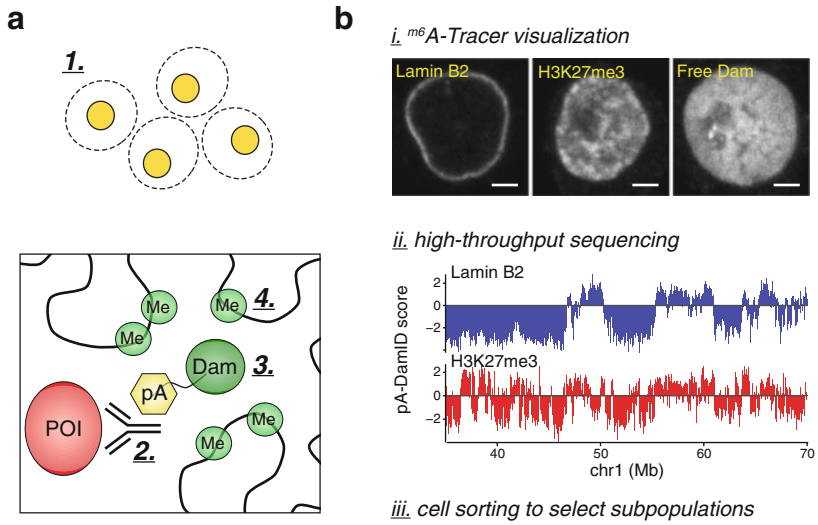

Fig. 1 Overview of the pA-DamID method. (Adapted with permission from [8]). (**a**) An overview of the steps in a pA-DamID experiment. Cells are permeabilized with digitonin (*step 1*) and incubated with a primary antibody against a protein of interest (POI) (*step 2*). This antibody is in turn bound by the pA-Dam fusion protein (*step 3*). Addition of SAM initiates adenine methylation deposition at nearby DNA (*step 4*). (**b**) An overview of the downstream possibilities after a pA-DamID experiment. The ^{m6}A modifications can be visualized in situ with the ^{m6}A-Tracer (*option i*). Example patterns are shown for Lamin B2 (Abcam ab8983, mouse, 1:100 dilution; combined with the bridging antibody Abcam ab6709 (*see* **Note 14**)) and H3K27me3 (CST C36B11, rabbit, 1:100 dilution) in human HAP-1 cells. Additionally, the ^{m6}A distribution of the free Dam control is shown (*see* **Note 9**). Scale bar corresponds to 2 μm. DNA can also be extracted and processed for high-throughput sequencing to infer protein interactions from the ^{m6}A pattern (*option ii*). Example data tracks are shown for the Lamin B2 and H3K27me3 experiments illustrated above. The pA-DamID score is defined as a $\log_2$-ratio of the target over the free Dam control. Finally, samples can be FACS sorted to select specific subpopulations before downstream sample processing (*option iii*) (*see* **Note 17**)

activation of the latter enzyme results in fragmentation of nearby DNA. These fragments are then identified by high-throughput sequencing. pA-DamID instead utilizes a fusion of protein A and DNA adenine methyltransferase (Dam). After addition of its methyl donor *S*-adenosyl-methionine (SAM) this results in ^{m6}A modifications on GATC sequences in proximity of the protein of interest (see Fig. 1a) [8].

The labeled cells can then be processed in three ways (see Fig. 1b). First, the methylated DNA can be visualized in situ with the ^{m6}A-Tracer, which consists of a ^{m6}A binding domain fused to a fluorescent protein [9]. This provides a quick visual check of the subnuclear location and intensity of the ^{m6}A signal. This serves as a quality control step, and in some applications can provide new biological insights. Second, isolated genomic DNA can be used to generate genome-wide binding profiles using the DamID library preparation [10]. Third, specific subpopulations of cells can be selected by fluorescence-activated cell sorting (FACS) prior to the

genome-wide mapping. Due to the sensitivity of the DamID library preparation protocol, only a few thousand sorted cells were sufficient for genome-wide binding profiles of nuclear lamina interactions [8].

pA-DamID should be performed with several controls, as described in more detail in the protocol below. The most important control is a separate sample incubated with free Dam enzyme in the presence of SAM, which is used to control for chromatin accessibility and unspecific binding of Dam. This control is used to normalize the data obtained with pA-Dam, and thus corrects for biases due to variation in chromatin accessibility across the genome, ensuring that pA-DamID specifically captures *bona fide* sites that interact with the protein of interest. A similar control is implemented in conventional DamID [10, 11], but not in alternative protein A-based methods [6, 7, 12]. This makes pA-DamID especially suited for antibodies that target inaccessible DNA such as heterochromatin marks.

Because Dam can only label GATC sequences, which occur in most genomes on average every ~200–400 bp, pA-DamID is particularly suitable for the mapping of proteins that form domains of at least several kb; it may be less suitable for proteins with narrow binding sites such as transcription factors. So far, we have used the method successfully with antibodies that target different types of heterochromatin [8]; other applications need to be tested.

Combined, the control for chromatin accessibility with free Dam, the visual readout of ^{m6}A-marked DNA and the low cell number requirement for library preparation make pA-DamID a powerful method to study protein–DNA interactions of nuclear compartments and chromatin proteins that generally bind in large domains.

2 Materials

Use nuclease free H_2O for all DNA steps.

2.1 pA-DamID Localization and Activation

1. Dam activity mix: 1× MethylTransferase buffer supplemented with 80 μM SAM (both shipped with Dam enzyme if purchased from New England Biolabs).
2. MboI digestion mix: 1× NEB buffer 3 supplemented with 10 mM $MgCl_2$ and 5 units of MboI restriction enzyme (suggested supplier, New England Biolabs).
3. 1 M HEPES–KOH, pH 7.5: dissolve 23.8 g HEPES in 90 mL demineralized H_2O. Mix and adjust pH with KOH pellets to 7.5. Make up to 100 mL with demineralized H_2O. Sterilize by filtration and store up to several months at 4 °C.

4. 5% w/v digitonin: dissolve 12.5 mg digitonin (Millipore) in 250 µL demineralized H_2O (*see* **Notes 1** and **3**). Keep on ice.
5. Dig-Wash buffer: 20 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 0.02% w/v digitonin (*see* **Note 2**), 1× EDTA-free Protease Inhibitor Cocktail in demineralized H_2O. Prepare 50 mL for a routine pA-DamID experiment and keep on ice (*see* **Note 3**).
6. pA-Dam protein: purified pA-Dam protein (*see* **Note 4**).

2.2 Enrichment of ^{m6}A Labeled DNA and Preparation of Illumina Sequencing Library

For all master mixes, prepare 1.1× the required volume.

1. 50 µM DpnI adapter: mix equal volumes of the two adapter oligonucleotides at 50 µM (5′- CTAATACGACTCACTA TAGGGCAGCGTGGTCGCGGCCGAGGA and 5′-TCCTC GGCCGCG) in a microcentrifuge tube. Incubate for 5 min at 95 °C in a heat block. Turn off the heat block while keeping the tube inside to slowly cool the sample to below 50 °C. Aliquot and store at −20 °C.
2. 50 µM Y-shaped adapter: mix equal volumes of the two adapter oligonucleotides at 50 µM (5′- ACACTCTTTCCCTACAC GACGCTCTTCCGATCT and 5′-P- GATCGGAAGAGCA CACGTCT (*see* **Note 5**)) and anneal as described for the DpnI adapter.
3. Indexed P7 primers: 10 µM Illumina TruSeq primers (5′-CA AGCAGAAGACGGCATACGAG [8xN] GTGACTGGAGTT CAGACGTGTGCTCTTCCGATCT , where [8xN] is the sample-specific index sequence).
4. PCR clean-up magnetic beads: magnetic beads for DNA purification steps, for example CleanPCR beads, CleanNA.
5. DpnI digestion mix: 1 µL 10× CutSmart buffer, 0.5 µL DpnI (20 units/µL, suggested supplier, New England Biolabs), and 2 µL H_2O per sample. DpnI may be swapped with shrimp alkaline phosphatase (rSAP, 1 unit/µL, suggested supplier, New England Biolabs) in this mix, see Subheading 3.3, **step 3**.
6. DpnI adapter ligation mix: 0.25 µL of 50 µM DpnI adapter, 2 µL 10× ligation buffer, 0.5 T4 DNA ligase (5 units/µL, suggested supplier, Roche), and 7.25 µL H_2O per sample.
7. Methylation-specific PCR mix: 20 µL 2× MyTaq (Bioline), 1 µL 50 µM PCR primer (5′-NNNNGTGGTCGCGGCCGAG GATC), and 15 µL H_2O per sample.
8. End repair mix: 5 µL 10× end repair buffer, 5 µL dNTP, 5 µL ATP, 1 µL end repair enzyme mix (all reagents from Lucigen), and 9 µL H_2O per sample.

9. Klenow 3′A-overhang mix: 0.1 μL 100 mM dATP, 5 μL NEB buffer 2, 0.5 μL 50 units/μL Klenow Fragment exo- (50 units/μL), and 19.4 μL H_2O per sample.
10. Y-shaped adapter ligation mix: 1 μL 10× ligase buffer, 0.5 μL Y-shaped adapter, 0.5 μL T4 DNA ligase (5 units/μL), and 1.5 μL H_2O per sample.
11. Index PCR mix: 10 μL 2× MyTaq (Bioline), 0.5 μL 10 μM P5-primer (5′- AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCCGATCT), and 1 μL H_2O per sample.

2.3 Visualization of ^{m6}A Labeled DNA

1. ^{m6}A-Tracer protein*:* purified ^{m6}A-Tracer protein (*see* **Note 4**).
2. 2% w/v formaldehyde: mix 13.25 mL PBS with 757 μL of 37% formaldehyde stabilized with methanol.
3. 0.5% v/v NP40: dilute 1 mL NP40 with 9 mL of PBS for a 10% NP40 stock that keeps several months at room temperature. Dilute 500 μL of this 10% stock with 9.5 mL PBS for a 0.5% NP40 solution.
4. 1% w/v BSA: dissolve 0.1 g BSA in 10 mL of PBS. Mix well to dissolve completely.
5. 1 mg/mL 4′,6-diamidino-2-phenylindole (DAPI): Dissolve 1 mg DAPI in 1 mL H_2O. Aliquot and store at −20 °C for several months.
6. Mounting medium: mounting medium to preserve fluorescence, for example Vectashield, Vector Laboratories.

2.4 Equipment and Labware

1. Refrigerated centrifuge (4 °C) for 1.5 mL microcentrifuge tubes.
2. Tube rotator for 1.5 mL microcentrifuge tubes at 4 °C, to gently rotate tubes during incubation steps (~20 rotations/minute).
3. Suction system to remove supernatant from 1.5 mL microcentrifuge tubes.
4. Heat block for 1.5 mL microcentrifuge tubes between 37 and 95 °C.
5. NanoDrop spectrophotometer or other labware to measure DNA concentration, for example a Qubit (Invitrogen).
6. PCR machine.
7. Illumina sequencing machine, for example, HiSeq 2500. Different adapters/primers may be required for different machines.

3 Methods

3.1 pA-Dam Activity Testing

1. The Dam activity of pA-Dam should be determined in activity units (*see* **Note 6**), and can be estimated by comparison with a calibration series of known activities. Incubate dilutions of NEB Dam enzyme (0.25, 1, 4 and 16 NEB units) and dilutions of pA-Dam protein (typically between 0.001 and 2 μL) with 500 ng of adenine unmethylated plasmid (*see* **Note 7**) in 20 μL of Dam activity mix for 30 min at 37 °C, followed by heat inactivation for 15 min at 65 °C.
2. To 10 μL of this reaction, add 40 μL MboI digestion mix. Incubate for 1 h at 37 °C and run 20 μL of the digestion on an 1% agarose gel.
3. Estimate the activity of pA-Dam by comparing the extent of MboI protection with NEB Dam units (*see* Fig. 2).

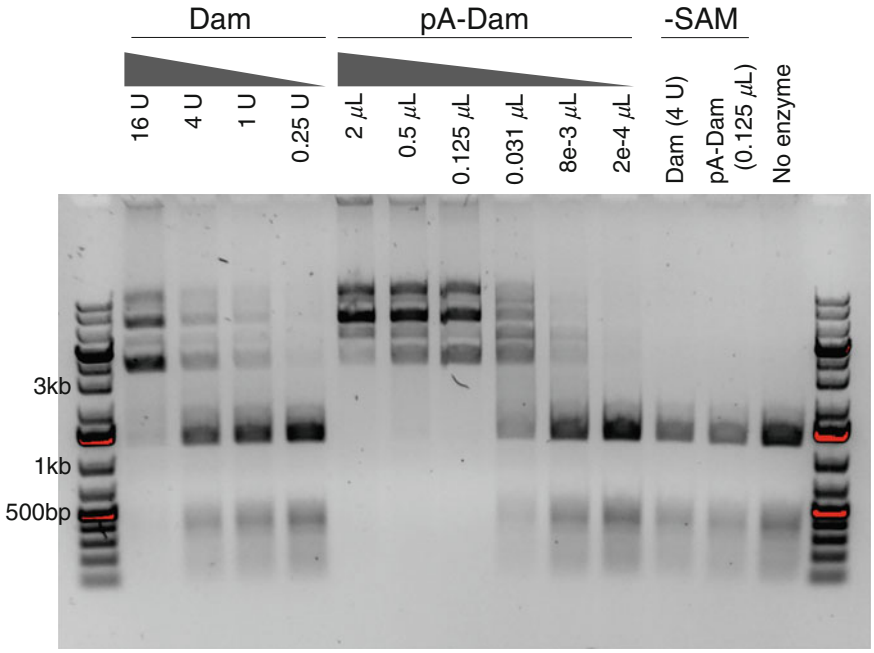

Fig. 2 Gel analysis to calibrate pA-Dam activity. An example gel image used to estimate Dam activity of purified pA-Dam protein. Unmethylated plasmid is incubated with consecutive dilutions of NEB Dam enzyme and pA-Dam protein (1.2 mg/μL). The amount of ^{m6}A methylation can be visualized by the extent of protection from the MboI restriction enzyme. A similar digestion pattern indicates a similar Dam activity, which for example can be seen between 1 unit of NEB Dam (U in the figure) and 8 × 10e−3 μL of pA-Dam. We thus estimated the activity of pA-Dam to be about 120 NEB units/μL. This corresponds to approximately 100 units/mg protein. Controls devoid of SAM should be unprotected and thus fully digested

3.2 pA-Dam Localization and Activation

Carry out all pA-DamID steps on ice unless otherwise specified. Use a P1000 pipette for 200 µL volumes.

1. Harvest cells (*see* **Note 8**).
2. For each epitope that needs to be mapped or visualized, prepare one million of unfixed cells (*see* **Notes 10** and **11**). Take control conditions into account and prepare additional cells accordingly (*see* **Note 9**). Centrifuge cells for 3 min at 500 × *g* in a precooled centrifuge (4 °C) and remove supernatant (*see* **Note 12**). Resuspend the cell pellet in 0.5 mL of ice-cold PBS in 1.5 mL microcentrifuge tubes. Repeat once, followed by resuspension in 0.5 mL of ice-cold Dig-Wash buffer. This washing procedure will be repeated throughout the protocol.
3. Centrifuge cells and resuspend the pellet in Dig-Wash buffer to reach a concentration of 5 M cells/mL. Divide samples between new tubes (200 µL for each antibody or control) and add the antibody of interest. For most commercial antibodies, a dilution of 1:100 is a good starting concentration for a strong signal, but can be optimized for a better dynamic range (*see* **Note 13**). Control experiments are incubated without any antibody. Rotate tubes for 2 h at 4 °C, followed by centrifugation and one wash with Dig-Wash.
4. Optionally, resuspend the cells in 200 µL Dig-Wash with a secondary anti-rabbit antibody (*see* **Note 14**), rotate for 1 h at 4 °C and wash once with Dig-Wash.
5. Next, resuspend the cells in 200 µL with 20–60 NEB units of pA-Dam. Rotate for 1 additional hour at 4 °C and wash two times with Dig-Wash.
6. Resuspend the cells in 100 µL of Dig-Wash supplemented with 80 µM SAM and incubate for 30 min at 37 °C to induce ^{m6}A methylation. For the Dam-only control, add an additional 0.5 µL of Dam enzyme to the sample and mix gently. At the end of incubation, return samples to 4 °C, centrifuge and remove supernatant (*see* **Note 15**).
7. Continue with library preparation in Subheading 3.3 (*see* **Note 16**), microscopy slides to visualize ^{m6}A methylation in Subheading 3.4 or selecting specific subpopulations by FACS before further sample processing (not discussed here, *see* **Note 17**).

3.3 Enrichment of ^{m6}A Methylated DNA and Preparation of Illumina Sequencing Library

1. Isolate genomic DNA (gDNA) and determine the concentration (*see* **Note 18**).
2. Mix between 10 and 500 ng of gDNA with H_2O to a total volume of 6.5 µL (*see* **Note 19**). Prepare at least one control with DpnI omitted and one control with Ligase omitted (Subheadings 3.3, **steps 3** and **5**). Additionally, include a negative control without any DNA.

3. (a) If cell death occurred or double strand DNA breaks were induced (*see* **Note 20**), replace DpnI in the DpnI digestion mix with 0.5 μL of rSAP and add 3.5 μL of this mix, incubate for 1 h at 37 °C and heat inactivate for 10 min at 65 °C. Then add 0.5 μL of DpnI and continue with the incubation in **step 4**.

   (b) If rSAP was not used, add 3.5 μL of the DpnI digestion mix and continue with the incubation in **step 4**.

4. Incubate for 8 h at 37 °C, followed by 20 min of heat inactivation at 80 °C.

5. On ice, add 10 μL of the DpnI adapter ligation mix to every sample and incubate for 16 h at 16 °C, followed by 10 min of heat inactivation at 65 °C.

6. Add 4 μL of the ligation reaction to 36 μL of Methylation-specific PCR mix in PCR tubes. Amplify for 13–23 PCR cycles with the following cycling scheme: 8 min of 72 °C, 13–23 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 20 s and a final extension at 72 °C for 2 min (*see* **Note 21**).

7. Run 4 μL of each sample on a 1% agarose gel to check for the presence of amplified material. This will appear as a smear between 250 and 1500 bp (*see* Fig. 3 and **Note 22**).

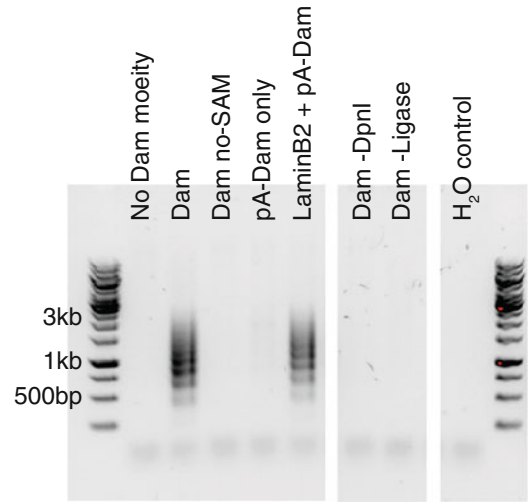

Fig. 3 Gel analysis of amplified ^{m6}A-labeled DNA fragments. (Reproduced with permission from [8]). DNA isolated from a typical pA-DamID experiment was digested with DpnI and ligated to PCR adapters. After 15 PCR cycles, samples were run on an agarose gel to visualize amplification. The negative control, no-SAM control and samples without DpnI or Ligase during the DNA processing should be devoid of signal, and the sample without primary antibody should be significantly weaker than with primary antibody. Samples with clear signal can be further processed for high-throughput sequencing

8. Purify the samples that show a stronger signal compared to the control without primary antibody with PCR clean-up magnetic beads. Add 1.8× volumes of beads, and follow manufacturer's instructions. Elute in 25 μL H_2O (*see* **Note 23**). Alternatively, use a spin column-based PCR purification kit to extract the DNA fragments from the PCR mixture.
9. Add 25 μL of End repair mix and incubate for 45 min at room temperature. Immediately proceed with DNA purification as described in **step 8**.
10. Add 25 μL of Klenow 3′A-overhang mix and incubate for 30 min at 37 °C, followed by 20 min of heat inactivation at 75 °C. Purify DNA as described in **step 8**, but elute in 20 μL of H_2O (*see* **Note 24**). Determine the DNA concentration.
11. Mix 220–250 ng of DNA from **step 10** with H_2O to a total volume of 6.5 μL. Add 3.5 μL of Y-shaped adapter ligation mix and incubate for 16 h at 16 °C, followed by 10 min of heat inactivation at 65 °C. As an additional negative control, include at least one sample with Ligase omitted. Purify DNA as described in **step 8** but elute in 20 μL of H_2O.
12. To 11.5 μL of Index PCR mix, add 8 μL of DNA from **step 11** and 0.5 μL of indexed P7 sequencing primer. Amplify for 9–-12 cycles with the following cycling scheme: 1 min of 94 °C, 9–12 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 2 min (*see* **Note 25**).
13. Run 4 μL of each sample on a 1% agarose gel to check for the presence of amplified DNA.
14. Estimate the intensity on gel and pool the samples accordingly to achieve an even representation in the sequencing library. Purify fragments with 1.6× beads as described in **step 8**. Run a small amount of purified DNA on gel to confirm removal of primers.
15. High-throughput sequencing on an Illumina machine (*see* **Note 26**).

3.4 Visualization of ^{m6}A Labeled DNA

All steps are performed at room temperature unless otherwise stated. Keep samples in the dark when fluorescent molecules are present. Prevent drying out of samples by using one hand to remove liquid (i.e., using suction) and adding new liquid with the other hand.

1. Resuspend cells from Subheading 3.2, **step 7** in PBS and incubate on poly-L-lysine–coated coverslips for 30 min on ice, at a cell density of approximately 0.1 million cells/cm^2 (*see* **Notes 27** and **28**).
2. At room temperature, remove PBS and add 2% formaldehyde in PBS, incubate for 10 min and wash with PBS (*see* **Note 29**).

3. Permeabilize cells with 0.5% NP40 in PBS for 20 min (*see* **Note 30**).
4. Block unspecific protein binding sites with 1% BSA in PBS for 30–60 min.
5. Optionally, incubate with primary antibodies in 1% BSA for 1 h (*see* **Note 31**) and wash 3 times with PBS.
6. Incubate with ^{m6}A-Tracer protein and optionally secondary antibodies for 30–60 min (*see* **Note 32**) and wash once with PBS.
7. Stain DNA with 1 μg/mL DAPI for 10 min and wash two times with PBS and one time with demineralized H_2O. Immediately continue with **step 8**.
8. Dry coverslips by touching the rim on a paper tissue and place upside down on a microscopy slides with a drop of mounting medium. Carefully remove excess mounting medium with a tissue.
9. Seal with nail polish and let dry.

4 Notes

1. Use prewarmed (~95 °C) H_2O to dissolve digitonin and mix by pipetting several times up and down. Wear gloves while handling digitonin and be aware of digitonin toxicity concerns.
2. We have successfully used 0.02% w/v digitonin in several human and mouse cell lines, but the optimal concentration can differ in other cell lines. Complete permeabilization is required for unbiased DNA labeling, while too much digitonin can result in nuclear disruption during the pA-DamID protocol. To determine the optimal concentration, incubate and wash cells once with Dig-Wash buffer with multiple digitonin concentrations. Induce ^{m6}A labeling as Dam control (see methods Subheading 3.2) and prepare microscopy slides to visualize ^{m6}A (see methods Subheading 3.4). The optimal digitonin concentration is the lowest concentration with complete and homogenous ^{m6}A labeling. Alternatively, DAPI entry can be used to quickly assess cell permeability, but given the small size of DAPI compared to antibodies this might overestimate the permeabilization.
3. Buffers should remain stable at 4 °C for up to a week, but we prefer to make fresh buffers for every experiment.
4. Plasmids encoding for the pA-Dam and ^{m6}A-Tracer proteins will be shared upon request for protein purification, for example as described in [8]. Additionally, an aliquot of purified protein for initial testing will be shared upon request.

5. This oligonucleotide requires 5′ phosphorylation for the ligation with A-tailed DNA.
6. NEB Dam units are defined as the amount of enzyme required to protect 1 μg of unmethylated Lambda DNA in 1 h at 37 °C in a total reaction volume of 10 μL against cleavage by MboI (see Dam NEB # M0222L). Dam activity units can also be estimated by a direct comparison with NEB Dam enzyme in different conditions as described here. We typically purify pA-Dam protein with an estimated Dam activity around 100 NEB units/mg protein.
7. Unmethylated plasmid DNA can be isolated from Dam-negative bacteria (i.e., NEB catalog # C2925H) and verified by DpnI and DpnII/MboI digestions. Alternatively, other large fragments of unmethylated DNA can be used to assess Dam activity, including mammalian genomic DNA.
8. Adherent cells can be harvested by trypsinization or scraping, while suspension cells can simply be collected and washed with PBS.
9. Three controls are important for pA-DamID experiments. First, a negative control without antibody or Dam added, which should be completely devoid of any ^{m6}A signal. Second, a pA-DamID sample without primary antibody added. This sample can be used to determine potential background binding of pA-Dam. Third, a sample without antibody or pA-Dam, but with Dam enzyme added during the activation step. This latter control should be included for every cell culture condition, as it will be used to control for DNA accessibility and other possible biases.
10. We have tried pA-DamID with formaldehyde fixation (1% formaldehyde for 5–15 min at room temperature) prior to permeabilization with digitonin. However, this resulted in unspecific binding of pA-Dam. pA-DamID might be compatible with other fixation protocols. A consequence of using unfixed cells is that some epitopes are difficult to map with pA-DamID when these are unstable and lost during the permeabilization and washing steps.
11. pA-DamID relies on multiple rounds of centrifugation and supernatant removal to wash the cells. It is possible to work with fewer than one million cells, but this results in near-invisible pellets. We have successfully performed pA-DamID with 0.1 million starting cells. Reduce the cell concentration in **step 3** accordingly to keep working with 200 μL volumes.
12. After 3 minutes of centrifuging, turn the tubes 180° and centrifuge for a few additional seconds at 500 × *g*. This prevents accumulation at the side of the tube and reduces loss of cells during suction.

13. The antibody optimization strategy depends on the protein of interest. Ideally, the enrichment of ^{m6}A-Tracer intensity is used if the protein is localized in distinct nuclear patterns. Alternatively, the optimal antibody dilution can be estimated by selecting the concentration that gives the highest dynamic range after high-throughput sequencing.
14. Protein A has a high binding affinity for rabbit IgG antibodies, but low affinity for antibodies from mouse and some other species. Binding of a secondary rabbit antibody is required in when Protein A has low binding affinity for the primary antibody. We have successfully used 1:100 dilutions of rabbit anti-mouse (Abcam # ab6709) and rabbit anti-goat antibodies (Abcam # ab6697) as bridging antibodies.
15. This is a good moment to take a few cells and look at them with a phase contrast microscope. Suspension cells and trypsinized cells should result in round and intact cells at this stage, while scraped cells remain clustered in aggregates.
16. The pellets can be frozen at −20 °C for several weeks before isolation of genomic DNA.
17. We have performed propidium iodide staining followed by FACS sorting to purify G1, mid-S and G2/M subpopulations [8]. A modified single-cell protocol was used to prepare sequencing libraries from 3000 sorted cells per condition. We assume that other sorting strategies will be feasible as well, for example based on fluorescent proteins, the intensity of ^{m6}A, or labeling of other epitopes.
18. We use commercial kits (Bioline, Qiagen and Invitrogen) to isolate gDNA and determine the concentration with a NanoDrop spectrophotometer.
19. When available, we recommend to use 500 ng of input DNA. A high-quality data set can be obtained from as little as 10 ng, corresponding to gDNA from roughly 1000 diploid human cells. However, the complexity of the sequencing library is generally lower with limited input DNA and results in fewer unique reads. This can be partially rescued by running multiple PCR reactions to utilize all input DNA (i.e., 4× reactions instead of 1×, see methods Subheading 3.3, **step 6**) and combining these afterward. For samples that will be compared directly in downstream analyses, it is important to use the same amount of starting DNA and an identical number of PCR cycles to prevent biases.
20. Apoptotic cells and cells treated with DNA-damaging agents contain fragmented DNA that will be ligated to the adapter and thus amplified independently of DpnI. Such undesired ligation events can be prevented by dephosphorylation of the genomic DNA prior to DpnI digestion.

21. For antibodies against broad histone modifications and the nuclear lamina, we generally achieve strong signals after 15 PCR cycles with 500 ng input DNA. The number of PCR cycles can be increased in case of reduced input DNA or the use of antibodies against less abundant epitopes. Note that the control without primary antibody generally results in a strong smear after 21 PCR cycles with 500 ng of input DNA. This control is thus particularly important for antibodies that bind sparsely on the genome or not efficiently to the epitope. If kept on ice, additional cycles can be added to the PCR reaction after running 4 μL on gel.
22. Here, it is important to verify that the negative control samples (those lacking any Dam or pA-Dam, and those lacking DpnI or Ligase) are devoid of signal, and that the no-antibody control is significantly weaker than the sample with antibody.
23. The amplified PCR fragments were originally ^{m6}A methylated and can be processed for Illumina sequencing as described in the steps below. Alternatively, the fragments can be quantified with quantitative PCR [4] or processed for other purposes.
24. A-tailing on DNA is unstable. Freeze and thaw cycles on A-tailed DNA should be avoided.
25. Optimization is required for this PCR. We typically use 10 PCR cycles, which gives a clear signal on gel. Too many cycles may negatively affect sequencing efficiency.
26. We typically sequence pA-DamID libraries with 65 bp single-end reads, which after adapter removal results in 46 bp for genomic mapping. The sequencing depth ranges between 10 million reads for exploratory analyses to 40 million reads for high-quality profiles.
27. Poly-L-lysine–coated coverslips can be bought or prepared from poly-L-lysine solutions. The electrostatic interactions of poly-L-lysine are required for adhesion of the processed cells to the coverslips.
28. Gently shake the plate under a phase contrast microscope to verify that a large fraction of the cells has attached. Increase binding time if cells are insufficiently attached.
29. After fixation and two PBS washes, cover slips can be stored in PBS at 4 °C for several days, although some epitopes might be too unstable for long-term storage.
30. Even though digitonin is used for cell permeabilization in **step 2** of Subheading 3.2, incubation with NP40 is done here to ensure complete permeabilization.
31. Antibodies are not required to visualize ^{m6}A methylation. For visualization of the targeted protein, it is advised to add more of the antibody used in **step 3** of Subheading 3.2, as pA-Dam

masks the majority of the original antibody. Additional antibody probably results in binding to epitopes that have lost antibody binding during the pA-DamID protocol. Antibodies can be used to visualize other nuclear proteins, but take care not to mix antibody species with the antibody used in **step 3** of Subheading 3.2 to prevent undesired mixing of the protein visualization.

32. We use ^{m6}A-Tracer with a stock concentration of 1.15 mg/mL in a 1:500 dilution [8]. This dilution should be optimized for different batches of ^{m6}A-Tracer, as the fraction of functional protein can differ. The optimal concentration can be determined by staining pA-DamID cells processed with an epitope against a nuclear compartment (i.e., the nuclear lamina) with different concentrations of ^{m6}A-Tracer protein, and selecting the concentration that gives the highest enrichment at the compartment.

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