

Apr 25, 2025

single-cell TChIC for Zebrafish

This protocol is a draft, published without a DOI.

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sc-tChIC



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Protocol Citation: Vivek Bhardwaj, Alberto Griffa, Helena Viñas Gaza, Peter Zeller, Alexander van Oudenaarden 2025. single-cell TChIC for Zebrafish . [protocols.io https://protocols.io/view/single-cell-tchic-for-zebrafish-cwubxesn](https://protocols.io/view/single-cell-tchic-for-zebrafish-cwubxesn)

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Protocol status: Working

We use this protocol and it's working

Created: July 06, 2023

Last Modified: April 25, 2025

Protocol Integer ID: 84579

Funders Acknowledgements:

EMBO

Grant ID: ALTF 1197–2019

ERC

Grant ID: ERC-AdG 101053581-scTranslatomics

Abstract

Establishing a cell-type-specific chromatin landscape is critical for the maintenance of cell identity during embryonic development. However, our knowledge of how this landscape is set during vertebrate embryogenesis has been limited, due to the lack of methods to jointly detect chromatin modifications and gene expression in the same cell. Here we present a multimodal measurement of full-length transcriptome and chromatin modifications in individual cells during early embryonic development in zebrafish using a novel protocol: single-cell transcriptome with chromatin immunoclevage (scTChIC)

Protocol materials

- ☒ RNasin Plus Ribonuclease Inhibitors **Promega Catalog #N2615**
- ☒ rat serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #R9759-5ML**
- ☒ Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb **Cell Signaling Technology Catalog #9733S**
- ☒ H3K4me1 Recombinant Polyclonal Antibody **Thermo Fisher Scientific Catalog #710795**
- ☒ Stericup® Quick Release Vacuum Driven Sterile Filters **Merck Millipore (EMD Millipore) Catalog #S2GPU05RE**
- ☒ Tris 1M pH 7.5 **Thermofisher Catalog #15567027**
- ☒ BSA 20mg/mL **New England Biolabs Catalog ##B9000**
- ☒ ATP Solution (100 mM) **Thermo Fisher Scientific Catalog #R0441**
- ☒ T4 DNA Ligase **New England Biolabs Catalog #M0202L**
- ☒ MgCl₂ 1M **Thermofisher Catalog #AM9530G**
- ☒ DTT 0.1M **Invitrogen - Thermo Fisher Catalog #15846582**
- ☒ Invitrogen™ Nuclease-Free Water (not DEPC-Treated) **Invitrogen - Thermo Fisher Catalog #AM9932**
- ☒ Hybridase Thermostable RNase H **Biosearch Technologies Catalog #H39500**
- ☒ RQ1 RNase-Free DNase **Promega Catalog #M6101**
- ☒ T4 PNK **New England Biolabs Catalog # M0201L**
- ☒ dNTPs Mix **Promega Catalog #U1515**
- ☒ DTT 0.1M **Invitrogen - Thermo Fisher Catalog #15846582**
- ☒ MgCl₂ 1M **Thermofisher Catalog #AM9530G**
- ☒ Invitrogen™ Nuclease-Free Water (not DEPC-Treated) **Invitrogen - Thermo Fisher Catalog #AM9932**
- ☒ DNA Polymerase I, Large (Klenow) Fragment **New England Biolabs Catalog # M0210L**
- ☒ Thermolabile Exonuclease I **New England Biolabs Catalog # M0568L**
- ☒ ATP Solution (100 mM) **Thermo Fisher Scientific Catalog #R0441**
- ☒ Tris 1M pH 7.5 **Thermofisher Catalog #15567027**
- ☒ BSA 20ng/mL **New England Biolabs Catalog #B9000S**
- ☒ SuperScript™ III Reverse Transcriptase **Fisher Scientific Catalog #18080085**
- ☒ dNTPs Mix **Promega Catalog #U1515**
- ☒ BSA 20ng/mL **New England Biolabs Catalog #B9000S**
- ☒ T4 PNK **New England Biolabs Catalog # M0201L**
- ☒ E. coli Poly(A) Polymerase **New England Biolabs Catalog #M0276L**
- ☒ Adenosine 5'-Triphosphate (ATP) **New England Biolabs Catalog # P0756L**

- ☒ RNaseOUT™ Recombinant Ribonuclease Inhibitor Thermo Fisher Scientific Catalog #10777019
- ☒ MgCl₂ 1M ThermoFisher Catalog #AM9530G
- ☒ BSA 20ng/mL New England Biolabs Catalog #B9000S
- ☒ NEB T4 RNA Ligase 2, truncated New England Biolabs Catalog #M0242L
- ☒ RNaseOUT™ Recombinant Ribonuclease Inhibitor Invitrogen - Thermo Fisher Catalog #10777019
- ☒ SuperScript™ III Reverse Transcriptase Fisher Scientific Catalog #18080085
- ☒ RNaseOUT™ Recombinant Ribonuclease Inhibitor Invitrogen - Thermo Fisher Catalog #10777019
- ☒ RNase A Thermo Scientific Catalog #EN0531
- ☒ Invitrogen™ Nuclease-Free Water (not DEPC-Treated) Invitrogen - Thermo Fisher Catalog #AM9932
- ☒ AmpliTaq™ 360 DNA Polymerase Thermo Fisher Scientific Catalog #4398828
- ☒ dATPs 100mM Promega Catalog #U1335
- ☒ KCl 1M ThermoFisher Catalog #AM9640G
- ☒ BSA 20mg/mL New England Biolabs Catalog ##B9000
- ☒ SuperScript™ III Reverse Transcriptase Fisher Scientific Catalog #18080085
- ☒ dNTPs Mix Promega Catalog #U1515
- ☒ NEBNext® High-Fidelity 2X PCR Master Mix New England Biolabs Catalog #M0541L
- ☒ VBLOK200 Reservoir, Case ClickBio, Inc. Catalog #CBVBLOK200-1
- ☒ Mineral Oil BioUltra, for molecular biology Merck MilliporeSigma (Sigma-Aldrich) Catalog #69794-500ML
- ☒ RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615
- ☒ CellTrace® CFSE Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34554
- ☒ CellTrace® Far Red Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34572
- ☒ CellTrace® Yellow Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34567
- ☒ RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615
- ☒ RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615
- ☒ CellTrace® CFSE Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34554
- ☒ cOmplete™, EDTA-free Protease Inhibitor Cocktail Merck Catalog #5056489001
- ☒ RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615
- ☒ Spermidine Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2626-5G
- ☒ FACSmax Cell Dissociation Solution amsbio Catalog #AMS.T200100
- ☒ rat serum Merck MilliporeSigma (Sigma-Aldrich) Catalog #R9759-5ML
- ☒ Pronase Merck MilliporeSigma (Sigma-Aldrich) Catalog #10165921001
- ☒ CellTrace® Far Red Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34572

☒ CellTrace® Yellow Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34567

☒ RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615

☒ Silverseal plate sealer greiner bio-one Catalog #676090

☒ Thermolabile Proteinase K New England Biolabs Catalog #P8111S

Before start

Reagents, tips and tubes should be RNAse and DNase-free, and ideally DNA-low-binding as well.

Embryo Dissociation

1 Obtain Zebrafish embryos. Grow them at  28.5 °C in a 10cm diameter petri dish in ~  10 mL of fresh E3 media in an incubator.

1.1 If planning to incubate the embryos for a long time, check the water level periodically for evaporation. You might want to add a beaker with water just to provide a bit more moisture.

1.2 Check on the embryos periodically. Count them and decide if and how to split them between one or multiple timepoints.

Optionally, take representative pictures of the embryos

1.3 Sort our the unfertilized embryos from the fertilized ones using a plastic Pasteur pipette. This can be done at any time between the collection of the eggs and their dissociation, but it's advisable to do it very early (~1hpf) because of the ease of distinguishing fertilized and unfertilized.

2 1 hour before the start of dissociation prepare and/or thaw the reagents required:

1. fresh E3 media ( 1 L or more)

2. Pronase solution (1mg/mL, dissolved in E3 media)

 Pronase Merck MilliporeSigma (Sigma-Aldrich) Catalog #10165921001

3. Calcium-free Ringer's solution (prepared following ZFIN recipe book, 5th Edition

<https://zfin.atlassian.net/wiki/spaces/prot/pages/362220041/RINGER+S+SOLUTIONS>

4. FACSmax (used for timepoints lower than 10hpf)

 FACSmax Cell Dissociation Solution amsbio Catalog #AMS.T200100

5. Protease solution (used for timepoints from 10hpf (included) onwards)

- 25mL 0.5% Trypsin-EDTA
- 5mL 10X PBS0
- 20mL sterile water

6. Protease Stop solution (used for timepoints from 10hpf (included) onwards)

- 3ml FBS
- 30µL of 2M CaCl₂
- 5.9mL sterile water
- 1mL 10X PBS0 (PBS0 is PBS without Ca+ and Mg+)

7. Suspension media (1mL FBS + 9ml PBS0). PBS0 is PBS without Ca+ and Mg+

Cell Tracer dyes and rat serum to stop staining (depending on whether multiple timepoints will be mixed in a single tube or not, see flowcharts below):

☒ CellTrace®; CFSE Cell Proliferation Kit, for flow cytometry Thermo
Fisher Catalog #C34554

☒ CellTrace®; Far Red Cell Proliferation Kit, for flow cytometry Thermo
Fisher Catalog #C34572

☒ CellTrace®; Yellow Cell Proliferation Kit, for flow cytometry Thermo
Fisher Catalog #C34567

☒ rat serum Merck MilliporeSigma (Sigma-Aldrich) Catalog #R9759-5ML

Equipment

CENTRIFUGE 5430 R

NAME

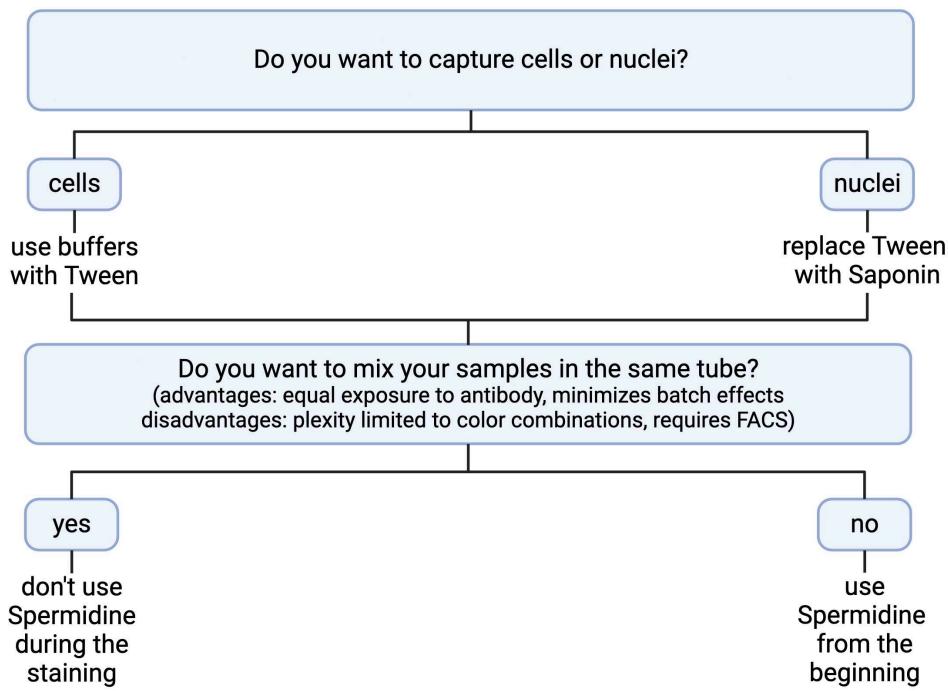
Eppendorf

BRAND

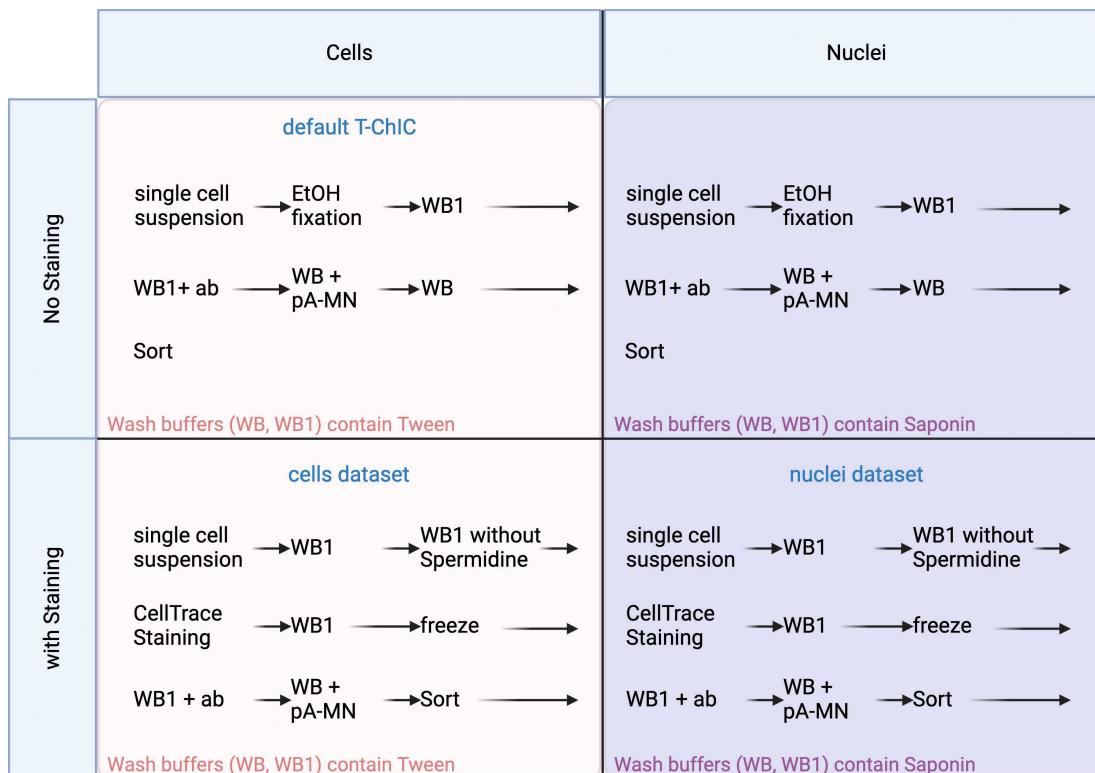
MP2231000510

SKU

After the dissociation is complete, there are a few possible routes that can be taken (requiring tweaks to the solutions). The flowchart below illustrates the 2 main choices and their required changes:



The choice of protocol will determine which solutions to prepare and whether to also procure the cell tracer dyes. Below is a more detailed overview of which solutions are needed and for which steps according to your choices. We used the two approaches in the bottom row in our datasets.



More detailed overview of which solutions are needed and for which steps according to your choices. We used the two approaches in the bottom row in our datasets. EtOH: Ethanol, WB1: Wash Buffer 1, WB: Wash Buffer, ab: antibody, pA-MN: protein A-MNase fusion protein

How to prepare the various Washing Buffers (CellTrace Staining included)

If you are not planning on using the Celltrace dyes to combine multiple samples, see alternative option below at step 2.1

Prepare Wash Buffer^(w/out Spermidine), regular Wash Buffer, Wash Buffer1^(w/out Spermidine) and regular Wash Buffer 1 (WB1). Not all of these need to be prepared now, but for the sake of completeness, they are all presented here.

We suggest preparing the Wash buffers in the following order: prepare 50mL of Wash Buffer^(w/out Spermidine) and take 10mL aside to make WB1^(w/out Spermidine). Use the remaining 40mL to make regular WB, and from regular WB, prepare WB1 as needed.

Depending on whether the objective is to capture cells or nuclei, switch out the Tween-20 for 10% Saponin.

(Dissolve fresh 10% Saponin in PBS0. Store at  4 °C , use within 2 days.)

For all Wash Buffers: prepare  On ice, store at  4 °C, use for up to 2 days

Wash Buffer(w/out Spermidine)

A	B
Wash Buffer (w/out Spermidine)	Volume 50 mL
H2O RNase free water	47.8 mL
HEPES pH 7.5 (1M)	1 mL
NaCl(5M)	1.5 mL
Saponin 10%	250 µl
Protease inhibitor	1 complete tablet

 cOmplete™, EDTA-free Protease Inhibitor Cocktail Merck Catalog #5056489001

from the Wash Buffer^(w/out Spermidine), prepare the **Wash Buffer(w/out Spermidine) 1**. We suggest preparing it on a per-needed basis to save on the RNase inhibitor.

A	B	C	D
Wash Buffer w/out Spermidine 1	Volume 1 mL	Volume 1.5mL	Volume 7,5
Wash Buffer MINUS	1mL	1.5 mL	7.5 mL
EDTA 0.5M	4 µl	6 µl	30 µl
RNAse inhibitor (1:40)	25 µl	37.5 µl	187,5 µl

 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615

From the remaining Wash Buffer^(w/out Spermidine), prepare 40mL of the **regular Wash Buffer** (by adding the Spermidine).

A	B
regular WB	Volume 40mL
Wash Buffer w/out Spermidine	40mL
Spermidine solution	2.88 µl

 Spermidine Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2626-5G

From the regular Wash Buffer, prepare the **Wash Buffer 1**. We suggest preparing it on a per-needed basis to save on the RNase inhibitor.

A	B
Wash Buffer1	Volume 2.5mL
regular WB	2.43 mL

A	B
EDTA 0.5M	10 µL
RNAse inhibitor (1:40)	62,5 µL

 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615

2.1 ALTERNATIVE: Preparing Wash Buffers (not for CellTrace staining)

For those not planning to do any stainings, they only need to prepare the regular Wash Buffer following this recipe and the WB1 by adding EDTA to the regular WB.

A	B	C	D
regular Wash Buffer	Concentration	Volume 50 mL	Volume 10 mL
H2O RNAse free water		47.8 mL	9.56 mL
HEPES pH 7.5	1M	1 mL	200 ml
NaCl	5M	1.5 mL	300 ml
Spermidine solution		3.6 µL	0.72 ml
Tween (10%)	0.05%	250 µL	50 ml
Protease inhibitor		1 complete tablet	

Store at  4 °C or in a cold room. Use for up to 2 days.

WB1

To an aliquot of WB, add  4 µL of [M] 0.5 Molarity (M) EDTA for each  1 mL of WB.

Store at  4 °C or in a cold room. Use for up to 2 days.

We suggest not turning all of the regular WB in WB1 immediately, as the regular WB will still be used during day 2.

3 Dechorionation

Transfer the embryos to a glass beaker with a plastic Pasteur pipette. Remove as much E3 as possible without exposing the embryos to air. Add ~  5 mL of

[M] 2 micromolar (µM) Pronase Solution. Incubate at  28.5 °C for  00:03:00 checking constantly for cracks in the chorion. If the chorions are still intact after the 3 minutes, incubate for longer ( 00:01:00 -  00:02:00). At the first sign of the chorions cracking, proceed to the next step.

3.1 Dilute the Pronase with serial dilutions with a large (enough to fill 90% the beaker) volume of E3 media. Pour away as much liquid as possible without leaving the embryos exposed to air, and repeat this washing step another 3-4 times. Swirl the beaker to release the embryos from their chorions.

6m



8m

- 4 Transfer the dechorionated embryos with a glass pipette to **1.5mL protein low-binding tubes**. Use multiple tubes so that each tube only contains at most ~50 embryo (less at later timepoints).

4.1 Deyolking

Remove as much E3 as possible (without leaving the embryos exposed to air). Add  200 µL of **Calcium-free Ringers solution**.

- 4.2 Gently pipette up and down with a P200 pipette to deyolk (~15 times).

Expected result

the solution should turn cloudy and slightly yellow-tinted.

- 4.3 Let sit in Ringer's solution for approximately  00:05:00 at  Room temperature . 

- 4.4 Centrifuge the samples at  600 rcf, 00:03:00 with a Swinging Buckets Centrifuge 

Equipment

CENTRIFUGE 5430 R

NAME

Eppendorf

BRAND

MP2231000510

SKU

- 5 Wash the samples with  500 µL of Suspension Media at  Room temperature 

- 5.1 centrifuge at  600 rcf, 00:03:00 with the swinging buckets centrifuge. 

6 Dissociation:

Resuspend cells in  200 µL of either FACSmax (for timepoints up to 8hpf (included)), or Protease Solution (for timepoints later than 10hpf (included)). Incubate on shaker at

600 rpm, 28°C, 00:06:00

Resuspend up and down gently every 2min.

Check under the microscope that the suspension is single cell! If not keep longer.

Expected result

the solution should contain single cells

If the solution is already single cell, interrupt the shaking and proceed to the next step.

6.1 If using the Protease Solution, at the end of the dissociation, add 34uL of Protease Stop Solution for each 200uL of Protease Solution.

7 Collect and filter the single cell solution into a 1.5 mL prot-low binding tube **using the blue cap from FACS tubes (35 um)** and centrifuge at 600 rcf, 00:03:00 with the swinging buckets centrifuge.

3m



8 Resuspend the cell pellet in 500 µL of Suspension media at Room temperature. Take a 10 µL aliquot to count the number of cells. Centrifuge the rest at 600 rcf, 00:03:00 with the swinging buckets centrifuge.

3m



9 Resuspend the pellet in 500 µL of **WB1 Full** (remember the 1:40 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615).

Keep on On ice until all the timepoints have been collected.

DAY 1: Cell Staining and Antibody Incubation

2h

10 Centrifuge all samples at 600 rcf, 00:03:00 with the swinging buckets centrifuge. Wash each sample with 200 µL of WB1 (w/out Spermidine) (with 1:200 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615), and centrifuge them again at 600 rcf, 00:03:00 with the swinging buckets centrifuge.

6m



Skip till Step 13 if you are not planning on staining your samples!

11 Resuspend each sample in 1 mL of WB1 (w/out Spermidine). Add to each sample 1 µL of one or a combination of the CellTrace Dyes:



CellTrace® CFSE Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34554

CellTrace® Far Red Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34572

CellTrace® Yellow Cell Proliferation Kit, for flow cytometry Thermo Fisher Catalog #C34567

We suggest consistently using the same staining setup between replicate experiments to speed up the configuration of the FACS Sorting Layout.

11.1 Vortex each sample well!

11.2 Incubate the samples in the dark  On ice for  00:20:00

20m



11.3 In the meantime, prepare/thaw the

 rat serum Merck MilliporeSigma (Sigma-Aldrich) Catalog #R9759-5ML (which will stop the staining)

12 Add  70 µL of Rat Serum for each  1 mL of the staining volume (7% of staining volume) to each sample and incubate at  Room temperature for  00:05:00 to stop the staining.

5m

12.1 Centrifuge the samples at  600 rcf, 4°C, 00:03:00 with the swinging buckets centrifuge.

3m

13 Wash each sample with  200 µL of WB1 Full (with 1:200

3m

 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615), and centrifuge the samples at  600 rcf, 4°C, 00:03:00 with the swinging buckets centrifuge.



14 Resuspend and collect the samples in 1 tube with WB1 Full (with 1:40



 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615). Calculate the final value so that the concentration of cells is approximately 1 Mil cells/mL, and the mixing ratios corresponds to the desired experimental mix.

If planning to measure more than one histone modification in the same experiment, split the sample in the corresponding number of tubes.

If using colors to mix samples, an unstained populations of carrier cells can be added to increase the cell number.

15 Add to each sample the appropriate primary antibody, at the appropriate dilution.

Incubate  Overnight on a roller in the dark and at  4 °C .

5m



Note

We used a 1:200 dilution for

 Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb Cell Signaling Technology Catalog #9733S

and a 1:100 dilution for

 H3K4me1 Recombinant Polyclonal Antibody Thermo Fisher Scientific Catalog #710795

DAY 2: Sort, Protein A-MN treatment and activation

1d

16

Note

From now on, reagents, cells, plates and machines should be kept cold,  On ice or at  4 °C as much as possible (including on the way from a machine to a centrifuge). Cells should ideally also be kept in the dark (until the sort).

17 **Prepare 384-wells Plates**

Before starting, make sure to have enough plates to sort into. These should be 384-wells hardshell plates (Biorad) containing at least 5uL of Mineral Oil

 Mineral Oil BioUltra, for molecular biology Merck MilliporeSigma (Sigma-Aldrich) Catalog #69794-500ML

and  0.05 µL of WB2 containing 28ng/uL of Celseq2 adapters.

Note

We suggest preparing the 384-wells plates in advance of the sort

17.1 You can prepare the plates by first **filtering** the mineral oil with a

 Stericup® Quick Release Vacuum Driven Sterile Filters Merck Millipore (EMD Millipore) Catalog #S2GPU05RE

and then dispensing several (between 5 and 10) μ Ls of the filtered mineral oil in each plate.

Note

We use a Tecan Freedom Evo for this step, but many other machines might be up to the task.

- 17.2 Next you should dispense in each plate, $\text{PCR tube } 0.05 \mu\text{L}$ of CelSeq2 adapters, diluted in WB2, in each well. We use a Mosquito Liquid Handling robot (SPT Labtech) to transfer $\text{PCR tube } 0.05 \mu\text{L}$ from each well of a master plate(containing the primers in WB2) to each well of the target plate(s) (up to 4 at the same time). To minimise evaporation, the Mosquito should be humidified to a humidity of ~ $\text{Humidity } 80 \%$
- Keep the plates On ice if you plan to use them the same day, otherwise store them at $\text{-20 } ^\circ\text{C}$. When needed, thaw them ~ 00:30:00 before the sort On ice or on thermocyclers cooled to $\text{4 } ^\circ\text{C}$.

30m

WB2

A	B	C	D
Wash Buffer 2	Concentration	Volume 50 mL	Volume 10 mL
H2O RNAse free water		47.8 mL	9.56 mL
HEPES pH 7.5	1M	1 mL	200 ml
NaCl	5M	1.5 mL	300 ml
Spermidine solution		3.6 μ L	0.72 ml
Tween (10%)	0.05%	250 μ L	50 ml

The CelSeq2 adapters were ordered from IDT and are distributed according to this layout
(same as CelSeq2 and VASA-seq)  Celseq2_primers.xlsx 19KB

- 18 Centrifuge the antibody stained sample at $\text{Centrifuge } 600 \text{ rcf, } 4^\circ\text{C, 00:03:00}$ with the swinging buckets centrifuge. Resuspend the sample in $\text{PCR tube } 500 \mu\text{L}$ of WB (with 1:200 ).

6m

Centrifuge the sample again at $\text{Centrifuge } 600 \text{ rcf, } 4^\circ\text{C, 00:03:00}$ with the swinging buckets centrifuge.

Note

It's important to use the correct buffer (WB) to wash away the EDTA that was previously present, in order for the pA-MNase not be hampered after sorting.

DAY 2: Sort, Protein A-MN treatment and activation 1d

- 19 Resuspend the sample in  500 µL of WB (with 1:40

 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615) + **ProteinA-MN** (600uL/mL, add 5uL every 1mL of WB) and **Hoechst** dye.

Note

We use our own in-house produced Protein A-MN. If producing your own, you might want to perform a titration the first time you use it.

For details of its production, see

Zeller, P., Yeung, J., Viñas Gaza, H. et al. Single-cell sortChIC identifies hierarchical chromatin dynamics during hematopoiesis. *Nat Genet* **55**, 333–345 (2023).

<https://doi.org/10.1038/s41588-022-01260-3>

Mix for  01:00:00 at  4 °C on a roller in the dark.

- 20 Centrifuge the sample at  600 rcf, 4°C, 00:03:00 with the swinging buckets centrifuge. Resuspend the sample in 500uL of WB (with 1:200

 RNasin Plus Ribonuclease Inhibitors Promega Catalog #N2615).

- 20.1 Repeat this wash again.  [go to step #20](#)

- 21 Filter the sample through the blue caps filter of FACS tubes. Proceed to sorting. Keep samples in the dark and  On ice

- 22 Sort single cells/nuclei in 384 well-plates containing mineral oil and Celseq2 adapters in WB2. Use the Hoechst and Celltracer dyes staining to identify the cells and distinguish each timepoints.
Label the plates as you sort them (We suggest adding Histone mark, Experiment, Sorting Order information in the labelling).

1d3m1h

Note

We suggest sorting all populations in the same plate, and to sort them by columns (population x in column 1, 2, 3; population y in column 4, 5, 6; etc), rather than rows. These two provisions help counter possible inter-plate and intra-plate batch effects (due to the fact that the robots we use dispense row-wise, rather than column-wise).

We also suggest leaving a few empty wells as Negative Controls (we typically leave wells O21 to O24 and P21 to P24).

22.1 Keep plates On ice

2m

Immediately after sorting, seal the plates with

 Silverseal plate sealer greiner bio-one Catalog #676090



!

Once sealed, immediately centrifuge  2000 rcf, 4°C, 00:02:00 the plate, then move it on coolblocks  On ice, or on 384-wells thermocyclers set at  4 °C.

23 Prepare the WB2 + 3 millimolar (mM) CaCl2 and the Stop Solution to start and (then) stop the MN digestion.

15m

WB2 + CaCl2:

Prepare WB2

A	B	C	D
Wash Buffer 2	Concentration	Volume 50 mL	Volume 10 mL
H2O RNase free water		47.8 mL	9.56 mL
HEPES pH 7.5	1M	1 mL	200 ml
NaCl	5M	1.5 mL	300 ml
Spermidine		3.6 µl	0.72 ml
Tween (10%)	0.05%	250 µl	50 ml

Dilute CaCl2 solution ~1:1000 (or more) in the WB2 solution, to a final concentration of 3mM.

MNase Stop Solution:

A	B	C	D
MNase Stop Solution	Concentration	Volume (1 plate)	N+1 plates
H2O RNase free water		50.25 µl	
EGTA	0.5 M	6 µl	
NP40	10%	11.25 µl	
Prot K Thermolabile	20 mg/mL	7.5 µl	
MgCl2	1M	0.94 µl	

Calculate margin when preparing the mix to account for the robot's dead volumes

 Thermolabile Proteinase K New England Biolabs Catalog #P8111S

Note

Depending on the number of plates to be processed, there might be enough time left to prepare the Stop solution during the Digestion. Even in such case, we would still suggest to prepare the MNase Stop solution (without the Prot K) before starting the Digestion anyway, and only adding the enzyme during the Digestion.

- 24 Dispense  0.1 µL of WB2 +  3 millimolar (mM) CaCl₂ in each well to activate the MN.

32m

We use a Nanodrop Liquid Handling machine for this step, and dispense into 2 plates, held in cool blocks, at the time.



As soon as plates have received the MNase, seal each plate with Aluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

Transfer the plates to 384-wells thermocyclers set at  4 °C for a  00:30:00 digestion.

Note

You should be able, with practice, to simultaneously dispense in a set of plates while centrifuging the pair that was just dispensed. If you can proceed at a steady pace (thus keeping the timing uniform), you would only need to time the first pair of plates, and then just proceed to the Digestion Stop in the same order and at the same pace.

Note

If timing allows, it would be good (and polite) to run a Daily Clean of the Nanodrop (~7/8 minutes) to remove any potential traces of the nuclease.

- 25 Stop the digestion of the plates by adding  0.1 µL of MNase Stop Solution to each well.

2m

(Remember to add the Thermolabile Prot K, if not done previously)



We use a Nanodrop Liquid Handling machine for this step, and dispense into 2 plates, held in cool blocks, at the time.

Once the dispersion is complete, seal each plate with Alluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

Once the centrifugation is complete, move plate to a 384-well thermocycler.

Note

Once done, you should run a Daily Clean of the Nanodrop (~7/8 minutes) to remove any potential traces of the Prot K for the next user(s).

- 25.1 Incubate the plate at  4 °C for  00:20:00 , then  37 °C for  02:00:00 , 2h 40m
then  55 °C for  00:20:00 and then hold at  4 °C .
- 26 Once done, freeze all plates in a  -80 °C . The plates can then be thawed and processed at any time. 

DAY 3: RNA and DNA Fragment processing and DNA adapter ligation 1d

- 27 Thaw the plates you plan to process  On ice or at  4 °C on pre-cooled thermocyclers.

Note

Reagents and plates should be kept cold,  On ice or at  4 °C as much as possible (including on the way from a machine to a centrifuge). Coolblocks are highly advisable.

28 Fragment RNA

Incubate the plates in a pre-warmed thermocycler at  85 °C for  00:02:00 .

Immediately move plates to a pre-cooled thermocycler at  4 °C . 

Note

Carefully time this step to correctly fragment the RNA.

29 Blunt End RNA fragments and Poly-A tailing

Prepare Mix1, and aliquot it in a 8-well strip tube to dispense with the Nanodrop. Keep  On ice 15m

A	B	C	D
Mix1	1 cell (nL)	1 plate (400 cells), uL	N plates + 10%
T4 PNK	1,2	12	
polyA polymerase	0,175	1,75	
ATP (0.1mM)(1 in 100)	0,88	8,75	
Tris-HCl 1M pH8	2,8	28	
50% PEG8000	0,75	7,5	
BSA 20mg/mL	0,075	0,75	
RNAse out	1	10	
KCl 1M	3	30	
DTT 0,1M	2	20	
MgCl2 1M	0,15	1,5	
H2O	3,12	31,2	
Per well in strip		18,93	

 T4 PNK New England Biolabs Catalog # M0201L

 E. coli Poly(A) Polymerase New England Biolabs Catalog #M0276L

 Adenosine 5-Triphosphate (ATP) New England Biolabs Catalog # P0756L

 RNaseOUT™ Recombinant Ribonuclease Inhibitor Thermo Fisher Scientific Catalog #10777019

 MgCl2 1M ThermoFisher Catalog #AM9530G

 BSA 20ng/mL New England Biolabs Catalog #B9000S

- 29.1 Dispense Mix1 with Nanodrop Liquid Handling Robot. Dispense  0.150 μ L of Mix1 per each well.
Once the dispensation is complete, seal each plate with Alluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

- 29.2 Once the centrifugation is complete, move plate to a 384-well thermocycler. Incubate the plate at  37 °C for  01:00:00 , and then hold at  4 °C .

30 First Strand cDNA synthesis of RNA fragments

Prepare Mix2, and aliquot it in a 8-well strip tube to dispense with the Nanodrop. Keep

 On ice

A	B	C	D
Mix2	1 cell (nL)	1 plate (400 cells), uL	N plates + 10%
SSIII	1	10	
KCl 1M	0,75	11,25	
DTT 0.1M	0,5	7,5	

2m



1h

15m

15m

A	B	C	D
dNTPs Mix (10mM)	0,75	11,25	
50% PEG8000	0,5	7,5	
BSA 20mg/mL	0,05	0,75	
H2O	6,45	96,75	
Per well in strip		18,125	

 SuperScript™ III Reverse Transcriptase Fisher Scientific Catalog #18080085

 dNTPs Mix Promega Catalog #U1515

 BSA 20ng/mL New England Biolabs Catalog #B9000S

- 30.1 Dispense Mix2 with Nanodrop Liquid Handling Robot. Dispense  0.100 µL of Mix2 per each well.

Once the dispensing is complete, seal each plate with Aluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

- 30.2 Once the centrifugation is complete, move plate to a 384-well thermocycler. Incubate the plate at  50 °C for  01:00:00 , and then hold at  4 °C .

31 DNA fragment Blunt Ending

Prepare Mix3, and aliquot it in a 8-well strip tube to dispense with the Nanodrop. Keep

 On ice

A	B	C	D
Mix3	1 cell (nL)	1 plate (400 cells), uL	N plates + 10%
Klenow large	0,325	3,25	
NaCl 5M	0,2	2	
T4 PNK	0,325	3,25	
TL Exo1	0,5	5	
dNTPs Mix (10mM)	0,325	3,25	
Tris-HCl 7,5pH	1,75	17,5	
DTT 0,1M	0,75	7,5	
ATP 100mM	0,65	6,5	
MgCl2 1M	0,25	2,5	
50% PEG8000	0,75	7,5	
BSA 20mg/mL	0,075	0,75	
H2O	9,1	91	
Per well in strip		18,75	

 DNA Polymerase I, Large (Klenow) Fragment New England Biolabs Catalog # M0210L

- ☒ T4 PNK New England Biolabs Catalog # M0201L
- ☒ Thermolabile Exonuclease I New England Biolabs Catalog # M0568L
- ☒ dNTPs Mix Promega Catalog #U1515
- ☒ ATP Solution (100 mM) Thermo Fisher Scientific Catalog #R0441
- ☒ DTT 0.1M Invitrogen - Thermo Fisher Catalog #15846582
- ☒ Tris 1M pH 7.5 ThermoFisher Catalog #15567027
- ☒ MgCl₂ 1M ThermoFisher Catalog #AM9530G
- ☒ BSA 20ng/mL New England Biolabs Catalog #B9000S
- ☒ Invitrogen™ Nuclease-Free Water (not DEPC-Treated) Invitrogen - Thermo Fisher Catalog #AM9932

31.1 Dispense Mix3 with Nanodrop Liquid Handling Robot. Dispense  0.150 µL of Mix3 per each well.
Once the dispensation is complete, seal each plate with Alluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00.

2m

31.2 Once the centrifugation is complete, move plate to a 384-well thermocycler. Incubate the plate at  37 °C for  00:30:00, then  75 °C for  00:20:00, and then hold at  4 °C.

50m

32 A-tailing DNA fragments

Prepare Mix4, and aliquot it in a 8-well strip tube to dispense with the Nanodrop. Keep

 On ice

15m

A	B	C	D
mix4	1 cell (nL)	1 plate (400 cells), uL	N plates + 10%
Taq 360	0,2	2,0	
dATP 100mM	0,2	2,0	
KCl 1M	1	10,0	
50% PEG8000	0,75	7,5	
BSA 20mg/mL	0,075	0,8	
H ₂ O	12,775	127,8	
Per well in strip		18,8	

- ☒ AmpliTaq™ 360 DNA Polymerase Thermo Fisher Scientific Catalog #4398828

- ☒ dATPs 100mM Promega Catalog #U1335

KCl 1M ThermoFisher Catalog #AM9640G

BSA 20mg/mL New England Biolabs Catalog ##B9000

Invitrogen™ Nuclease-Free Water (not DEPC-Treated) Invitrogen - Thermo Fisher Catalog #AM9932

- 32.1 Dispense Mix4 with Nanodrop Liquid Handling Robot. Dispense  0.150 μ L of Mix4 per each well.

Once the dispensing is complete, seal each plate with Aluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

2m

- 32.2 Once the centrifugation is complete, move plate to a 384-well thermocycler. Incubate the plate at  72 °C for  00:15:00 , and then hold at  4 °C .

15m

- 32.3 Start thawing the adaptor master plate ( On ice). It's advisable to centrifuge it at  2000 rcf, 4°C, 00:02:00 once it's thawed.

2m

Start humidifying the Mosquito Liquid Handling robot (SPT Labtech).

33 Adaptor Ligation DNA fragments

10m

Using a Mosquito Liquid Handling robot (SPT Labtech) transfer 50nL of 5uM paired forked adaptors from each well of the adaptor master plate to the plate(s) being processed.

Note

Humidify the Mosquito to ~  80 % to minimise the evaporation from the adaptor master plate!

These adaptors were purchased from IDT, paired (top and bottom strand) and annealed, diluted to the working concentrations and stored at -20C till used. Sequences and layout are in this table:  ChIC_forked_adaptors.xlsx 24KB

A more complete explanation of their design is in the Supplementary Information of the sortChIC paper from where they are derived:

[Single-cell sortChIC identifies hierarchical chromatin dynamics during hematopoiesis](#)

- 33.1 Once the dispensing is complete, seal each plate with Aluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

2m

- 33.2  go to step #33 Repeat the dispensing with the other plates.

Note

Once the Mosquito is done transferring the adaptors to one set of plates, you can immediately start transferring the adaptors to a second set of plates while dispensing Mix5 to the set of plates that just finished.

- 33.3 Prepare Mix5, and aliquot it in a 8-well strip tube to dispense with the Nanodrop. Keep

 On ice

15m

A	B	C	D
Mix5	1 cell (nL)	1 plate (400 cells), uL	N plates + 10%
T4 ligase (40000U)	3,5	35	
MgCl2 1M	0,35	3,5	
DTT 0,1M	6,75	67,5	
ATP 100mM	0,35	3,5	
Tris pH7.5 1M	0,45	4,5	
50% PEG8000	0,75	7,5	
BSA 20mg/mL	0,075	0,75	
H2O	2,85	28,5	
Per well in strip		18,84	

 T4 DNA Ligase New England Biolabs Catalog #M0202L

 MgCl2 1M ThermoFisher Catalog #AM9530G

 DTT 0.1M Invitrogen - Thermo Fisher Catalog #15846582

 ATP Solution (100 mM) Thermo Fisher Scientific Catalog #R0441

 Tris 1M pH 7.5 ThermoFisher Catalog #15567027

 BSA 20mg/mL New England Biolabs Catalog ##B9000

 Invitrogen™ Nuclease-Free Water (not DEPC-Treated) Invitrogen - Thermo Fisher Catalog #AM9932

- 33.4 Dispense Mix5 with Nanodrop Liquid Handling Robot. Dispense  0.150 μ L of Mix5 per each well.

2m

Once the dispensation is complete, seal each plate with Alluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

- 33.5 Once the centrifugation is complete, move plate to a 384-well thermocycler. Incubate the plate at  4 °C for  00:20:00, then at  16 °C for  16:00:00, then  65 °C for  00:10:00, and then hold at  4 °C.

16h 30m



DAY 4: Pooling, Second Strand Synthesis, Amplification

18h 4m

34 Pooling:

5m

Add 1uL of nuclease-free Water in each well of each plate.

Note

We use a Nanodrop Liquid Handling Robot for this, but an iDOT or similar machines should be capable of the same movement.

- 34.1 Once the dispension is complete, seal each plate with Alluminium plate sealer and proceed to centrifuge them at  2000 rcf, 4°C, 00:02:00 .

2m

35 Prepare Pooling Plate(s)

15m

Label each pooling plates so that they can be identified and connected with the plate they are to collect.

Pre-wet each pooling plate with  1 mL or  2 mL of filtered Mineral Oil. Tilt the plates until the oil has spread over the entire collection surface. We use

 VBLOK200 Reservoir, Case ClickBio, Inc. Catalog #CBVBLOK200-1 , but a clean, upside-down pipette tips box lid works as well .

- 35.1 Place each plate upside down over a pooling plate.

2m

Centrifuge them at up to  2000 rcf, 4°C, 00:02:00 . Do not stack pooling plates on top of each other in the centrifuge.



Expected result

the plate should be empty, and the pooling plate should contain 2 separate liquids, with the aqueous phase below and the oil above.

- 36 For each plate, pool the aqueous phase in 2mL DNA-low-binding tubes. Centrifuge the tubes at  2000 rcf, 00:01:00 (this is just to separate the aqueous phase form the oil).

5m

37 Take the lower aqueous phase and move it into a 1.5 mL DNA-low binding tube.

1m

1m

37.1 Centrifuge the tubes  2000 rcf, 00:01:00 again.



37.2 Take the lower aqueous phase and move it into a 1.5 mL DNA-low binding tube. During this pipetting step measure the volume.

2m

38 Clean up DNA using DNA beads

Add 0.8X μ L (depending on volume of each sample) of DNA beads diluted 1 in 8 with bead binding buffer (1 M NaCl, 20% PEG8000, 20mM Tris pH8, 1 mM EDTA) and mix by pipetting.

5m

Take the beads out of the fridge ~  00:30:00 before using them

38.1 Incubate for  00:15:00 at  Room temperature .

15m

38.2 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant.

5m

38.3 Add  1 mL of 80% EtOH to each sample. Incubate for  00:00:30

30s

38.4 Remove the supernatant.

1m

38.5 Repeat the 80% EtOH wash  [go to step #38.3](#)

38.6 Dry up the beads (until they should lose their shimmer, up to  00:10:00 . Drying time is influenced by the humidity. Over and under drying leads to material loss!!)

10m

39 Resuspend in  17 μ L of nuclease-free water (away from the magnet).

4m

Let elute for  00:02:00 , then move back onto the magnet. Once the beads have coalesced on the side of the tube and the eluant is clear, move the eluant to a new tube.

40 Second Strand Synthesis:

1m

 On ice !

Add  2 μ L of 10X SSS buffer. Mix by vortexing and spin down.

40.1 Add  1 μL of SSS Enzyme and mix by gently flicking the tube. Spin down

1m

40.2 Incubate at

 16 °C for  02:30:00,  65 °C for  00:20:00, then hold at  4 °C

2h 50m

41 Add 1.3X μl of undiluted DNA beads ( 26 μL) and incubate for  00:15:00 at
 Room temperature

15m

41.1 Take out of the freezer the IVT reagents (except the enzyme) so they start thawing ~30min in advance of when they will be used.

41.2 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant.

41.3 Add  1 mL of 80% EtOH to each sample. Incubate for  00:00:30

30s

41.4 Remove the supernatant.

1m

41.5 Repeat the 80% EtOH wash  [go to step #41.3](#)

41.6 Dry up the beads (until they should lose their shimmer, up to  00:10:00). Drying time is influenced by the humidity. Over and under drying leads to material loss!!)

10m

41.7 Resuspend in  8 μL of nuclease-free water (away from the magnet). Let elute for ~
 00:02:00

2m

42 IVT

Prepare the IVT mix (thaw the reagents (except the T7 enzyme) ~  00:30:00 in advance):

13h 30m



A	B	C
IVT Mix	1 tube	N + 1 tube
ATP	2 μL	
GTP	2 μL	
CTP	2 μL	
UTP	2 μL	
T7 Buffer	2 μL	

A	B	C
T7 Enzyme	2 uL	

Add 12 μ L of IVT mix to each tube of eluate with beads.

Incubate on a thermocycler at 37°C for 13:00:00, with lid at 70°C . Then, hold at 4°C

DAY 5: rRNA depletion, RNA adapter ligation, RT, PCR and final library

6h 29m 30s

43 IVT bead cleanup

10m

Move samples onto magnet rack.

Move supernatant to a new tube, and add 1.3X Ampure RNA beads (26 μ L) and incubate for 00:10:00 at Room temperature

43.1 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant.

43.2 Add 1 mL of 80% EtOH to each sample. Incubate for 00:00:30

30s

43.3 Remove the supernatant.

43.4 Repeat the 80% EtOH wash  [go to step #41.3](#)

43.5 Dry up the beads (until they should lose their shimmer, up to 00:10:00). Drying time is influenced by the humidity. Over and under drying leads to material loss!!

10m

43.6 Resuspend in 10 μ L of nuclease-free water (away from the magnet). Let elute for ~ 00:02:00

43.7 Place sample in magnet rack, once the beads have coalesced on the side of the tube and the eluant is clear, move the supernatant to a new tube.

44 rRNA depletion

Transfer 6 μ L of the aRNA to a new tube On ice, and freeze the remaining eluant at -80°C

45 Prepare the Hybridisation buffer - oligos mix:



45.1 Prepare the Hybridisation Buffer.

Note

We suggest preparing a large volume of the Hybridisation buffer once, aliquoting and storing it at -20 °C until needed.

A	B
Hybridisation Buffer	
NaCl 5M	200 uL
Tris-HCl (1M), pH 7.5	500uL
H2O	300 uL

45.2 Mix the Hybridisation Buffer with the rRNA depletion Oligos (On ice)

2m

A	B	C
	1 tube	n+1 tubes
Hybridization Buffer	2 uL	
rRNA-depletion oligos, 25uM	2 uL	

The rRNA depletion oligos that we used for the Zebrafish Ribosomes are these (ordered from IDT and pooled together): Zf_rRNA_dep_oligos.xlsx 15KB

45.3 Add 4 µL of the Hybridisation Buffer + rRNA depletion Oligos mix to each tube.

1m

Spin down and cool 0 °C .

45.4 Incubate tubes on a thermocycler at 95 °C for 00:02:00 , then decrease the temperature to 45 °C at a rate of **0.1C/s**, then hold at 45 °C

2m



46 Prepare the RNase mix:

46.1 Prepare the RNase Buffer.



A	B
RNase Buffer	
Tris-HCl (1M), pH 7.5	500 uL

A	B
NaCl (5M) 200µL	200 uL
MgCl ₂ (1M) 200µL	200 uL
H ₂ O 3100µL	3100 uL

Note

We suggest preparing a large volume of the RNase buffer once, aliquoting and storing it at  -20 °C until needed.

46.2 Prepare the RNase Mix.

2m

A	B	C
	1 tube	n+1 tubes
Epicentre (Lucigen) RNaseH (Thermostable)	2 uL	
RNAse buffer	8 uL	

 [Hybridase Thermostable RNase H Biosearch Technologies Catalog #H39500](#)

46.3 Incubate the RNase Mix at 45 °C for a few minutes (we suggest placing it in the same thermocycler as samples, once they are holding at 45C)

5m

46.4 While keeping both samples and Hybridise Mix on the thermocycler at 45 °C , add 10 µL of the RNAse mix to each sample.

1m

46.5 Incubate the samples at 45 °C for 00:30:00 .

30m

46.6 Spin down and cool On ice .

47 DNAse treatment (removal of depletion oligos)

47.1 Prepare the DNAse Mix:

A	B	C
	1 tube	n+1 tubes
Nuclease-free Water	21 uL	

A	B	C
CaCl ₂ (10mM)	5 μ L	
Promega RQ1 DNase	4 μ L	

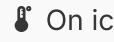
 [RQ1 RNase-Free DNase Promega Catalog #M6101](#)

- 47.2 Add  30 μ L of DNAse Mix to each tube. Incubate at  37 °C for  00:30:00 , then hold at  4 °C . 30m
- 47.3 Start thawing Ampure RNA beads at  Room temperature ~30 min before the Bead clean up.
- 48 **Bead Cleanup** 10m
Add  80 μ L of undiluted, pre-warmed RNA beads to each sample and incubate at  Room temperature for  00:10:00 .
- 48.1 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant. 5m
- 48.2 Add  1 mL of 80% EtOH to each sample. Incubate for  00:00:30 30s
- 48.3 Remove the supernatant. 1m
- 48.4 Repeat the 80% EtOH wash  [go to step #41.3](#)
- 48.5 Dry up the beads (until they should lose their shimmer, up to  00:10:00 . Drying time is influenced by the humidity. Over and under drying leads to material loss!!) 10m
- 48.6 Resuspend in  6 μ L of nuclease-free water (away from magnet). Let elute for ~  00:02:00
- 48.7 Place sample in magnet rack, once the beads have coalesced on the side of the tube and the eluant is clear, transfer  5 μ L of eluant to a new tube ( On ice).
- 49 **Adapter ligation**
Pre-heat a thermocycler to  70 °C (with heated lid at  105 °C).
Add to each sample  1 μ L of RA3($[M]$ 20 micromolar (μ M)).

RA3 (ordered from IDT): /5rApp/TGGAATTCTCGGGTGCCAAGG/3SpC3/

- 49.1 Heat adapter-sample-mix at  $70\text{ }^{\circ}\text{C}$ for  00:02:00 and then directly put  

- 49.2 Pre-heat a thermocycler to  $25\text{ }^{\circ}\text{C}$ (with lid heated at  $25\text{ }^{\circ}\text{C}$ or without the lid closed).

Prepare the Ligation Mix ( On ice):

A	B	C
	1 tube	$n+1$ tubes
NEB 10x T4 RNA Ligase Reaction Buffer	1.1 μl	
NEB T4 RNA Ligase 2, truncated	1.1 μl	
Invitrogen RNaseOUT	1.1 μl	
Nuclease-free H ₂ O	1.1 μl	

 NEB T4 RNA Ligase 2, truncated **New England Biolabs Catalog #M0242L**

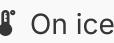
 RNaseOUT™ Recombinant Ribonuclease Inhibitor **Invitrogen - Thermo Fisher Catalog #10777019**

- 49.3 Add  4 μl of the Ligation mix to each tube, mix well by pipetting and spin down (keep  On ice)

- 49.4 Incubate on thermocycler at  $25\text{ }^{\circ}\text{C}$ for  01:00:00 (lid heated at 25°C or without the lid closed), then hold at  $4\text{ }^{\circ}\text{C}$.

50 cDNA synthesis

Pre-heat a thermocycler to  $65\text{ }^{\circ}\text{C}$ (with heated lid at  $105\text{ }^{\circ}\text{C}$).

- 50.1 Prepare the dNTPs Mix ( On ice):

A	B	C
	1 tube	$n+1$ tubes
Promega dNTP Mix (10 mM each)	1 μl	
RTP (20 μM)	2 μl	

 SuperScript™ III Reverse Transcriptase **Fisher Scientific Catalog #18080085**

 dNTPs Mix **Promega Catalog #U1515**

RTP (ordered from IDT): GCCTTGGCACCCGAGAATTCCA

50.2 Add  3 μ L of the dNTPs to each tube.

5m

Incubate on thermocycler at  65 °C for  00:05:00, then transfer tubes directly  On ice.

50.3 Prepare the Reverse Transcription Mix ( On ice):

A	B	C
	1 tube	n+1 tubes
Invitrogen 5x FS Buffer	4 μ L	
H ₂ O	1 μ L	
Invitrogen 0.1M DTT	1 μ L	
Invitrogen RNaseOUT (40U/ μ L)	1 μ L	
Invitrogen SS III (200U/ μ L)	1 μ L	

 RNaseOUT™ Recombinant Ribonuclease Inhibitor Invitrogen - Thermo Fisher Catalog #10777019

 SuperScript™ III Reverse Transcriptase Fisher Scientific Catalog #18080085

50.4 Add  8 μ L of the Reverse Transcription mix to each tube, mix well by pipetting and spin down (keep  On ice).

1h 15m

50.5 Incubate in a thermocycler at  50 °C for  01:00:00, then  70 °C for  00:15:00, and then hold at  4 °C (with lid heated at  85 °C).

51 RNA degradation

30m

Add  1 μ L of  RNase A Thermo Scientific Catalog #EN0531 to each tube.

Incubate on thermocycler at  37 °C for  00:30:00, then hold at  4 °C (with heated lid at  70 °C)

51.1 Start thawing Ampure DNA beads at  Room temperature ~30 min before the next Bead clean up.

52 Bead Cleanup

10m

Add  22 μ L of undiluted, pre-warmed Ampure XP beads to each sample and incubate at  Room temperature for  00:10:00.

52.1 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant.

52.2 Add  1 mL of 80% EtOH to each sample. incubate for  00:00:30

30s

- 52.3 Remove the supernatant. 1m
- 52.4 Repeat the 80% EtOH wash 1m
- 52.5 Dry up the beads (until they should lose their shimmer, up to 00:10:00). Drying time is influenced by the humidity. Over and under drying leads to material loss!! 10m
- 52.6 Resuspend in 20 µL of nuclease-free water (away from magnet). Let elute for ~ 00:02:00 2m
- 52.7 Place sample in magnet rack, once the beads have coalesced on the side of the tube and the eluant is clear, transfer 20 µL of eluant to a new tube (On ice). 2m
- 52.8 Split the eluant in 2 tubes. Store half (10 µL) at -20 °C, and continue with the other 10 µL. 2m

53 PCR amplification

Prepare the PCR Mix:

A	B	C
	1 tube	n+1 tubes
NEBNext High-Fidelity 2X PCR Master Mix	25 µl	
RP1	2 µl	
H2O (nuclease free)	11 µl	

 NEBNext® High-Fidelity 2X PCR Master Mix New England Biolabs Catalog #M0541L

RP1 (Illumina RNA PCR Primer 1):

5' AATGATACGGCGACCACCGAGATCTACACGTTAGAGTTCTACAGTCCGA

- 53.1 Add 36 µL of the PCR Mix to each 10uL sample. 1m
- 53.2 To each sample, add 2 µL of one Illumina TruSeq Small RNA Index Adapter (RPI Series). When choosing indexes, keep in mind your sequencing arrangements (it's not advisable to have 2 samples with the same index, even if they come from different organisms). 1m

Note

We usually avoid the following indexes: RPI10, RPI24, RPI41

Adaptor sequences (Illumina TruSeq Small RNA Index Adapter, RPI Series) are here :

 Illumina_RPI_sequences.xlsx 9KB

- 53.3 Incubate at  98 °C for  00:00:30 ,  30m
then run either 7 or 8 cycles of:  98 °C for  00:00:10 ,  60 °C for   00:00:30 ,  72 °C for  00:00:30 ;
then  72 °C for  00:10:00 ,
and finally hold at  4 °C 

54 **Bead Cleanup**  15m

Add  40 µL of undiluted, pre-warmed Ampure XP beads to each sample and
incubate at  Room temperature for  00:15:00 .

- 54.1 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant.
- 54.2 Add  1 mL of 80% EtOH to each sample. Incubate for  00:00:30  30s
- 54.3 Remove the supernatant.
- 54.4 Repeat the 80% EtOH wash  [go to step #54.2](#)
- 54.5 Dry up the beads (until they should lose their shimmer, up to  00:10:00 . Drying time is influenced by the humidity. Over and under drying leads to material loss!!)
- 54.6 Resuspend in  25 µL of nuclease-free water (away from magnet). Let elute for ~  00:02:00
- 54.7 Place sample in magnet rack, once the beads have coalesced on the side of the tube and the eluant is clear, transfer  25 µL of eluant to a new tube.

55 **Bead Cleanup**

Add  20 μL of undiluted, pre-warmed Ampure XP beads to each sample and incubate at  Room temperature for  00:15:00.

15m

55.1 Move eppendorfs on a magnet rack and, once the beads have coalesced on the side of the tube and the supernatant is clear, remove the supernatant.

55.2 Add  1 mL of 80% EtOH to each sample. Incubate for 

30s

55.3 Remove the supernatant.

55.4 Repeat the 80% EtOH wash  [go to step #55.2](#)

55.5 Dry up the beads (until they should lose their shimmer, up to  00:10:00). Drying time is influenced by the humidity. Over and under drying leads to material loss!!)

55.6 Resuspend in  10 μL of nuclease-free water (away from magnet). Let elute for ~
 00:02:00

55.7 Place each sample in magnet rack. Once the beads have coalesced on the side of the tube and the eluant is clear, transfer  10 μL of eluant to new tubes.



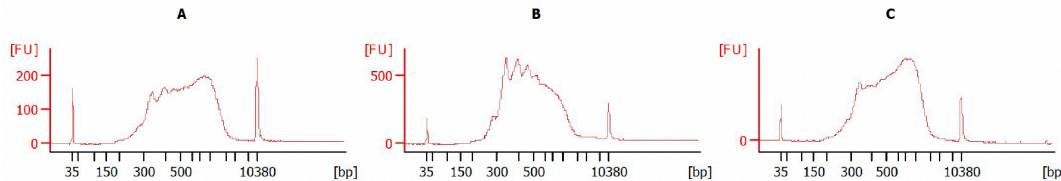
Label these tubes carefully for they contain the final library. Store at -20C if not planning on measuring concentration and size distribution soon,

56 Measure the concentration with a Qubit High Sensitivity DNA assay.

Measure the distribution of lengths of the library with a High Sensitivity DNA Bioanalyser. Store at -20C if not planning on sequencing soon.

Expected result

the concentration should be greater than 1ng/uL, and the average length should be around 500-600nt.



Example library size distribution with a High Sensitivity DNA Bioanalyser.
The peaks, particularly evident in B, are due to the ChIC fraction of the library. The smoother curve is due to the RNA fraction of the library.

- 57 Proceed to sequence each library at a depth of ~60M reads per plate.

Note

We have used Illumina NextSeq 2000 or Novaseq 6000, 2 x 100 bp, with 10% PhiX spike in, low complexity region: NNNNNNNNNNNNTNNNNNNNNNNNN .