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Dam&ChIC in bulk

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Dam&ChIC



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We use this protocol and it's working

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Abstract

Bulk Dam&ChIC is an adaptation of single-cell Dam&ChIC (Kefalopoulou et al., 2025) tailored to sorted populations of cells (e.g. 20c-10000c). The protocol doesn't require the specialized robotic liquid handlers used in its single-cell version. Ideally, cell populations are sorted by FACS to ensure good cell/nuclei quality and selection based on cell-cycle state. If that is not possible, the protocol could also be performed on crude bulks of cells as long as cell/nuclei quality is confirmed to be high.

Besides being easier to implement and more accessible, this protocol can be useful for multifactorial profiling of targets of interest to derive preliminary data prior to single-cell experiments, for experiments in which single-cell resolution is not per se necessary, or for testing new Dam-expressing cell lines in combination with a target of interest.



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 for DamID2, top and bottom oligonucleotides, set of 384

 DamID2_adapters_384_top_bottom... 36.1KB

- Adapters for DamID2, top and bottom oligonucleotides, set of 96

 DamID2_adapters_96_top_bottom... 15.9KB

- Random hexRT primer

GCCTTGGCACCCGAGAATTCCANNNNNN

- Illumina RNA PCR primer 1 (RP1)

- Illumina RNA PCR index primers (RPI series)

 RPI_primers.xlsx 10.1KB

Chemicals and Buffers

For sample preparation:

- PBS0
- Ethanol
- Saponin (Sigma, 47036-50G-F)
- Tween 20 (Sigma, P9416)
- cComplete 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:

- CellTraceTM 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)
- 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



- Protein LoBind tubes 0.5 ml (Eppendorf, 0030108094)
- Low-retention pipette tips (Greiner Bio-One)
- Polypropylene round bottom tubes 5 ml (Corning, 352002)
- Qubit 4 fluorometer (Invitrogen)
- Agilent 2100 Bioanalyzer platform

FACS

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

Sequencing

- Illumina NextSeq500 sequencing platform
- Illumina NextSeq2000 sequencing platform



Before start

Considerations

Prior to implementation of bulk Dam&ChIC some relevant expertise is required in order to engineer Dam-POI fusions into cell lines or model organisms (or having direct access to such engineered systems).

Advantages of FACS-based sorting

Similarly to single-cell Dam&ChIC, FACS 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. Multiplex different samples/conditions (e.g. differentiation timepoints; drug treatments) using CellTrace or equivalent fluorescent dyes (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 during sample preparation.
3. Preferentially sort populations of cell types expressing known cell (surface) markers (Zeller et al., 2023, Gaza et al., 2024).

NOTES on handling and equipment during the protocol

- Upon permeabilization or fixation, samples should be kept at 4°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.
- Ensure that both bench and pipettes are decontaminated from RNases and DNases when handling pre-amplified material, and especially when handling amplified RNA.
- 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 **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

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°C.
- ChIC buffers are preferably made fresh and used within 24 hours. Buffers made for the overnight antibody staining can be stored at 4°C and used the following day.

2 Preparation of sorting tubes

Prior to the sorting populations by FACS, prepare PCR strips or 0.5mL tubes with 5 uL WB0 or WB0f.

Optionally, the tubes can also contain 10-50 uL mineral oil, similarly to the 384-well plates in the single-cell sortChIC protocol, but it is not necessary in our experience.

NOTE:

If no mineral oil is used, make sure all incubations in PCR machines are done with heated lid.

Sample preparation for antibody staining

3 Harvest cells and Wash

1. Harvest 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*.

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)

4 **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°C. Proceed to *step 6*.

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

1. Pre-cool 100% Ethanol, by placing it at -20°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°C. It is also possible to fix overnight.
5. Spin tubes at 300rcf for 4 minutes in a cooled centrifuge (4°C) and remove supernatant. If none of the optional following steps are desired, proceed to *step 7*.

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.

5.1 (optional)

Cell-Trace stainings for sample multiplexing

To enable parallel processing of multiple samples/conditions, and thereby minimize batch effects, cells can be stained with CellTrace dyes. Samples stained uniquely with CellTrace dyes can be mixed together right before the antibody staining step and their respective populations will be distinguished during FACS.

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°C protected from light.
6. Quench the staining with the addition of 50 uL rat serum.
7. Incubate for 10 minutes at 4°C protected from light.
8. Spin down at 300rcf for 4 minutes at 4°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 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 5.2*), or immediately used for antibody staining (*step 7*)

5.2 (optional)**Cryopreservation of EtOH-fixed samples**

1. Resuspend cells in WB1f.
2. Spin down at 300rcf for 4 minutes at 4°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°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**6 Antibody staining of nuclei**

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

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

7 Antibody staining of fixed cells

For cryopreserved EtOH-fixed cells:

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

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

1. Thaw cells from -80°C
2. Mix together the different populations in equal cell numbers in a 0.5mL or 1mL low-binding tube to make a "super-sample". 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°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 μL of WB1f containing the primary antibody.
7. Incubate overnight at 4°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 μL of WB1f containing the primary antibody.
5. Incubate overnight at 4°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 to make a "super-sample". 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°C
4. Remove supernatant.
5. Transfer to a 0.5mL low-binding tube (optional) and repeat wash with WB1f.
6. Resuspend in 400 μL of WB1f containing the primary antibody.
7. Incubate overnight at 4°C on a roller.

8 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 here or previously by Zeller et al., 2023 or 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°C, right before pA-MNase tethering (*step 9*).

pA-MNase tethering

9 pA-MNase tethering and parallel staining for DNA content

1. Spin the nuclei or fixed cells at 300 rcf for 4 minutes at 4°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°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°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).

10 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.

Bulk sort (FACS)



- 11
 1. Analyze samples by FACS to set the desired gates. See example strategy below.
 2. Sort the desired cell number (e.g. 100, 1000, 10000 cells) in PCR strips or 0.5mL tubes prefilled with 5 uL WB0 or WB0f (*step 2*). Sorting should be done on cooling settings.
 3. After sorting of each strip or tube is complete, perform a quick spin and keep samples on cool blocks at 4°C at all times.

Digestion, end-processing and barcoding

- 12 **pA-MNase activation**
 1. Add 5 uL of Activation solution (*step 1*) per bulk sample, while keeping the samples at 4°C.
 2. Do a quick spin.
 3. Digest for exactly 30 minutes at 4°C.

NOTES:

- It is important to precisely time the activation of pA-MNase and keep it constant across samples, especially if they represent technical replicates.

- 13 **Proteinase K treatment and Lysis**

Stop digestion by adding 10 uL of Stop Solution (*step 1*) to each bulk sample.
The total volume at this point is 20 uL.

Incubate with the following program in a PCR machine:

65°C for 6 hours

80°C for 20 minutes

Hold at 4°C

Samples can be frozen at -20°C for long-term storage.

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

Add 10 uL of Blunt-ending mix to each bulk sample:

Reagent	Volume (uL) per sample
Klenow Large 3'-5' exo (5000 U/uL)	0.2
dNTPs (10 mM)	0.5



Reagent	Volume (uL) per sample
T4 PNK (10000 U/uL)	0.2
ATP (10 mM)	3
PNK buffer 10X	3
MgCl ₂ (25 mM)	1
PEG8000	0.5
BSA (20 mg/mL)	0.3
H ₂ O	1.3
TOTAL	10
Cumulative volume	30

Incubate with the following program in a PCR machine:

37°C for 30 minutes

75°C for 20 minutes

Hold at 4°C

NOTE:

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

15 DpnI digestion

Add 20 uL of DpnI mix to each bulk sample:

Reagent	Volume (uL) per sample
DpnI (20U/uL)	0.5
PNK buffer 10X	2
BSA (20 mg/mL)	0.5
water	17
TOTAL dispensation	20
Cumulative volume	50

Spin plates at 2000g for 1-2 minutes at 4°C.



Incubate with the following program in a PCR machine:

37°C for 8 hours

80°C for 20 minutes

Hold at 4°C

16 **Barcoding/Adapter ligation**

Add **2 uL** of a unique DamID2 adapter per bulk sample
(final adapter concentration can be in the range of 20-50nM)

Add 48 uL of Ligation mix to each bulk sample:

Reagent	Volume (uL) per sample
T4 ligase (5U/uL)	1
T4 ligase buffer 10X	8
H2O	39
TOTAL	48
FINAL cumulative volume	100

Incubate with the following program in a PCR machine:

4°C for 20 minutes

16°C for 16 hours

65°C for 10 minutes

Hold at 4°C

NOTE:

We provide both the set of 384 and the set of 96 DamID2 adapter sequences in Materials, and either of them can be used for bulk experiments. We prefer using the set of 96, so to keep reagents for single-cell and bulk experiments separate.

Pooling and amplification

17 **Pooling**

Pool uniquely-barcoded bulk samples in one eppendorf tube for downstream processing.

NOTES:



- We especially recommend pooling when handling technical replicates and samples for titrations of antibodies. In general, it makes the process more efficient and limits variation/batch effects during downstream steps.
- Do a couple of transfers to clean tubes, by leaving behind the oil phase in case the samples were sorted in tubes containing mineral oil.

18 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 6 uL ultra-pure water (DNase/RNase-free) for 10 minutes.
9. Transfer the eluted samples to PCR strips for the IVT reaction (*step 18*)

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.

19 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



Reagent	Volume (uL)
T7 enzyme	1.5

Total reaction volume is 15 uL

Incubate with the following program in a PCR machine:

37°C for 14 hours

Hold at 4°C

The amplified RNA (aRNA) can be stored long-term at -80°C.

20 **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.

21 (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°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 19.

22 **aRNA quantification**

1. Measure 1 uL of aRNA on the Nanodrop to estimate total amount of product. In case this measurement gives a total yield of higher than 1 ug per 100c population sample, we know empirically that the sample contains too much free adapter and most likely



requires extra clean-ups. In that case, repeat *step 19* and measure on Nanodrop again.

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, 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 19* and make sure to do a quantification of the final aRNA product, before proceeding with library preparation.

Library preparation

23 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°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°C for 10 minutes

42°C for 1 hour

Hold at 4°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.

24 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°C for 30 seconds

8-11 cycles of:

- 98°C for 10 seconds
- 60°C for 30 seconds
- 72°C for 30 seconds

72°C for 10 minutes

Hold at 4°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.



25 **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.

26 **Library quantification and Sequencing**

1. Measure 1-2 uL of library with dsDNA Qubit 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

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