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Dam&ChIC

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Samy Kefalopoulou[1], Peter Zeller[1,2]

[1]Hubrecht Institute for Developmental Biology and Stem Cell Research;
[2]Aarhus University, Department of Molecular Biology and Genetics

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Samy Kefalopoulou
Hubrecht Institute for Developmental Biology and Stem Cell R...

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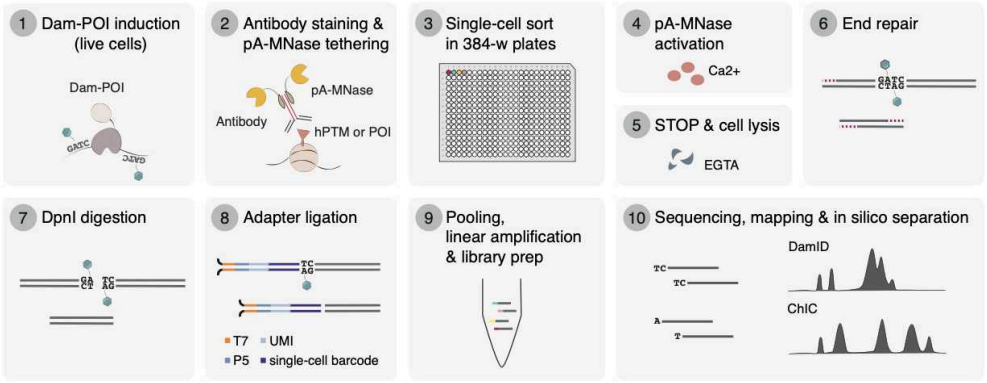
Abstract

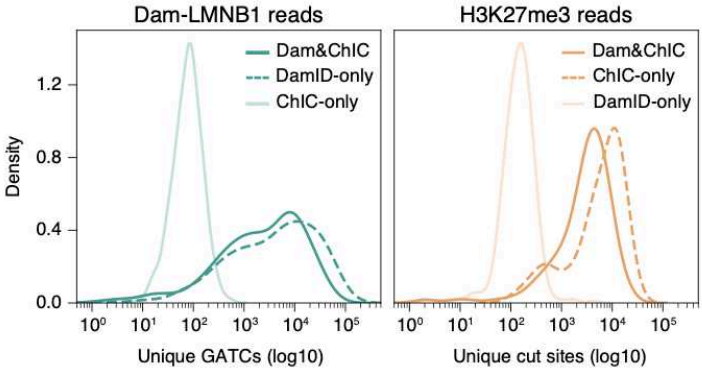
Dam&ChIC is a single-cell chromatin profiling method that allows for multifactorial and retrospective measurements of chromatin factors. It builds-upon and integrates scDamID and sortChIC, which are both methods for profiling of chromatin factors at high resolution in single cells.

Dam&ChIC combines the properties of its predecessor approaches, which are fundamentally different. In DamID chromatin states are recorded over time in living cells, due to the capacity of Dam to methylate the chromatin in its proximity with a stable exogenous to eukaryotes modification (m6A). On the other hand, ChIC captures the most recent chromatin state in fixed cells using antibody-mediated targeting of pA-MNase on the chromatin. Thus, Dam&ChIC provides a cumulative past readout by its DamID modality, coupled to a present-state readout by its ChIC modality. As such, the method can be used to (i) profile two different chromatin factors in single cells to dissect their interplay, or (ii) profile the same chromatin factor retrospectively and obtain insights into its dynamics over time.

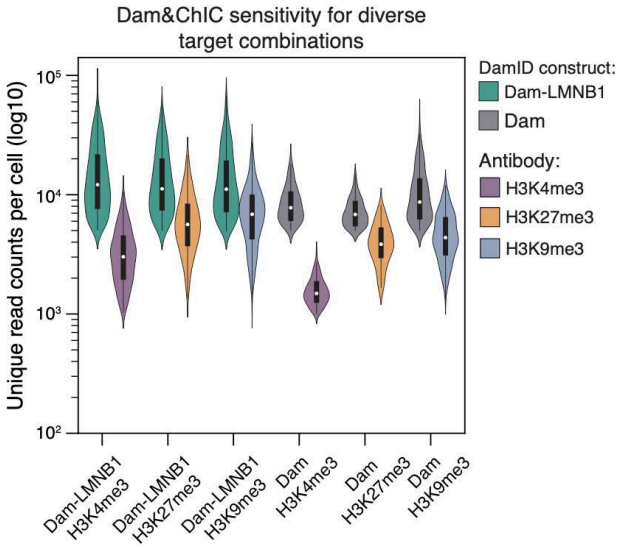
Here we describe the workflow of single-cell Dam&ChIC, including a strategy for fluorescence-based sample multiplexing ("hashing") with CellTrace dyes, that enables parallel processing of cells derived from multiple experimental conditions.



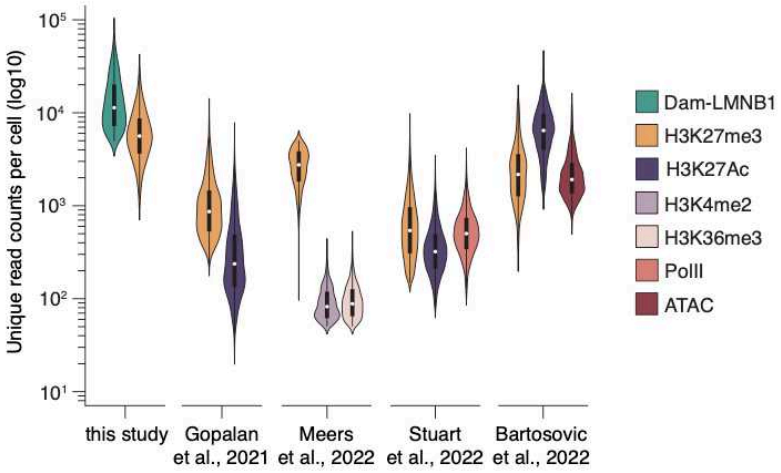
Schematic overview of Dam&ChIC



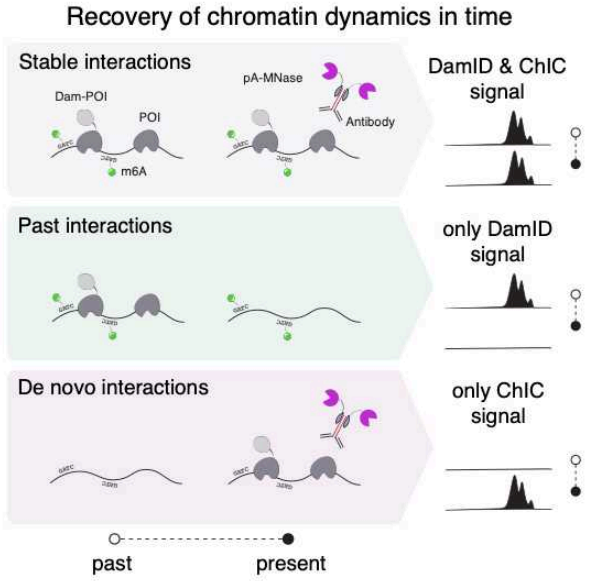
Confident read separation between the two readouts of Dam&ChIC. Control experiment in which Dam&ChIC, DamID-only and ChIC-only were performed in parallel against Dam-LMNB1 and H3K27me3.



Number of unique counts recovered per cell for multiple combinations of targets



Comparison of the recovery of unique reads per cell between Dam&ChIC and other single-cell multifactorial methods



Schematic showing the expected outcome of retrospective measurements with Dam&ChIC in different scenarios of chromatin dynamics

Attachments

DamChIC_Dispensions_..

21KB

Materials

Antibodies

- Rabbit polyclonal anti-Lamin B1 (Abcam, ab16048)
- Rabbit monoclonal anti-H3K27me3 (Cell Signaling Technologies, 9733S)
- Rabbit monoclonal anti-H3K9me3 RM389 (Thermofisher, MA5-33395)
- Rabbit monoclonal anti-H3K4me3 (Thermofisher, MA5-11199)
- Rabbit monoclonal anti-Histone H3 (Abcam, ab176842)
- Rabbit monoclonal anti-H2AK119Ub (Cell Signaling, D27C4)

Oligonucleotides

- Adapters, top and bottom oligonucleotides, set of 384 (Markodimitraki et al., 2020)

DamID2_adapters_384_top_bottom... 36KB

- Random hexRT primer

GCCTTGGCACCCGAGAATTCCANNNNNN

- Illumina RNA PCR primer 1 (RP1)
- Illumina RNA PCR index primers (RPI series)

RPI_primers.xlsx 10KB

Chemicals and Buffers

For cell culture/Dam-POI induction:

- Shield-1 (Glixx Laboratories Inc, GLXC-02939)
- Indole-3-acetic acid (IAA; Sigma, I5148)
- 4-Hydroxytamoxifen (4-OHT; Sigma, SML1666)

For sample preparation:

- PBS0
- Ethanol
- Saponin (Sigma, 47036-50G-F)
- Tween 20 (Sigma, P9416)
- cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, 11836170001)
- HEPES 1M (Gibco, 15630080)
- Spermidine Solution (Sigma, S2626-1G)
- Sodium Chloride (NaCl) 5M
- EDTA 0.5M
- Hoechst 34580 (Sigma-Aldrich, 63493-5MG)

For CellTrace stainings:

- CellTrace™ CFSE Cell Proliferation kit (Invitrogen, C34570)
- CellTrace™ Far Red Cell Proliferation kit (Invitrogen, C34572)
- CellTrace™ Yellow Cell Proliferation kit (Invitrogen, C34573)
- Rat Serum (Sigma, R9759)
- DMSO

For molecular processing:

- Calcium Chloride (CaCl2) 1M
- EGTA 0.5M
- Igepal CA-630 (Sigma, I8896-50ML)
- DTT 1M (Invitrogen, Y00147)
- 10X PNK buffer (NEB, B0201S)
- T4 Ligase buffer (Roche)
- dNTP set 100mM (Invitrogen, 10297018)
- Magnesium Chloride (MgCl2) 25mM (NEB, B9021S)
- ATP 10mM (NEB, P0756L)
- BSA Molecular Biology Grade 20 mg/ml (NEB, B9000S)
- PEG8000 50% (Promega, V3010)
- Mineral Oil (Sigma-Aldrich, M8410)
- Bead-binding buffer (1M NaCl, 20% PEG8000, 20mM Tris-HCl pH = 8, 1mM EDTA)
- Fragmentation buffer (500mM potassium acetate, 150mM magnesium acetate, 200mM Tris-acetate)
- CleanNGS DNA and RNA purification beads (GC Biotech, CNGS-0050)

Enzymes

- PA-MNase fusion recombinant protein, self-produced
- Proteinase K solution 20 mg/ml (Ambion, AM2548)
- DNA polymerase I, Large (Klenow) Fragment (NEB, M0210L)
- T4 Polynucleotide Kinase (NEB, M0201L)
- DpnI (NEB, R0176L)
- T4 DNA Ligase (Roche, DNALIG-RO)
- Superscript II Reverse Transcriptase (Thermofisher, 18064071)
- RNAseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
- Phusion High Fidelity 2X PCR mastermix (NEB)

Commercial Assays

- MEGAscript T7 transcription kit (Invitrogen, AMB13345)
- Agilent High Sensitivity DNA Assay (Agilent, 5067-4626)
- Agilent RNA 6000 Pico Assay (Agilent, 5067-1513)
- Qubit sdDNA High Sensitivity Assay (Invitrogen, Q32854)

Other

- Hard-Shell 384-well PCR plates (Bio-Rad, HSP3801 or PerkinElmer, 6008910)
- 384 PCR machines
- VBLOK200 Reservoir (Click-Bio, CBVBLOK200S)
- Protein LoBind tubes 0.5 ml (Eppendorf, 0030108094)
- Low-retention pipette tips (Greiner Bio-One)
- Polypropylene round bottom tubes 5 ml (Corning, 352002)
- PCR plate seals (Greiner, 676090)
- Qubit 4 fluorometer (Invitrogen)
- Agilent 2100 Bioanalyzer platform

FACS

- BD FACS Influx Cell Sorter System
- BD FACS Jazz Cell Sorter System

Robotic liquid handling

- Nanodrop II liquid handling platform (Innovadyne)
- Mosquito LV liquid handling platform (STP Labtech)
- Freedom EVO liquid handling platform (Tecan)

Sequencing

- Illumina NextSeq500 sequencing platform
- Illumina NextSeq2000 sequencing platform

Troubleshooting

Before start

Requirements prior to implementation of single-cell Dam&ChIC

- Expertise to engineer Dam-POI fusions into cell lines or model organisms (or access to such systems)
- Specialized equipment, such as robotic liquid handlers and FACS machines for single-cell sorting.

Considerations on throughout

As a plate-based method, Dam&ChIC enables parallel processing of thousands of cells with the use of liquid handlers. It has lower cell throughput compared to equivalent multifactorial chromatin methods that are based on microfluidics or combinatorial indexing, but it outperforms many of them in terms of target sensitivity (Kefalopoulou et al., 2025).

Advantages of FACS-based sorting

Dam&ChIC retains information on various cellular characteristics measured and recorded during FACS for each sorted cell. This offers a few advantages:

1. Selection of cells/nuclei of good quality, confident exclusion of doublets and, if desired, selection of cells at certain cell-cycle states defined by DNA staining (eg Hoechst).
2. DNA content of any sorted cell is recorded and can be later integrated with Dam&ChIC data to derive insights into the timing of chromatin dynamics relative to cell cycle state (Kefalopoulou et al., 2025)
3. Different samples/conditions (e.g. differentiation timepoints; drug treatments) can be multiplexed using CellTrace or equivalent fluorescent dyes, and sorted in appropriate formats to enable their parallel processing (Zeller et al., 2023, Yeung et al., 2023, Gaza et al. 2024, Kefalopoulou et al., 2025). This approach can considerably reduce batch effects and hands-on time.
4. Cell populations expressing known cell (surface) markers can be preferentially sorted (Zeller et al., 2023, Gaza et al., 2024).

Considerations on the time resolution provided by Dam&ChIC

Dam&ChIC's unique feature is measuring chromatin states in single cells in a retrospective manner. Retrospective measurements can be done for various chromatin targets undergoing dynamics that span a defined time window - from one replication cycle to the next (see Kefalopoulou et al. 2025 for a proof of principle application during mitosis). This unique feature of Dam&ChIC is useful, for example, when one wants to study the gradual binding or release of factors over time.

On the other hand, the different time frames recovered by the two modalities in Dam&ChIC might be a disadvantage for some applications. Because of the induction kinetics of Dam-POI fusions, certain chromatin events that happen extremely rapidly (i.e. in the time frame of minutes) may not be recovered by the DamID modality, but can always be recovered by ChIC or other snapshot-based methodologies.

Therefore prior to implementing Dam&ChIC, it is important to evaluate suitability to the research question, as well as accessibility to related equipment and expertise.

Depending on the research question, Dam&ChIC can be used in bulk to profile small cell populations of sorted cells (e.g. 20c-10000c), without the need of specialized equipment. The bulk protocol can be found in the Dam&ChIC collection.

NOTES on handling and equipment during the protocol:

- Upon permeabilization or fixation, samples should be kept at 4^{o}C, and whenever washes are involved, they should be done in a cooled centrifuge.
- To increase nuclei or fixed cell recovery after washes, we recommend the use of a swing-out cooled centrifuge instead of conventional one.
- Use low-binding tubes and low-retention pipette tips at all times when handling nuclei or fixed cells, in order to prevent losses.
- With every spin and discarding supernatant of nuclei or fixed cells, make sure to pipette carefully without disturbing the pellet. We do not recommend aspirating with a vacuum.
- This protocol makes use of self-produced pA-MNase. Targeting of the protein may need further optimizations when using different batches or a commercial enzyme.
- This protocol involves the use of a few robotic liquid handlers. Make sure you are trained for the use of such robots, and if using robots with fixed pipetting setups (e.g. fixed needles), make sure to follow cleaning procedures to prevent contaminations.
- Ensure that both bench and pipettes are decontaminated from RNases and DNases when handling pre-amplified material, and especially when handling amplified RNA.
- Ensure that stocks of reagents used for single-cell experiments are stored separately from others. Ideally, these should be opened under a benchtop hood that is used solely for handling of non-amplified single-cell material or related reagents, and can be UV-decontaminated.
- Programs on 384 PCR machines do not need a heated lid, as the reactions are done enclosed in mineral oil, which prevents evaporation.
- Make sure you are familiar with the process and principles of DNA/RNA cleanups using beads (eg AMPure/SPRI), as they are used extensively in this protocol.

Steps from nuclei isolation/cell fixation to FACS are common to **sortChIC** developed by **Zeller et al., 2023**, also described in detail by **Gaza et al., 2024**.

Preparation

1 **Preparation of ChIC buffers**

Wash buffer (WB0): the basic ChIC buffer for nuclei

	solution	volume	concentration in ChIC buffer
	Ultra-pure water	47.5 mL	
	1M HEPES pH 7.5	1 mL	20 mM
	5M NaCl	1.5 mL	150 mM
	pure spermidine solution	3.6 uL	66.6 ug/ml
	10% Saponin	250 uL	0.05%

Wash buffer 0f (WB0f): the basic ChIC buffer for fixed cells

	solution	volume	concentration in ChIC buffer
	Ultra-pure water	47.5 mL	
	1M HEPES pH 7.5	1 mL	20 mM
	5M NaCl	1.5 mL	150 mM
	pure spermidine solution	3.6 uL	66.6 ug/ml
	10% Tween-20	250 uL	0.05%

Wash buffer 1 (WB1): the antibody incubation buffer for nuclei
WB0 + protease inhibitors + 4 uL/mL 0.5M EDTA
Wash buffer 1f (WB1f)*: the antibody incubation buffer for fixed cells
WB0f + protease inhibitors + 4 uL/mL 0.5M EDTA
*a variation of WB1f is used specifically during Cell-Trace stainings of fixed cells, in which the spermidine is omitted

Wash buffer 2 (WB2): the pA-MNase incubation buffer for nuclei
WB0 + protease inhibitors
Wash buffer 2f (WB2f): the pA-MNase incubation buffer for fixed cells
WB0f + protease inhibitors

Activation solution: the buffer used to activate pA-MNase
WB0 or WB0f containing 4 mM CaCl2

STOP solution: the buffer used to stop pA-MNase activity and lyse the cells
For 100uL:
67 uL nuclease-free water
8 uL 0.5M EGTA
15 uL 10% NP40
10 uL ProtK (Ambion, AM2548)

NOTES:

- Saponin and Tween 10% stock solutions should be put on a roller to dissolve properly.
- Saponin solution should be always made fresh, Tween can be aliquoted and frozen at -20^{o}C.
- Mix the STOP solution with very soft pipetting
- ChIC buffers are preferably made fresh and used within 24 hours. Buffers made for the overnight antibody staining can be stored at 4^{o}C and used the following day.

2 **Preparation of other reagents/buffers**

Bead binding buffer:
1M NaCl
20% PEG8000
20mM Tris-HCl pH = 8
1mM EDTA

Filtered mineral oil:
Filter the mineral oil (Sigma-Aldrich, M8410) using a vacuum filter system (eg Stericup from Millipore or similar). Filtered oil can be stored in room temperature protected from light for a few weeks.

3 **Preparation of 384-well plates for single-cell sorting**
Single cells will be sorted in 384-well plates that are pre-filled with filtered mineral oil + WB0 or WB0f.

To prepare these plates:

- Using the Freedom EVO liquid handling platform (Tecan) or equivalent liquid handler, dispense 384-well plates with **5 uL per well** filtered mineral oil. Plates prefilled with

filtered mineral oil can be stored at room temperature, protected from light. We recommend using them within a month or so.

- Using the Nanodrop (Innovadyne) or equivalent liquid handler, dispense **100 nL per well** WB0 or WB0f. Dispense WB0/WBOf on the day of the sort, or the day before. Plates with filtered mineral oil + WB0/WB0f can be kept at 4^{O}C until sorting.

4 **Preparation of adapter plate**

The double-stranded adapter sequences used for ligation/barcoding in single-cell Dam&ChIC are the same as described previously by Markodimitraki et al., 2020. In short, these adapters contain a T7 promoter, the P5 Illumina sequence, a UMI sequence and a unique cell-specific barcode. The adapters will ligate to both DamID- and ChIC-derived fragments, which will be *in silico* separated upon sequencing based on their distinct sequence context.

- For a detailed explanation on adapter design, as well as, mixing and annealing top and bottom oligos check the protocol by Markodimitraki et al., 2020.
- Upon annealing top and bottom oligos, we recommend limiting the usage of the annealed adapter plate and instead making copies of diluted working plates that can be used multiple times. We call these "motherplates", they can be used multiple times and stored at -20^{O}C.
- The working motherplates used for adapter dispension/barcoding during Dam&ChIC have a concentration of adapters ranging from 0.5 uM to 1 uM (diluted in ultra-pure water). Generally, the concentration of a working motherplate depends on the final concentration of adapters in the ligation reaction (in the present protocol this is 25nM, but can be optimized depending on target abundance).
- To make a "motherplate" in the most accurate way possible, we recommend using robotic liquid handlers. For example with the Freedom EVO liquid handling platform (Tecan) or equivalent, you can dispense ultra-pure water and with the Mosquito LV (STP Labtech) or equivalent, you can copy adapters from a more concentrated ("grandmother") plate to a "motherplate".

NOTES:

- **"Age" the adapter plates:**

While the DamID2 adapters are designed to contain forks on one side, they have the tendency to ligate to each other forming dimers because their other side is blunt-ended. Additionally, some non-ligated adapter can be seen together with the aRNA product after IVT. Getting rid of these adapters is essential for a library to be sequenced as efficiently as possible. When such adapter peaks are disproportionally high, extra bead purification

steps are necessary prior to library prep, and this can increase hands-on time in the protocol.
We have empirically observed that adapter peaks are decreased the more freeze-thaw cycles the motherplates have gone through, probably due to gradual loss of phosphorylation on their ends. Because this effect is so strong when using freshly-made adapter motherplates in Dam&ChIC, we recommend some freeze-thaw cycles before the first time a motherplate will be used (for example, by moving the plate back and forth between a PCR block set to 18^{O}C and dry ice). "Aging" the adapter motherplate before first use will help not only decrease hands-on time, but also decrease the chance of losing part of the aRNA product when doing more than necessary purification steps.
The proportion of free adapter to product in Dam&ChIC can also be decreased by using a more optimal final adapter concentration, relative to the abundance of targets of interest.

- **Proper sealing to prevent evaporation:**

After every use and before storing at -20^{O}C, it's important that adapter plates are sealed (preferably with aluminum seals) as air-tight as possible. Pay special attention to the sides and corners of the plate. Tight sealing helps prevent evaporation, which usually starts at the edges of a plate.

Induction of the Dam-POI fusion protein in live cells

5 Induce expression of Dam fused to a protein of interest (POI) for the appropriate time (live cells). This can be highly variable and dependent on (i) the Dam-POI construct and (ii) the experimental design. We recommend testing induction times and resulting m6A levels for every new Dam-POI cell line.

Design of Dam-POI constructs, engineering of cell lines and clone screening has been previously described here:

Induction of the different cell lines used in the Dam&ChIC manuscript (Kefalopoulou et al., 2025):

- Human KBM7 Dam-LMNB1; addition of 0.5 nM Shield-1 for 15h (data in Figures 1 and 2) or 13h concurrently with second thymidine block (data in Figure 3)
- Human KBM7 Dam untethered; addition of 0.5 nM Shield-1 for 15h
- F1 hybrid mouse ESCs Dam-LMNB1; wash off IAA for 6h

- F1 hybrid mouse ESCs Dam-scFv-H3K27me3; addition of 1 uM 4-OHT for 20h, wash off IAA for the last 6h.

Sample preparation for antibody staining

6 **Harvest cells and Wash**

1. Harvest Dam-induced cells and make a nice single-cell suspension.
2. Wash cells two-three times with room-temperature PBS0 (without Ca^{2+} and Mg^{2+})
3. Count on a cytometer.

There are two alternative downstream steps:
(i) To permeabilize the cells and isolate nuclei in their native condition, proceed to *step 7*.
(ii) To fix the cells with 70% Ethanol, then permeabilize, proceed to *step 8*.

We have performed Dam&ChIC in both native and fixed conditions, and we find that they give similar data quality. Data in Figures 1 and 2 of the manuscript are obtained from nuclei. Data in Figures 3-6 are obtained from fixed cells.

Fixation gives the advantage of long-term sample storage, particularly useful for samples that are challenging to obtain (e.g. from a differentiation time-course)

7 **Alternative 1: Nuclei isolation**

1. Depending on your cell count, split the sample into multiple 0.5mL low-binding tubes if multiple stainings will be done. Use around 0.5 million cells per antibody staining.
2. Spin down at 300rcf for 4 minutes and resuspend in 400 uL Wash Buffer 1 (WB1) per tube/staining, while keeping the sample at 4^{o}C. Proceed to *step 9*.

8 **Alternative 2: Cell fixation with Ethanol**

1. Pre-cool 100% Ethanol, by placing it at -20^{o}C a few hours beforehand.
2. Resuspend 1 million cells in 300 uL ice-cold PBS0 in a 15mL tube (ideally in a low-binding tube to prevent cell loss)
3. While vortexing, add drop-by-drop 700 uL ice-cold 100% Ethanol.
4. Fix for 1-2 hours at -20^{o}C. It is also possible to fix overnight.
5. Spin tubes at 300rcf for 4 minutes in a cooled centrifuge (4^{o}C) and remove supernatant. If none of the optional following steps are desired, proceed to *step 10*.

NOTES:

- It is important that fixation is done properly and drop-wise in order to avoid formation of clumps.
- Scale the volume accordingly if cell numbers are higher or lower. E.g. fix 0.5 million cells in 500uL total volume, 2 million cells in 2 mL total volume etc.

8.1 (optional)

Cell-Trace stainings for sample multiplexing

To enable parallel processing of multiple samples/conditions and minimize batch effects, we use a sample hashing strategy, using CellTrace Cell Proliferation kits (Invitrogen) that enable staining of fixed cells. Samples stained uniquely with CellTrace dyes can be mixed together right before antibody staining into a "super-sample". The populations of interest will be distinguished during FACS and sorted in the desired proportions and format.

1. Resuspend cells in Wash Buffer 1f (WB1f), in which no spermidine is added.
2. Transfer cells in low-binding 1.5mL tubes.
3. Wash cells once more with WB1f (-sperm).
4. Stain 1 million cells in 1 mL WB1f (-sperm) with 0.25 uL Cell-Trace dye. If necessary to stain with multiple non-overlapping dyes, add 0.25 uL of each.
5. Incubate for 20-30 minutes at 4^{O}C protected from light.
6. Quench the staining with the addition of 50 uL rat serum.
7. Incubate for 10 minutes at 4^{O}C protected from light.
8. Spin down at 300rcf for 4 minutes at 4^{O}C. Remove supernatant.

NOTES:

- Two washes are required to remove as much EtOH as possible prior to stainings.
- We have successfully used Cell-tracer dyes CFSE (C34570, Invitrogen), Yellow (C34573, Invitrogen) and Far-Red (C34572, Invitrogen), and combinations of them, to multiplex up to 8 different samples together. The use of other colors is possible and will increase the range of multiplexing but they should not overlap with Hoechst 34580, which will be used to measure DNA content during FACS.
- Cells stained with Cell-Trace dyes can be cryopreserved (*step 8.2*), or immediately used for antibody staining (*step 10*)

Data generated with sample multiplexing in the Dam&ChIC manuscript (Kefalopoulou et al., 2025)

- Figure 3: samples from G2 and G1 phase were uniquely-stained with CellTracers, mixed and sorted in the same plates.

- Figures 4-6: samples collected throughout the differentiation timecourse (d0-d6) were uniquely-stained with CellTracers, mixed and sorted in the same plates. This was the case for all the different Dam&ChIC experiments presented in these figures.

8.2 (optional)

Cryopreservation of EtOH-fixed samples

1. Resuspend cells in WB1f.
2. Spin down at 300rcf for 4 minutes at 4^{o}C. Remove supernatant.
3. Repeat with a 2nd wash in WB1f.
4. If desired, make aliquots of cells in 0.5mL low-binding tubes.
5. Add the same volume of WB1f containing 20% DMSO (final 10% DMSO per sample)
6. Freeze at -80^{o}C. Cryopreservation is long-term and samples can remain in good quality for several years.

NOTE:

- If Cell-Trace stainings were done prior to cryopreservation, it is handy to make aliquots of 100-200K cells per staining. Plan this accordingly to how many samples will be mixed and stained together for sorting.

Antibody staining

9 **Alternative 1: Antibody staining of nuclei**

At this point each sample is resuspended in 400uL WB1.

1. Spin down at 300rcf for 4 minutes at 4^{o}C. Remove supernatant.
2. Resuspend in 400 uL of WB1 containing the primary antibody.
3. Mix overnight at 4^{o}C on a roller.

10 **Alternative 2: Antibody staining of fixed cells**

For cryopreserved EtOH-fixed cells:

1. Thaw cells from -80^{o}C
2. Spin down at 300rcf for 4 minutes at 4^{o}C
3. Wash cells twice with WB1f to remove DMSO.
4. Resuspend in 400 uL of WB1f containing the primary antibody.
5. Incubate overnight at 4^{o}C on a roller.

For multiplexing of cryopreserved EtOH-fixed Cell-Tracer stained cells:

1. Thaw cells from -80^{o}C
2. Mix together the different populations in equal cell numbers in a 0.5mL or 1mL low-binding tube. E.g. 200K cells stained with CT CFSE + 200K cells stained with CT Far-Red + 200K cells stained with CT Yellow.
3. Spin down at 300rcf for 4 minutes at 4^{o}C
4. Wash once with WB1f to remove DMSO.
5. Transfer to a 0.5mL low-binding tube (optional) and repeat wash with WB1f.
6. Resuspend in 400 uL of WB1f containing the primary antibody.
7. Incubate overnight at 4^{o}C on a roller.

For cells freshly-fixed with EtOH:
At this point EtOH-fixed cells are in a 15ml tube, spun down once and supernatant is removed.

1. Resuspend cells in WB1f.
2. Transfer 0.5 million cells to a 0.5mL low-binding tube
3. Wash cells twice with WB1f to remove all EtOH.
4. Resuspend in 400 uL of WB1f containing the primary antibody.
5. Incubate overnight at 4^{o}C on a roller.

For cells freshly-fixed with EtOH and stained with Cell Tracers:

1. Resuspend cells in WB1f.
2. Mix together the different populations in equal cell numbers in a 0.5mL or 1mL low-binding tube. E.g. 200K cells stained with CT CFSE + 200K cells stained with CT Far-Red + 200K cells stained with CT Yellow
3. Spin down at 300rcf for 4 minutes at 4^{o}C
4. Remove supernatant.
5. Transfer to a 0.5mL low-binding tube (optional) and repeat wash with WB1f.
6. Resuspend in 400 uL of WB1f containing the primary antibody.
7. Incubate overnight at 4^{o}C on a roller.

11 NOTES:

- The ideal amount of primary antibody to be used for stainings differs per antibody (even per LOT number for polyclonal antibodies) and should be titrated to ensure proper enrichment and low signal to noise ratio.

- We recommend testing a series of concentrations around the recommended by the manufacturer, e.g. 1:200, 1:400, 1:1000 etc using bulk sortChIC, as described in the related protocol in this collection or previously by Zeller et al., 2023 and Gaza et al., 2024.
- pA-MNase has high affinity for rabbit IgG. If the primary antibody is not raised in rabbit, a secondary antibody staining has to be done for 1 hour at 4^{o}C, right before pA-MNase tethering (*step 12*).

Antibody concentrations used in the Dam&ChIC manuscript:

- anti-histone H3 (Abcam, ab176842) at 1:400
- anti-LMNB1 (Abcam, ab16048) at 1:200 or 1:400 (polyclonal antibody, different LOTs)
- anti-H3K27me3 (Cell Signaling Technologies, 9733S) at 1:200
- anti-H3K9me3 RM389 (Thermofisher, MA5-33395) at 1:200
- anti-H3K4me3 (Thermofisher, MA5-11199) at 1:400
- anti-H2AK119Ub (Cell Signaling, D27C4) at 1:400

pA-MNase tethering

12 **pA-MNase tethering and parallel staining for DNA content**

1. Spin the nuclei or fixed cells at 300 rcf for 4 minutes at 4^{o}C.
2. Resuspend the pellet with Wash Buffer 2 (WB2) for nuclei, or Wash Buffer 2f (WB2f) for fixed cells.
3. Spin again at 300 rcf for 4 minutes at 4^{o}C.
4. Resuspend nuclei in 500 uL WB2 or WB2f containing pA-MNase.
5. In the same mix add Hoechst 34580 at a final concentration of 2.5 ug/mL.
6. Incubate for 1 hour at 4^{o}C on roller

NOTES:

- The ideal amount of pA-MNase depends on the batch of protein production (for self-produced protein) and on the cell type.
- In the Dam&ChIC manuscript, KBM7 samples (data in Figures 1, 2 and 3) were incubated with pA-MNase at a final concentration of 3 ng/uL (1:200), while mouse ESCs and Vitamin C samples at a final concentration of 0.6 ng/uL (1:1000).

13 **Washes and transfer to FACS tubes**

1. Wash the nuclei twice with WB2 or WB2f, like above.
2. After the final wash, resuspend in 600 uL WB2 or WB2f.
3. Pipette the suspension in a FACS tube through a filter cap to get rid of clumps.

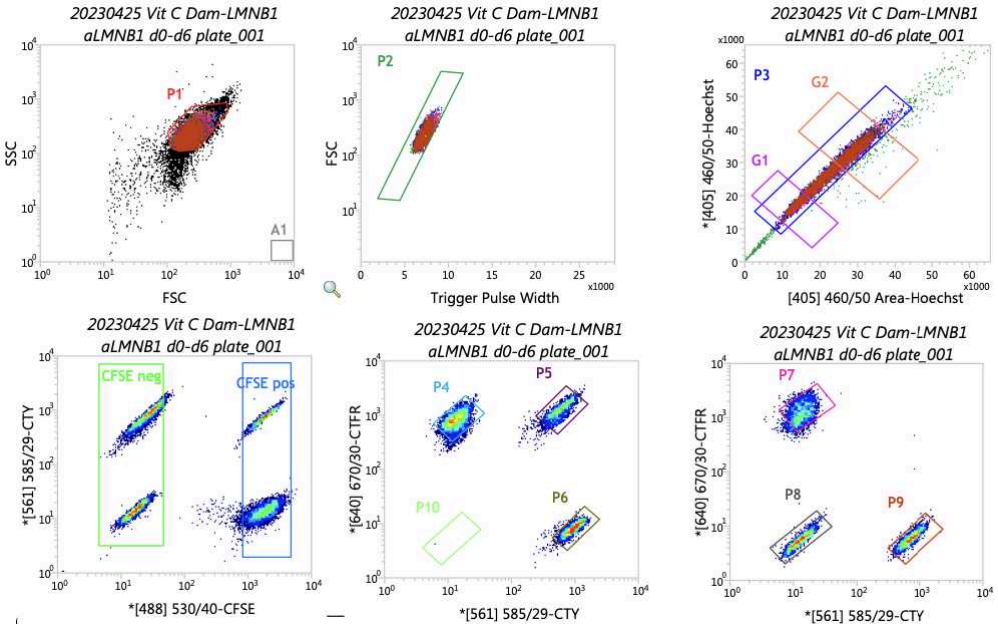
Single-cell sorting (FACS)

14

1. Analyze samples by FACS to set the desired gates. See example strategy below.
2. Sort one cell per well in 384-well plates prefilled with mineral oil and WB0 or WB0f (*step 3*). Sorting should be done with sample and plate cooling.
3. After sorting of each plate is complete, seal it with proper aluminum seals (e.g. Greinier, 676090) and spin at 2000g for 1-2 minutes at 4^{O}C.

NOTE:

- Keep samples and (sorted) plates cooled at 4^{O}C during the full duration of the sort and until the subsequent step of pA-MNase activation.



Example sorting strategy, including gating for Cell-Tracer stained populations. Here, cells from different timepoints during differentiation were stained with unique Cell-Tracer dyes or combinations of them and mixed together in one "super-sample". Gating was done hierarchically based on size (FSC/SSC), single cells, Hoechst profile (405), and subsequently for combinations of fluorescence by CellTracers CFSE (488), Yellow (561) and Far-Red (640). Single cells from sub-populations P4-P10 were sorted in the same 384-w plate based on a particular layout.

Digestion, end-processing and barcoding using robotic liquid handling

15 In the Dam&ChIC workflow, liquid dispensions of reaction mixes are performed using the Nanodrop II liquid handler (Innovadyne) and adapter dispensions are performed using the Mosquito LV (STP Labtech). It is recommended to use liquid handlers of similar range of dispension volumes for the respective steps.

Note that many handlers require liquid "dead volume", which should be taken into account when preparing the master mix of each reaction. An example calculation sheet for all Dam&ChIC liquid dispensions with the Nanodrop II robot is provided here.

DamChIC_Dispensions_Robots.xlsx 21KB

16 **pA-MNase activation**

1. Dispense **100 nL per well** Activation solution (*step 1*).
2. Spin plates at 2000g for 1-2 minutes at 4^{o}C.
3. Digest for exactly 30 minutes at 4^{o}C, with plates kept in 384 PCR machines set at 4^{o}C (or on cooling blocks).

NOTES:

- It is important to precisely time the activation of pA-MNase and keep it constant across plates, especially if they represent technical replicates.
- We recommend to keep the plate temperature constant at 4^{o}C at all times, also during dispension of the Activation solution on the liquid handler. For example, in the Nanodrop II the position of the needles can be set higher to allow for the use of cooling blocks under the plates. We recommend the use of equivalent cooling settings, if the liquid handler of choice allows that.
- If using the Nanodrop II, keep in mind that it performs dispensions of two plates at a time with high speed, which allows the sequential activation of multiple plates (up to 16) within 30 minutes, depending on the familiarity and hands-on experience of the user. This can be handy when processing plates at scale, as it makes the process time-efficient.
- Regardless of the used setup, we recommend noting the precise dispension time for each plate and performing test rounds before the actual experiment.
- Activation time can be decreased down to 10 minutes, which can be handy when using liquid handlers with lower dispension speeds.

17 **Proteinase K treatment and Lysis**

1. Stop digestion by dispensing **200 nL per well** Stop Solution (*step 1*).

2. Spin plates at 2000g for 1-2 minutes at 4^{O}C.
3. Incubate with the following program in a 384 PCR machine:

65^{O}C for 6 hours

80^{O}C for 20 minutes

Hold at 4^{O}C

Plates with lysed material can be frozen at -20^{O}C for long-term storage.

18 **End-repair of fragments produced by pA-MNase**

Dispense **200 nL per well** of Blunt-ending mix:

	Reagent	Volume (nL) per well
	Klenow Large 3'-5' exo (5000 U/uL)	4
	dNTPs (10 mM)	10
	T4 PNK (10000 U/uL)	4
	ATP (10 mM)	60
	PNK buffer 10X	60
	MgCl2 (25 mM)	20
	PEG8000	10
	BSA (20 mg/mL)	6
	H2O	26
	TOTAL	**200**
	Cumulative volume	600

Spin plates at 2000g for 1-2 minutes at 4^{O}C.
Incubate with the following program in a 384 PCR machine:

37^{O}C for 30 minutes

75^{O}C for 20 minutes

Hold at 4^{O}C

NOTE:

- During the end-repair program we recommend moving the plates on a cool block at 4^{O}C directly when 37^{O}C incubation is finished, and move them back to the PCR machine once the block reaches 75^{O}C.

19 **DpnI digestion**

Dispense **400 nL per well** of DpnI mix:

	Reagent	Volume (nL) per well
	DpnI (20U/uL)	20
	PNK buffer 10X	40
	BSA (20 mg/mL)	10
	water	330
	TOTAL	**400**
	Cumulative volume	1000

Spin plates at 2000g for 1-2 minutes at 4^{O}C.
Incubate with the following program in a 384 PCR machine:
37^{O}C for 8 hours
80^{O}C for 20 minutes
Hold at 4^{O}C

20 **Adapter ligation**

Dispense **100 nL** per well DamID2 adapters from a 0.5uM motherplate, to a final adapter concentration of 25nM.
NOTE: This step is done with the Mosquito (STP Labtech) or equivalent liquid handler.

Dispense **900 nL per well** of Ligation mix:

	Reagent	Volume (nL) per well
	T4 ligase (5U/uL)	50
	T4 ligase buffer 10X	200
	H2O	650
	TOTAL	**900**
	FINAL cumulative volume	**2000**

Spin plates at 2000g for 1-2 minutes at 4^{O}C.

Incubate with the following program in a 384 PCR machine:

4°C for 20 minutes

16°C for 16 hours

65°C for 10 minutes

Hold at 4°C

NOTE:

- While the final adapter concentration in the ligation reaction is 25nM, during optimizations we tested concentrations as low as 1.25 nM. Optimizing the final adapter concentration might be necessary depending on the abundance of certain targets.

Pooling and amplification

21 **Pooling**

1. Pool the content of all wells of a 384-well plate by spinning it inverted on top of a collection reservoir at 500g for 2 minutes.
2. Using a p1000, carefully pipette the content from the collection plate to a 1.5mL or 2mL tube, trying to get as much as possible of the aqueous phase, which contains the barcoded DNA fragments. The oil phase can be pipetted out and thrown away after spinning the tube at max speed for 20-30 seconds. This should be repeated a few times until all the content of the plate is collected.
3. After all the content is collected, spin for 5-10 minutes at high speed.
4. Pipette out and discard any remaining oil phase from the top.
5. Pipette the aqueous phase and transfer to a clean tube, ensuring that you leave behind as much oil and debris as possible (debris usually remains as loose white precipitate at the bottom).
6. Spin the pooled material at high speed.
7. Repeat the transfer to a clean tube two more times.
8. During the last pipetting step measure the volume of the pooled material for each sample (henceforth one sample ~ material from one pooled plate).

NOTES:

- The final recovered volume (aqueous phase) should be at least 85% of the expected volume from a 384-w plate processed with Dam&ChIC (2 uL/well x 384 wells = 768 uL). Recovery of lower volumes does not necessarily indicate a failed experiment, but rather that dispension errors may have occurred during processing, which may have affected the overall efficiency.

- To minimize loss of material when handling non-amplified samples (*steps 21-25*), we recommend using **low-retention pipette tips**.

22 **DNA purification**

1. Per sample, add 0.8 volume beads diluted 1:10 in bead-binding buffer (see recipe in Materials).
2. Incubate for 20-30 minutes at room temperature.
3. Put samples on magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear, around 20 minutes).
4. Remove unbound liquid.
5. Wash three times with freshly-made 80% ethanol.
6. During the last wash with ethanol, use a stronger hand magnet to concentrate the beads as much as possible at one place in the tube (they tend to be dispensed across the length of the tube)
7. Remove ethanol and let the beads air-dry until they look matte.
8. Elute in 7 ul ultra-pure water (DNase/RNase-free) for 10 minutes.
9. Transfer the eluted samples to PCR strips for the IVT reaction (*step 23*)

NOTES:

- It is important that the beads don't over-dry before elution, as this can result in irreversible binding of fragments.
- It is not necessary to separate the eluted material from the beads for the IVT reaction that follows.

23 **Linear amplification by *in vitro* transcription (IVT)**

Per sample add 9 uL of IVT mix, according to manufacturer instructions (MEGAScript T7 transcription kit):

	Reagent	Volume (uL)
	A	1.5
	U	1.5
	G	1.5
	C	1.5
	T7 buffer	1.5
	T7 enzyme	1.5

Total reaction volume is 16 uL

Incubate with the following program in a PCR machine:
37^{O}C for 14 hours
Hold at 4^{O}C

The amplified RNA (aRNA) can be stored long-term at -80^{O}C.

24 **aRNA purification**

1. Measure the exact volume of each aRNA sample with a pipette (should be around 15 uL) and transfer to a 1.5 mL tube.
2. Add ultra-pure water up to 30 uL.
3. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
4. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).
5. Remove unbound liquid.
6. Wash three times with freshly-made 80% ethanol.
7. Air-dry beads until they look matte.
8. Elute in 13 uL ultra-pure water. Leave for 10 minutes.
9. Put on magnetic stand until clear and transfer the eluate in a clean tube.

aRNA samples can be stored long-term at -80^{O}C.

25 (optional)
aRNA fragmentation

1. Add ultra-pure water up to 20 uL.
2. Add 0.2 volume fragmentation buffer.
3. Incubate in a pre-heated block at 94^{O}C for 90-120 seconds.
4. Transfer on ice and stop fragmentation with 0.1 volume 0.5M EDTA.

Repeat purification of the aRNA, like in *step 24*.

NOTE:

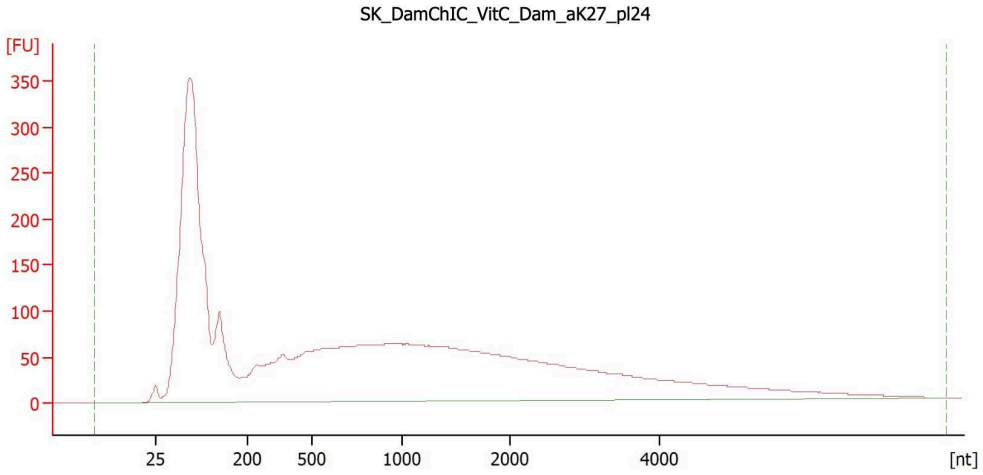
- We have tested single-cell Dam&ChIC with and without fragmentation for the same targets and saw extremely similar results. We thus do not regularly perform fragmentation and the end libraries produced by Dam&ChIC have a long average length (600-700bp).

26 **aRNA quantification**

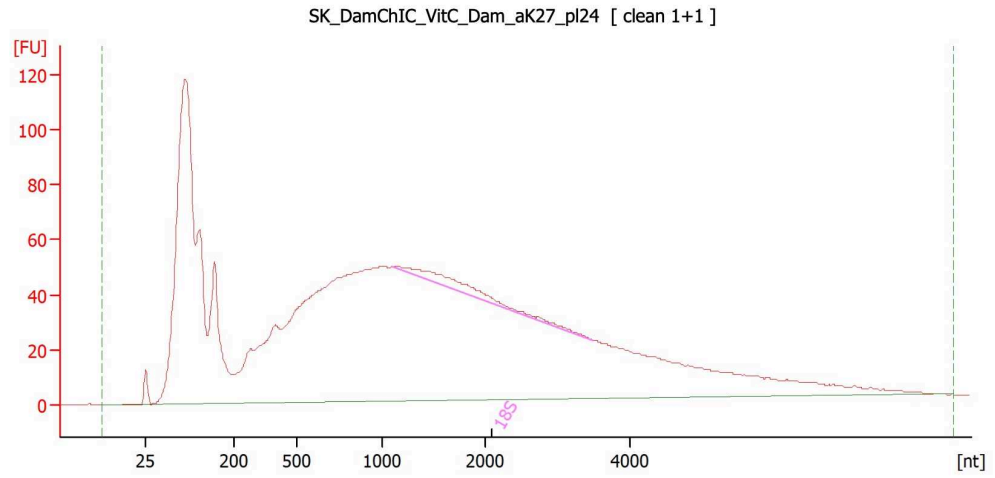
1. Measure 1 uL of aRNA on the Nanodrop to estimate total amount of product.
2. Run 1 uL of aRNA on the Bioanalyzer (Total Eukaryote RNA Assay) or equivalent.

NOTES:

- Based on the size distribution of the product, determine the ratio between aRNA and free adapter. Empirically, we know that if the adapter peak is more than two to three times as big as the aRNA product (see example below), it is necessary to perform extra bead clean-ups (1 to 3 extra), to remove as much as possible of the adapters. This is important as high amount of adapters may be amplified during library preparation and negatively affect the sequencing.
- Keep in mind that extra bead clean-up may entail some loss of product, so the decision whether and how many extra to perform should also depend on the amount of actual aRNA product you see on the Bioanalyzer at this first quantification.
- In case more bead clean-ups are needed, repeat *step 24* and make sure to do a quantification of the final aRNA product, before proceeding with library preparation.



Example of high adapter to aRNA product ratio that needs extra bead purifications. Product is non-fragmented



Example of okay adapter to aRNA product ratio that can be used for library prep. Product is non-fragmented

Library preparation

27 **Reverse transcription**

1. Take 100ng of aRNA product diluted in 5 uL ultra-pure water. This amount can definitely be lower, in case the aRNA product is not abundant.
2. Add a mix of 1 uL Random Hexamer primer (20 uM) + 0.5 uL dNTPs (10mM).
3. Incubate @65^{O}C for exactly 5 minutes.
4. Quickly transfer samples on ice.
5. Add 4 uL of RT mix:

	Reagent	Volume (uL)
	5X First-Strand buffer	2
	DTT 0.1M	1
	RNAse OUT	0.5
	Superscript II	0.5

Total reaction volume is 10.5 uL

Incubate with the following program in a PCR machine:

25^{O}C for 10 minutes

42^{O}C for 1 hour

Hold at 4^{O}C

NOTE:

- The Random Hexamer primer sequence is GCCTTGGCACCCGAGAATTCCANNNNNN (Markodimitraki et al., 2020) and it includes the Illumina P7. Check guidelines on illumina.com for design.

28 **Indexing PCR**

1. Add 2 uL of a unique RPi primer (10uM) in each library.
2. Add 37.5 uL of PCR mix:

	Reagent	Volume (uL)
	2X NEBNext High Fidelity mastermix	25
	RP1 primer 10uM	2
	ultra-pure water	10.5

Total reaction volume is 50 uL

Incubate with the following program in a PCR machine:
98^{O}C for 30 seconds
8-11 cycles of:

- 98^{O}C for 10 seconds
- 60^{O}C for 30 seconds
- 72^{O}C for 30 seconds

72^{O}C for 10 minutes
Hold at 4^{O}C

NOTES:

- Each of the RPi primers (index primers) contains a unique index from the Illumina Truseq small RNA series (RPI series) and an overlapping sequence to the Illumina P7, introduced to the molecules during the previous RT step. Follow guidelines on illumina.com for design.
- The RP1 primer (universal primer) contains an overlapping sequence to the Illumina P5, which is part of the DamID2 adapters sequence. Follow guidelines on illumina.com for design.
- The exact number of cycles for the library PCR depends on the amount of input aRNA. We decide this empirically by comparing the height (FU) of the marker peak to the highest peak of the aRNA product distribution (excluding the adapter peak). For example, if the marker peak and the product are in similar FU levels, or if the product is much higher, we recommend 8 PCR cycles. Increase cycles accordingly for lower amounts.

29 **Library purification**

1. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
2. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).
3. Remove unbound liquid.
4. Wash two times with freshly-made 80% ethanol.
5. Air-dry beads until they look matte.
6. Elute in 25 uL water. Leave for 10 minutes.
7. Put on magnetic stand until clear and transfer eluate in a clean tube.

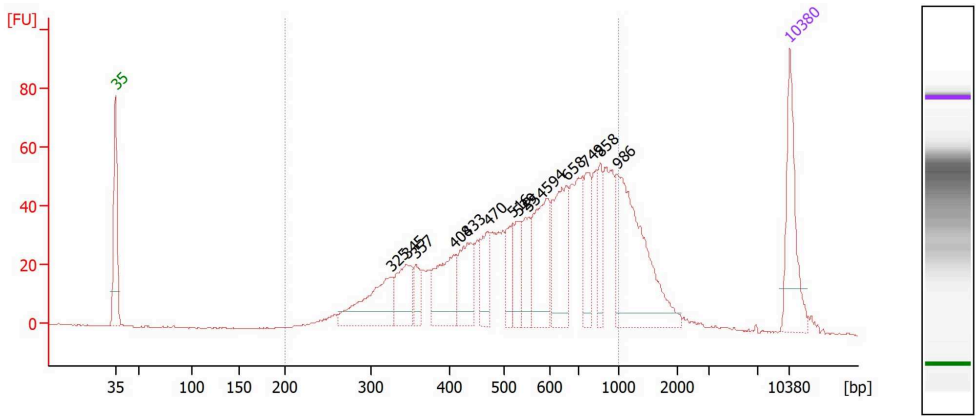
(REPEAT)

1. Add 0.8 volume of undiluted beads. Incubate for 10 minutes.
2. Put samples on a magnetic stand and incubate until all beads are bound to the magnet (liquid should look clear).
3. Remove unbound liquid.
4. Wash two times with freshly-made 80% ethanol.
5. Air-dry beads until they look matte.
6. Elute in 13 uL water. Leave for 10 minutes.
7. Put on magnetic stand until clear and transfer eluate in a clean tube.

Purified DNA libraries can be stored long-term at -20^{o}C.

30 **Library quantification and Sequencing**

1. Measure 1-2 uL of library with Qubit dsDNA High Sensitivity assay to determine total library amount.
2. Run a max of 2ng of library in a Bioanalyzer (High Sensitivity DNA Assay) or equivalent to estimate size distribution.
3. Calculate library molarity based on Qubit concentration and size distribution.
4. Sequence with single-end or paired-end sequencing.



Example of successful Dam&ChIC library, with at least 90% of fragments within 300-1000bp. The library was not fragmented, therefore showing a tendency to longer fragments.

NOTES:

- We recommend sequencing 75bp or 100bp reads at a starting sequencing depth of 10M reads per plate for test experiments, to check quality and complexity of the data.
- In the Dam&ChIC manuscript, libraries from haploid KBM7 cells (Figures 1-3) were sequenced at 20-30M reads/plate. Data from hybrid mES cells undergoing XCI (Figures 4-6) required allele-specific mapping, thus libraries were sequenced at 30-40M reads/plate.

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