

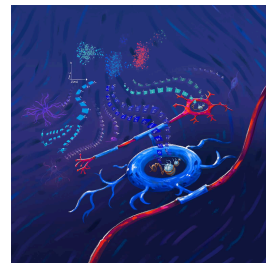
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Version 1

🌐 Nano-CUT&Tag for multimodal profiling of the chromatin V.1

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We use this protocol and it's working

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Abstract

Nano-CUT&Tag is a multimodal technology to profile several histone modifications at the same with single-cell resolution. Nano-CUT&Tag implements a novel Tn5 fusion proteins to anti-mouse and anti-rabbit secondary nanobodies. Optionally, ATAC-seq can be performed prior on the same sample to profile open chromatin at the same time. Novel tagmentation protocol, which involves two-step tagmentation by MeA and MeB oligonucleotides yields increased number of fragments per cell comparing to previous single-cell CUT&Tag protocols on 10x Genomics platform.

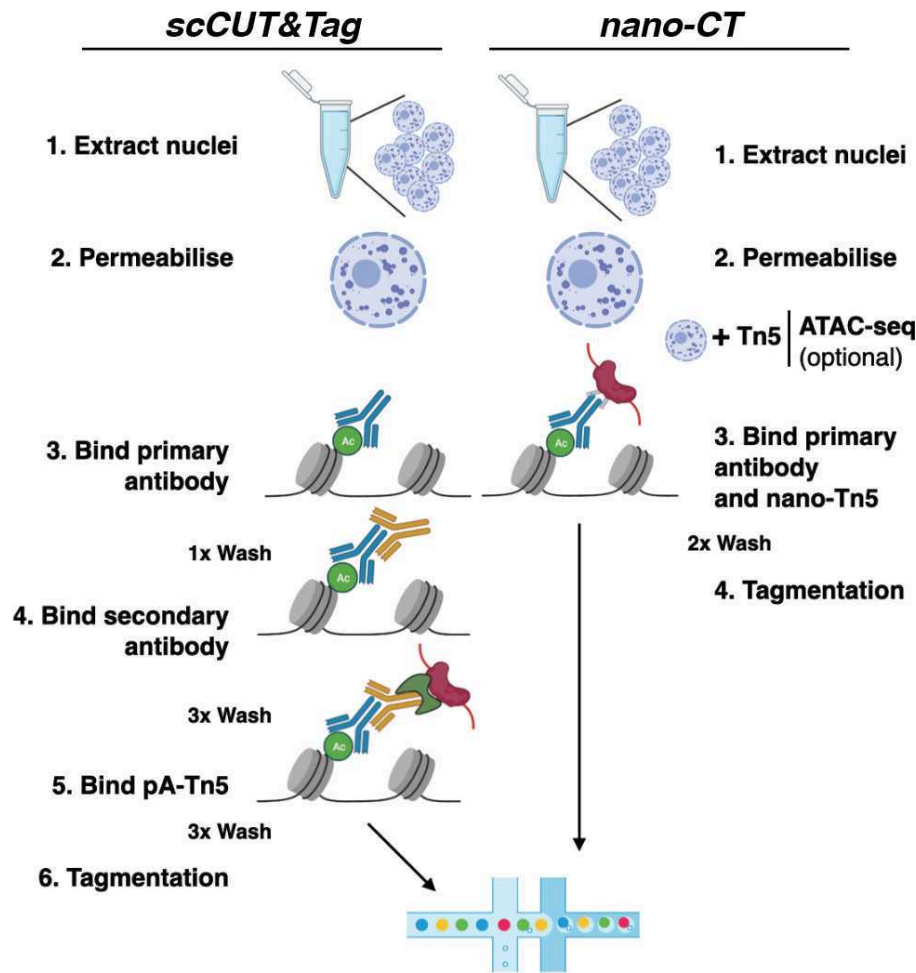


Figure 1. Comparison of scCUT&Tag and nano-CT tagmentation protocol

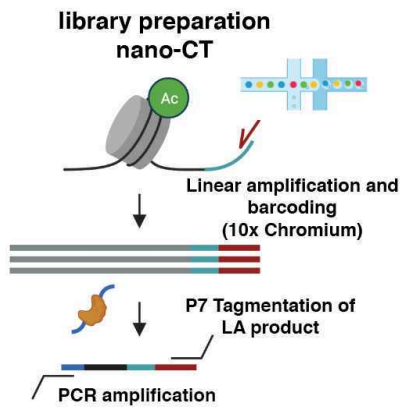


Figure 2. Depiction of the new tagmentation strategy of nano-CT. First nuclei are tagged by nano-Tn5 loaded with MeA oligonucleotides. After single-cell barcoding and DNA recovery/purification, it is tagmented randomly with standard Tn5 loaded with MeB oligonucleotides.

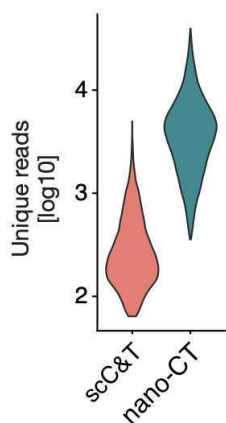


Figure 3. Number of fragments per cell in nano-CT comparing to single-cell CUT&Tag

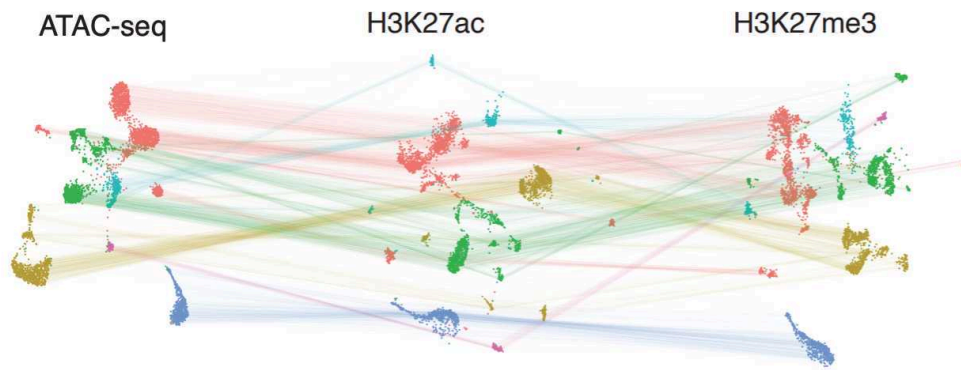


Figure 4. Multimodal UMAP projection of 3 epigenetic modalities measured in the mouse brain

Guidelines

This protocol involves large number of centrifugation steps, and it is critical to prevent nuclei loss and/or clumping. Therefore, always use **swinging bucket rotor centrifuge** for all centrifugation steps, include **2% BSA** in all buffers which come in contact with nuclei and **carefully aspirate the supernatant**. It is better to leave cca 5-10 ul of supernatant in the tube rather than lose the nuclei.

Clumping of nuclei can be a problematic, but generally, having cleaner nuclei helps to prevent clumping. Removing of debris from your sample is highly recommended. For mouse brain Debris Removal Solution has proven to be effective in removing excess debris (Miltenyi, 130-109-398).

Some steps in this protocol involve usage of in-house produced Tn5/nano-Tn5. Individual tagmentation steps should be optimised according to specific batch of the proteins.

The two-step tagmentation protocol can also be performed with pA-Tn5 protein. Load pA-Tn5 only with MeA/Me-Rev oligos and follow the protocol as described here. pA-Tn5 profiling should be compatible with ATAC-seq in the same sample, but this was not tested.

Materials

Preparation of buffers:

nano-CUT&Tag buffers preparation

5% Digitonin	Prepare aliquotes and store at -20
1g	Digitonin powder
20 ml	DMSO

20 % BSA	Filter through 0.45 um filter	BSA can be used within 2 days and stored at 4C
2g	BSA	Filter through
10ml	water	

2x Wash buffer (25ml)		Store at 4C and use within one week	
Final		Stock	Amount
40 mM	Hepes pH 7.5	1 M	1 ml
300 mM	NaCl	5 M	1,5 ml
1 mM	Spermidine	2 M	13 ul
2x	Protease inh.	tablet	1 tablet
	water		23 ml

Antibody buffer (2ml/sample)		Prepare fresh		
		1 sample	2 samples	4 samples
2x		1 ml	2 ml	4ml
2 mM		8ul	16 ul	32 ul
0.05%		20ul	40 ul	80 ul
0.01 %		2ul	4 ul	8ul
2%		200ul	400 ul	800ul
		770 ul	770ul x2	3080ul
				Water

Dig300 -Wash-BSA (2ml/sample)		Prepare fresh		
		1 sample	2 samples	4 samples
1x		1ml	2 ml	4ml
0,05%		20 ul	40 ul	80 ul
0,01%		2 ul	4 ul	8 ul
2%		200 ul	400 ul	800 ul
300 mM		60 ul	120 ul	240 ul
		718 ul	718 ul x2	2872 ul
				water

Tagmentation buffer (200 ul/sample)		Prepare fresh		
		for up to 6 samples		
1x		750 ul	2x wash buffer	
10 mM		15 ul	MgCl2 (1M)	
0.05%		15 ul	Digitonin (5%)	
0.01 %		1.5 ul	NP-40 (10%)	
300 mM		45 ul	NaCl (5M)	
2%		150 ul	BSA (20%)	
		523.5 ul	Water	



ATAC-seq buffers

ATAC resuspension buffer

final conc.		stock	for 50 ml
10 mM	Tris pH 7.5	1M	500 ul
10 mM	NaCl	5M	100 ul
3 mM	MgCl ₂	1M	150 ul
	water		49.25 ml

Store at room temperature long term

Cell lysis buffer

Prepare fresh

final conc		stock	for 200 ul
	ATAC resuspension buffer	1x	195.6 ul
0.1 %	10% NP-40	10%	2 ul
0.1 %	10% Tween-20	10%	2 ul
0.01%	5% Digitonin	5%	0.4 ul

ATAC wash buffer

Prepare fresh

final conc		stock	for 1ml
	ATAC resuspension buffer	1x	990 ul
0.1 %	10% Tween-20	10%	10 ul

2x TD buffer

Can be stored in aliquotes at -20

final conc	stock	stock	for 1ml
20 mM	Tris pH 7.5	1M	20 ul
10 mM	MgCl ₂	1M	10 ul
20%	Dimethylformamide	100%	200 ul
	water		770 ul

2x TD buffer is used in both ATAC transposition and MeB library prep

2x Tn5 loading buffer

Can be stored at 4C without DTT *

final conc	stock	stock	for 10ml
100 mM	HEPES pH 7.2	250 mM	4 ml
200 mM	NaCl	5M	400 ul
0.2 mM	EDTA	0.5M	4ul
0.2 %	Triton-X	10%	200 ul
20%	Glycerol	100%	2ml
2 mM	DTT	200 mM	*
	water		3.4 ml

* Add DTT fresh just before loading the Tn5 to small aliquot of 2x Tn5 loading buffer; e.g. 99ul of 2x Tn5 loading buffer + 1 ul of 200 mM DTT

2x Tn5 loading buffer can be stored at room temperature without DTT

oligonucleotide sequences:

Tn5_MeA_P5_noBCD.

5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

Tn5_MeA_P5_bcdA.

5'-TCGTCGGCAGCGTCT TATAGCCT GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Tn5_MeA_P5_bcdB

5'-TCGTCGGCAGCGTCT ATAGAGGC GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Tn5_MeA_P5_bcdC

5'-TCGTCGGCAGCGTCT CCTATCCT GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Tn5ME-B:

5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

Tn5MErev.

5'-[phos]CTGTCTCTTATACACATCT-3'

Additional barcodes can be selected based on:

Amini, S., Pushkarev, D., Christiansen, L. et al. Haplotype-resolved whole-genome sequencing by contiguity-preserving transposition and combinatorial indexing. Nat Genet 46, 1343–1349 (2014). <https://doi-org.proxy.kib.ki.se/10.1038/ng.3119>



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Material:

Tn5 loading:

250mM Hepes pH 7.2, homemade or commercial alternative

Glycerol (ThermoFischer 327255000)

5M NaCl (Invitrogen, AM9759)

0.5M EDTA (Invitrogen, AM9260G)

1M DTT (ThermoFisher, P2325)

10% Triton-X (ThermoFisher, 85111)

anti-rabbit-Tn5 (nano-Tn5, Addgene #183637) in house purified, no commercial option to date

anti-mouse-Tn5 (nano-Tn5, Addgene #183638) in house purified, no commercial option to date

unloaded Tn5 enzyme- in house purified (There are several commercial options available for example from lucigen or diagenode)

ATAC-seq

1x PBS

10% Tween-20 (Bio-Rad, 1706531)

5% Digitonin in DMSO (EMD Milipore, 300410)

Tn5 transposase protein (for ATAC-seq)



Nuclease free water (ThermoFisher, 10977015)
0.5M EDTA (Invitrogen, AM9260G)
1M Tris pH7.5
5M NaCl (Invitrogen, AM9759)
1M MgCl₂ (Invitrogen, AM9530G)
10%NP-40 (Thermo, 85124)
Dimethylformamide (Sigma, 227056)

nano-CUT&Tag

5% Digitonin in DMSO (EMD Milipore, 300410)
BSA (Sigma, A9418)
1M Hepes pH7,5 (Alfa Aesar, J60712)
5M NaCl (Invitrogen, AM9759)
Spermidine (ThermoFisher, A19096.06)
Complete EDTA-free protease inhibitors (Roche, 11873580001)
0.5M EDTA (Invitrogen, AM9260G)
10%NP-40 (Thermo, 85124)
1M MgCl₂ (Invitrogen, AM9530G)
Counting chambers (VWR, 630-1893)
Chromium Next GEM Chip H Single Cell Kit
Chromium Next GEM Single Cell ATAC Library & Gel Bead Kit v1.1
Single Index Kit N Set A
1M Tris pH7.5
Dimethylformamide (Sigma, 227056)
Zymo DNA Clean and concentrator-5 kit
SPRI beads (Beckman Coulter, B23318)
Ethanol, Pure (200 Proof, anhydrous, Milipore Sigma, E7023)
10% Tween-20 (Bio-Rad, 1706531)
Qiagen Buffer EB (Qiagen, 19086)
Bioanalyser or Tapestation with appropriate kits

Swinging bucket rotor centrifuge
Appropriate adaptors for 0.5 ml tubes

Custom sequencing primers:

>Custom_primer_R1
GCGATCGAGGACGGCAGATGTGTATAAGAGACAG
>Custom_primer_L2
CTGTCTCTTATACACATCTGCCGTCTCGATCGC

Validated primary antibodies in nano-CT



rabbit:

H3K27ac (Abcam, Ab177178)

mouse:

H3K27me3 (Abcam, Ab6002)

Validated primary antibodies for scCUT&Tag

H3K27ac (Abcam, Ab177178)

H3K4me3 (Diagenode, C15410030)

H3K27me3 (Cell Signalling, 9733T)

H3K36me3 (Abcam, Ab9050)

Safety warnings

⚠ Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves while handling any amount of digitonin.

Before start

Before starting, make yourself familiar with the 10x genomics Chromium Single Cell ATAC Reagent Kits User Guide (v1.1 Chemistry).

Follow all the best practices and tips given in the 10x genomics Chromium Single Cell ATAC Reagent Kits User Guide.

This protocol is compatible with Chromium Next GEM Single Cell ATAC Library & Gel Bead Kit v1.1 has not been tested with Chromium Next GEM Single Cell ATAC Kit v2.



CG000209_Chromium_NextGEM_Si...



Tn5 loading

2h 45m

1 Annealing adaptor sequences:

Tn5_MeA_P5_noBCD.

5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

Tn5_MeA_P5_bcdA.

5'-TCGTCGGCAGCGTCT **TATAGCCT** GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Tn5_MeA_P5_bcdB

5'-TCGTCGGCAGCGTCT **ATAGAGGC** GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Tn5_MeA_P5_bcdC

5'-TCGTCGGCAGCGTCT **CCTATCCT** GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Tn5ME-B:

5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

Tn5MErev.

5'-[phos]CTGTCTCTTATACACATCT-3'

Note

Nano-CUT&Tag can be performed in its barcoded (multimodal) and unbarcoded version (single histone modality)

**Note**

Optionally, extra nucleotides can be included to prevent low complexity in sequencing of adaptor part of the library, if using sequencing platform sensitive to low complexity regions.

Equimolar ratio of the 4 following oligonucleotides should then be used.

For example for barcode A:

```
>Tn5_P5_MeA_BcdA_0N
TCGTCGGCAGCGTCTCCACGCTATAGCCTGCGATCGAGGACGGCAGATGTGTATAAGAGACA
G
>Tn5_P5_MeA_BcdA_1N
TCGTCGGCAGCGTCTCCACGCTATAGCCTNGCGATCGAGGACGGCAGATGTGTATAAGAGAC
AG
>Tn5_P5_MeA_BcdA_2N
TCGTCGGCAGCGTCTCCACGCTATAGCCTNNGCGATCGAGGACGGCAGATGTGTATAAGAGA
CAG
>Tn5_P5_MeA_BcdA_3N
TCGTCGGCAGCGTCTCCACGCTATAGCCTNNNGCGATCGAGGACGGCAGATGTGTATAAGAG
ACAG
```

This should not be necessary for NovaSeq and HiSeq platforms, but sequence on your own risk.

- 2 Resuspend the oligonucleotides (Tn5ME-A, Tn5ME-B, Tn5MErev) in water to a final concentration of 100µM each. 10m
- 3 Mix equimolar amounts of Tn5MErev/Tn5ME-A and Tn5MErev/Tn5ME-B in separate 200 µl PCR tubes. 5m

e.g:

tube1: 10 µL Tn5ME-A + 10 µL Tn5MErev
tube2: 10 µL Tn5ME-B + 10 µL Tn5MErev
- 4 Denature in the thermocycler for 00:05:00 at 95 °C , and cool down slowly on the thermocycler by ramping down to 20 °C by 0.1 °C per second 1h

Pause point: Store the annealed oligos at -20 °C



5 Mix annealing mix.

30m

The final Tn5 concentration is [M] 2 micromolar (μM) of Tn5 dimer

Use unique barcodes for specific nano-Tn5 or WT-Tn5.

Mouse Nano-Tn5 (MeA/P5 loaded):

 4 μL Annealed, barcoded MeA/Me-Rev oligos [M] 50 micromolar (μM) (e.g. barcode A, Me-A/Me-Rev)

 21 μL Glycerol

 3 μL Nano-Tn5 (mouse, 5mg/ml, MW = 73941 g/mol;
[M] 67.6 micromolar (μM))

 22 μL 2x Tn5 loading buffer

Rabbit Nano-Tn5 (MeA/P5 loaded):

 4 μL Annealed, barcoded MeA/Me-Rev oligos [M] 50 micromolar (μM)
(e.g. barcode B, Me-A/Me-Rev)

 21 μL Glycerol

 2.2 μL Nano-Tn5 (rabbit, 6.8mg/ml, MW = 73013 g/mol
[M] 93.1 micromolar (μM))

 22.8 μL 2x Tn5 loading buffer

WT Tn5-MeA with barcode (for ATAC-seq)

 4 μL Annealed, barcoded MeA/Me-Rev oligos [M] 50 micromolar (μM)
(e.g. barcode C, Me-A/Me-Rev)

 21 μL Glycerol

 3.1 μL Tn5 (3.5 mg/ml, MW = 53300 g/mol [M] 65.7 micromolar (μM))

 21.9 μL 2x Tn5 loading buffer

**WT Tn5-MeB unbarcoded (for 2nd tagmentation)**

4 μL	Annealed MeB/Me-Rev oligos	1M 50 micromolar (μM) (un-barcoded, Me-B/Me-Rev)
21 μL	Glycerol	
3.1 μL	Tn5 (3.5 mg/ml, MW = 53300 g/mol	1M 65.7 micromolar (μM))
21.9 μL	2x Tn5 loading buffer	

Note

Adjust the volumes for specific nano-Tn5 protein batch for final 1M 2 micromolar (μM) nano-Tn5 dimer.

The volume of nano-Tn5 and 2x Tn5 loading buffer should add up to 25 μL and volume of oligos and glycerol also adds up to 25 μL .

Optionally scale up or down the reactions as necessary.

2x Dialysis buffer

100 mM HEPES-KOH pH7.2, 200 mM NaCl, 0.2 mM EDTA, 0.2% Triton-X, 20% Glycerol,

Store at 4 $^{\circ}\text{C}$;

Add DTT fresh to the 2x dialysis buffer just before loading (2mM final). Keep 200 mM

DTT stock at -20 $^{\circ}\text{C}$

For details on buffer preparation see Materials section

6 Incubate for 01:00:00 at Room temperature

1h

7 Store the loaded nano-Tn5 at -20 $^{\circ}\text{C}$



ATAC-seq (optional)

1h 30m

- 8 Dissociate tissues/cells of interest by desired method and obtain single-cell suspension. Wash the cells once with 1x PBS 10m

Note

ATAC-seq protocol is based on the Omni-ATAC protocol (Corces et al., 2017).

- 9 Centrifuge the cells for 00:10:00 at 300x g at 4 °C . Discard the supernatant. 15m

- 10 Add 200 µL of ATAC lysis buffer (for 200,000 cells, see materials section for buffer recipe). Pipette up and down gently 3x and incubate on ice for 00:03:00 5m

Note

Scale the volume of lysis buffer down for lower cell input (e.g. 50ul for 50,000 cells)

- 11 Add 1 mL of ATAC wash buffer and gently invert the tube 3x.

- 12 Centrifuge at 500x g for 00:10:00 . Discard the supernatant. 10m

- 13 Prepare transposition mix: 10m

100 µL 2X TD Buffer (see materials section)

66 µL 1X PBS

2 µL 10% Tween-20 (final 0.1% v/v)

2 µL 1% Digitonin (final 0.01% v/v)

 10 μL

Tn5 Transposase (loaded with uniquely barcoded oligonucleotides-MeA only)

 20 μL nuclease-free H₂O

- 14 Resuspend the nuclei in 200 μL of transposition mix (for 200,000 cells).

5m

Note

Scale the volume of transposition mix down for lower cell input (e.g. 50ul for 50,000 cells)

- 15 Incubate for 00:30:00 at 37 °C in thermomixer at 1000 rpm.

30m

- 16 Stop the tagmentation by adding 10 μL of 500 mM EDTA. Mix by pipetting up and down 3x.

- 17 Centrifuge for 00:10:00 at 500x g

10m

- 18 Remove the supernatant. Resuspend in 200 μL of CUT&Tag Antibody buffer.

- 19 Centrifuge for 00:03:00 at 600x g. Remove the supernatant.

3m

Proceed to CUT&Tag Antibody binding (**Step 23**).**CUT&Tag Nuclei isolation (nano-CUT&Tag without ATAC)**

15m

- 20 Dissociate tissues/cells of interest by desired method and extract nuclei by incubation for 00:03:00 in 200 μL of Antibody buffer on ice.

5m

**Note**

Amount of input material can range from 25,000 - 200,000, depending on tissue or cell type and whether ATAC-seq is performed together with nano-CUT&Tag

Note

If ATAC-seq is desired, good starting point is 200,000 cells.

In case ATAC-seq is not done, good starting point is 100,000 cells.

- 21 Centrifuge the nuclei for  00:03:00 at 600x g.

5m

- 22 Remove the supernatant.

Note

To prevent loss of nuclei, all centrifugations and incubations should be done in 0.5 ml micro-tubes. This makes it possible to see the nuclei better even for low input samples (e.g. 50,000 cells).

All centrifugation steps should be performed in swinging bucket rotor centrifuge with the appropriate adaptors.

When removing supernatant, it is ok to leave ~10ul of the supernatant in the tube, to prevent loss of nuclei.



Swinging bucket rotor centrifuge



Adaptor for 1.5 ml tubes



Adaptor for 0.5 ml tubes

Antibody binding

23 Prepare **antibody mix**

Starting concentrations (can be further optimised, depending on the antibody)

1:100 primary antibody

1:100 nano-Tn5

Final volume 100ul per sample.

24 Resuspend the nuclei pellet in the prepared **antibody mix** (1:100 primary antibody, 1:100 nano-Tn5) by pipetting up and down 5x.

25 Incubate Overnight on with rotation on a horizontal roller at 4 °C



Roller for overnight incubation

Note

Make sure the liquid does not reach the cap of the tube during the incubation, as nuclei can get stuck in the cap.

Washing and Tagmentation

1h 34m

26 The next day, centrifuge for 00:03:00 at 600xg.

10m

27 Remove the supernatant and resuspend in 200 ul of **Dig-300 wash buffer**.

10m

**Note**

Eject the buffer, with medium speed, so the pellet is dispersed, but do not pipette-mix (unless necessary, this might lead to loss of nuclei) or do not create excessive bubbles.

28 Repeat the steps 27-28 for total of 2 washes.

29 Resuspend the nuclei pellet in 200 ul of **Tagmentation buffer**. Pipette mix 5x to resuspend the pellet.

5m

30 Incubate for  01:00:00 at  37 °C

1h

Note

Pipette mix the nuclei ~30 minutes into the incubation to prevent sedimentation.

If there is no suitable adapter for 0.5 ml tubes in the thermomixer, use 1.5ml eppendorf tubes with ~800ul of water as adapters or use water bath.

31 During the incubation prepare tagmentation **STOP buffer**.

Prepare 2x diluted nuclei buffer (DNB) from 20x nuclei buffer (10x scATAC-seq, PN: 2000207). Store the aliquots of 2x DNB at  -20 °C

Stop buffer:

 100 µL 2x Diluted Nuclei buffer

 20 µL 20% BSA

 70 µL water

 10 µL 500 mM EDTA).

1x DNB + BSA buffer:

 500 μL

2x Diluted Nuclei buffer (10x scATAC-seq kit, dilute down from 20x (PN: 2000207))

 100 μL

20% BSA

 400 μL

water

32 Stop the tagmentation by removing from 37 thermoblock and adding 200 μL of **STOP buffer**. Mix well by pipetting up and down 3x.

33 Centrifuge for 00:03:00 at 300x g. Remove supernatant

3m

34 Resuspend the nuclei in 200 μL of 1x **DNB+BSA buffer**.

35 Centrifuge for 00:03:00 at 300x g. Remove supernatant

3m

36 Resuspend the nuclei in 200 μL of 1x **DNB+BSA buffer**.

37 Centrifuge for 00:03:00 at 300x g.

3m

38 Remove the most of the supernatant, leave the nuclei in cca 10 μL of remaining **1xDNB+BSA buffer**.

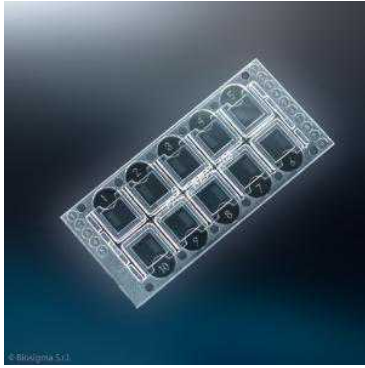
39 Add 10 μL of 1xDNB+BSA buffer, for final 20 μL of nuclei suspension. Measure the exact volume using P20 pipette.

Nuclei counting)

45m

40 Count the concentration of nuclei. Use 2 μL of nuclei suspension and mix with 8 μL of trypan blue. For counting use manual counting chambers (VWR, 630-1893). Do the counting in two replicates for more accuracy.

30m



- 41 To aim for cca 5,000 recovered nuclei, load cca 16,000 nuclei or determine ratio of loaded/recovered nuclei empirically.

Note

In our experience, the recovery of nuclei after 10x barcoding is lower, comparing to loading recommendations from 10x genomics for scATAC-seq.

The ratio of loaded/recovered nuclei can vary based on specific biological sample and nuclei preparation.

Chromium Next GEM barcoding

1h 40m

42

Note

The next part of the protocol follows closely the **Chromium Next GEM Single Cell ATAC Reagent Kits v1.1** Steps 2.0-2.5

Note

Make yourself familiar with the 10x Genomics scATAC-seq kit manual before proceeding.



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- 43 Mix the GEM generation and barcoding mix. Use the desired volume of nuclei and fill up to  15 μL with **1x DNB+BSA buffer**.

15m

Prepare **nuclei mix** and keep it on ice:

 7 μL	ATAC buffer B
 X μL	Nuclei (Use $\leq 8\mu\text{L}$ of nuclei)
up to  15 μL	1x DNB+BSA buffer

keep on ice

- 44 Prepare **barcoding mix** and :

 56.5 μL	Barcoding reagent B
 1.5 μL	Reducing agent B
 2 μL	Barcoding enzyme

keep it on ice

- 45 Assemble the Chromium Next GEM Chip H according to the manufacturer's instructions.

5m

Assemble Chromium Next GEM Chip H

After removing the chip from the sealed bag, use the chip in ≤ 24 h.



See Tips & Best Practices for chip handling instructions.

- Align notch on the chip (upper left corner) and the holder.
- Insert the left-hand side of the chip under the guide. Depress the right-hand side of the chip until the spring-loaded clip engages.
- Close the lid before dispensing reagents into the wells.
- The assembled chip is ready for loading the indicated reagents. Refer to step 2.2 for reagent volumes and loading order.



For GEM generation, load the indicated reagents only in the specified rows, starting from row labeled 1, followed by rows labeled 2 and 3. DO NOT load reagents in the bottom row labeled NO FILL. See step 2.2 for details.

Chromium Next GEM Chip H**Chromium Next GEM Secondary Holder****Assembled Chip**

46 Load the Chromium Next GEM Chip H according to the manufacturer's instructions. Run the droplet generation.

45m

Step 2

Chip Assembly & Loading

2.2

Load Chromium
Next GEM Chip H



! After removing the chip from the sealed bag, use in ≤ 24 h. For all chip loading steps, raising and depressing the pipette plunger should each take ~ 5 sec. When dispensing, raise the pipette tips at the same rate as the liquid is rising, keeping the tips slightly submerged.

- a. Dispense 50% Glycerol into Unused Chip Wells** (if < 8 samples per chip)
- 70 μ l to unused wells in row labeled 1.
 - 50 μ l to unused wells in row labeled 2.
 - 40 μ l to unused wells in row labeled 3.
- DO NOT add 50% glycerol solution to the bottom row of NO FILL wells. DO NOT use any substitute for 50% glycerol solution.

- b. Prepare Master Mix + Transposed Nuclei**
Add 60 μ l Master Mix to each tube containing Transposed Nuclei for a total of 75 μ l in each tube.

- c. Load Row Labeled 1**
Gently pipette mix the Master Mix + Transposed Nuclei 5x. Using the same pipette tip, dispense 70 μ l Master Mix + Transposed Nuclei into the bottom center of each well in row labeled 1 without introducing bubbles.



The illustrated chip is being loaded for 8 samples.

- d. Prepare Gel Beads**
Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex 30 sec. Centrifuge the Gel Bead strip for ~ 5 sec. Confirm there are no bubbles at the bottom of the tubes and the liquid levels are even. Place the Gel Bead strip back in the holder. Secure the holder lid.



- e. Load Row Labeled 2**
Puncture the foil seal of the Gel Bead tubes. Slowly aspirate 50 μ l Gel Beads. Dispense into the wells in row labeled 2 without introducing bubbles. Wait 30 sec.



- f. Load Row Labeled 3**
Dispense 40 μ l Partitioning Oil into the wells in row labeled 3 from a reagent reservoir. Failure to add Partitioning Oil to the top row labeled 3 will prevent GEM generation and can damage the Chromium Controller.



! Attach the gasket and run the chip in the Chromium Controller immediately after loading the Partitioning Oil.

- g. Attach 10x Gasket**
Align the notch with the top left-hand corner. Ensure the gasket holes are aligned with the wells. Avoid touching the smooth surface.



Keep horizontal to avoid wetting the gasket. DO NOT press down on the gasket.

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Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide • Rev F

27

47 Recover droplets from the Next GEM Chip H according to the manufacturer's instructions.

5m

Step 1

GEM Generation & Barcoding

2.4
Transfer GEMsNext
GEM

- a. Place a PCR 8-tube strip on ice.
- b. Press the eject button of the Controller to remove the chip.
- c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees.



- d. Check the volume in row labeled 1-2. Abnormally high volume in any well indicates a clog.

- e. Slowly aspirate **100 µl** GEMs from the lowest points of the recovery wells in the top row labeled 3 without creating a seal between the pipette tips and the wells.



- f. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels. Excess Partitioning Oil (clear) in the pipette tips indicates a potential clog.

- g. Over the course of **~20 sec**, dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the wells.

- h. If multiple chips are run back-to-back, cap/cover the GEM-containing tube strip or plate and place on ice for no more than 1 h.

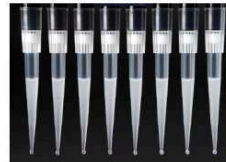
Expose Wells at 45 Degrees



Transfer GEMs



GEMs



48 Incubate in PCR cycler according to the manufacturer's instructions.

30m

The linear amplification (LA) and single-cell barcoding occurs at this step.

Use a thermal cycler that can accommodate at least 100 μ l volume. A volume of 125 μ l is the preferred setting on Bio-Rad C1000 Touch. In alternate thermal cyclers, use highest reaction volume setting.

a. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
105°C	100 μ l	30 min
Step	Temperature	Time
1	72°C	00:05:00
2	98°C	00:00:30
3	98°C	00:00:10
4	59°C	00:00:30
5	72°C	00:01:00 Go to step 3, repeat 11X (Total 12 cycles)
6	15°C	Hold



b. Store at 15°C for up to 18 h or at -20°C for up to 1 week, or proceed to the next step.

Barcoding and linear amplification temperature program.

Post-GEM incubation cleanup

1h

49 Perform post-GEM incubation cleanup according to manufacturer's instructions - Steps 3.1-3.2

45m

Step 3

Post GEM Incubation Cleanup

3.1 Post GEM Incubation Cleanup – Dynabeads

a. Add 125 μ l Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Gently invert tube 10x to mix. Centrifuge briefly.

The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).



A smaller aqueous phase volume indicates a clog during GEM generation.



b. Slowly remove and discard 125 μ l Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.

c. Prepare Dynabeads Cleanup Mix.

Biphasic Mixture



Remove Recovery Agent





Dynabeads Cleanup Mix <i>Add reagents in the order listed</i>	PN	1X (μl)	4X + 10% (μl)	8X + 10% (μl)
● Cleanup Buffer	2000088	182	800.8	1601.6
Dynabeads MyOne SILANE Vortex thoroughly (≥30 sec) immediately before adding to the mix.				
Aspirate the full liquid volume with a pipette tip to verify that the beads have not settled in the bottom of the tube. If clumps are present, pipette mix to resuspend completely. DO NOT centrifuge before use.	2000048	8	35.2	70.4
○ Reducing Agent B	2000087	5	22	44
Nuclease-free Water	-	5	22	44
Total	-	200	880	1760



- d.** Vortex and add 200 μl to each sample.
Pipette mix 5x (pipette set to 200 μl).
- e.** Incubate 10 min at room temperature.

Add Dynabeads Cleanup Mix





f. Prepare Elution Solution I. Vortex and centrifuge briefly.

Elution Solution I* <i>Add reagents in the order listed</i>	PN	1X (μl)	4X + 10% (μl)	8X + 10% (μl)
Buffer EB	-	98.0	431.2	862.4
10% Tween 20	-	1.0	4.4	8.8
☐ Reducing Agent B	200087	1.0	4.4	8.8
Total	-	100.0	440.0	880.0

*Elution Solution I will be used in steps 3.1o and 3.2j



- g. At the end of 10 min incubation, place on the 10x Magnetic Separator, high position (magnet•High) until the solution clears.
- h. Remove the supernatant.
- i. Add 300 μl freshly prepared 80% ethanol to the pellet while on the magnet•High. Wait 30 sec.
- j. Remove the ethanol.
- k. Add 200 μl 80% ethanol to pellet. Wait 30 sec.
- l. Remove the ethanol.
- m. Centrifuge briefly. Place on the magnet•Low.
- n. Remove remaining ethanol.
- o. Remove from the magnet. **Immediately** add 40.5 μl Elution Solution I to avoid clumping.
- p. Pipette mix (pipette set to 40 μl) without introducing bubbles.
- q. Incubate 1 min at room temperature.
- r. Centrifuge briefly. Place on the magnet•Low until the solution clears.
- s. Transfer 40 μl sample to a new tube strip.



Step 3

Post GEM Incubation Cleanup

3.2

Post GEM Incubation
Cleanup – SPRIselect

- a. Vortex the SPRIselect reagent until fully resuspended. Add **48 µl** SPRIselect reagent to each sample. Pipette mix thoroughly.
- b. Incubate **5 min** at **room temperature**.
- c. Centrifuge briefly. Place on the magnet•**High** until the solution clears.
- d. Remove the supernatant.
- e. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- f. Remove the ethanol.
- g. **Repeat** steps e and f for a total of 2 washes.
- h. Centrifuge briefly. Place on the magnet•**Low**.
- i. Remove any remaining ethanol.
- j. Remove the tube strip from the magnet. **Immediately** add **40.5 µl** Elution Solution I.
- k. Pipette mix (pipette set to 30 µl) without introducing bubbles.
- l. Incubate **2 min** at **room temperature**.
- m. Centrifuge briefly. Place on the magnet•**Low** until the solution clears.
- n. Transfer **40 µl** sample to a new tube strip.
- o. Store at **4°C** for up to **72 h** or at **-20°C** for up to **2 weeks**, or proceed to the next step.



50 Use 2ul of the purified **DNA** to measure the concentration (optional) using Qubit high sensitivity dsDNA kit.

15m

Note

We recommend measuring the DNA concentration at this stage, during optimisations experiments. The library is linearly amplified at this stage, so minor loss of sample is acceptable.

Optimal ratio of LA barcoding product and MeB Tn5 is **critical** to achieve maximum complexity of the library.

The DNA:Tn5 ratio can be optimised also in bulk experiments. Briefly:

1. perform this protocol until step 33.
2. Resuspend the nuclei in 100ul of Dig-300 buffer. A
3. Add 500ul of Zymo DNA clean and concentrator-5 binding buffer to the nuclei
4. Purify the gDNA using the zymo DNA clean and concentrator-5 kit
5. Elute DNA in 25 ul of elution buffer.
6. Use ~5ul of eluted DNA for linear amplification reaction (5ul DNA, 2ul 10x_LA primer_noBCD, 25ul 2x NEBnext mastermix, 18 ul water)
7. Run the program from 10x sATAC protocol - Step 2.5 GEM incubation (1. 72C 5min, 2. 98C 30s, 3. 98C 10s, 4. 59C 30s, 5. 72C 1min, 6. GOTO 3, 11x, 7. 15C hold infinite.)
8. Purify the product using 1.2x SPRI beads. Elute in 25 ul of EB buffer.
9. Measure concentration of DNA.
10. Mix 10ng of DNA with varying amounts of MeB-Tn5 (0.05ul-2ul) in 1x TD buffer final
11. incubate for 30 minutes at 37C
12. Purify using Zymo DNA clean and concentrator-5 kit. use 1:5 ratio of binding buffer to the sample. Elute the DNA in 25 ul
13. Use 10 ul of the eluted DNA for PCR (10 ul eluted DNA, 2ul PCR_FW primer 2ul Rev primer, 11ul water, 25ul 2x NEBnext MM).
14. Run PCR program as in step 53 PCR library amplification (1. 72C 5min, 2. 98C 45s, 3. 98C 20s, 4. 67C 30s, 5. 72C 20s, 6. GOTO 3, 7x, 7. 72C 1min, 8. 4C hold infinite.)
15. Run Bioanalyser/Tapestation to identify the optimal Tn5-MeB ratio to DNA

>10x_LA_primer_noBCD

AATGATACGGCGACCGAGATCTACACTCGTCGGCAGCGTC

>PCR_FW_primer

AATGATACGGCGACCGAGA,

>PCR_Rev_primer

CAAGCAGAAGACGGCATACGAGAT [8-bp sample index] GTCTCGTGGGCTCGG

**Note**

Optimal ratio of our home-made WT Tn5 is 0.7ul of MeB-Tn5 to 10ng of barcoded LA product. This ratio should be determined specifically for each batch of Tn5.

Typical yield of multimodal nanoCT barcoded LA product is ~5-10 ng in our hands.

Good starting point for optimisation of MeB Tn5 amount is  0.5 µL for our Tn5

Commercial Tn5 enzymes (e.g. EZ-Tn5) can be more active than a typical homemade Tn5 and therefore might require higher dilution.

MeB tagmentation and library preparation

1h 45m

51 Mix MeB tagmentation reaction:

45m

 38-40 µL

Barcoded LA product (from previous step, typically 5-10 ng)

 50 µL

2x TD buffer

 0.5 µL

MeB-loaded Tn5 (starting point, should be adjusted for specific batch of Tn5 and yield of DNA)

up to  100 µL

Water

Incubate in PCR cycler  00:30:00 at  37 °C →  4 °C holdHeated lid at  50 °C**52 Purify the DNA using Zymo DNA Clean and concentrator-5 kit according to manufacturer's instructions.**

15m

Transfer the sample into 1.5 ml eppendorf tube.

Add  500 µL of zymo binding buffer to your sample.

Wash 2x with  200 µL of zymo wash buffer.

Perform one more dry spin 1min at max speed to remove residual liquid.

Elute the DNA in  40 µL of DNA elution buffer (Zymo kit) . Incubate 2minutes on column, then centrifuge.



_D4003T_D4003_D4004_D4013_D...



53 Run PCR library amplification:

 40 μ L	Purified DNA from previous step (sample)
 7.5 μ L	SI-PCR primer B (10x ATAC-seq kit; PN: 2000128)
 2.5 μ L	Individual Single Index N Set A primer (product code: 1000212)
 50 μ L	AMP mix (10x ATAC-seq kit; PN: 2000047/ 2000103)

Incubate in PCR cycler with the following program:

Lid temperature  105 °C , volume  100 μ L .

1.  72 °C 5min
2.  98 °C 45sec
3.  98 °C 20sec |
4.  67 °C 30sec | Repeat 13x
5.  72 °C 20sec |
6.  72 °C 1min
7.  4 °C hold

Note

Typically we use 13 PCR cycles for library amplification, which is a good starting point, but the number of cycles should be adjusted according to typical yield for a specific combination of antibodies and input material.

We typically aim for 10nM library in the range of 300-700bp

54 Purify the final library using according to Step 4.2 in Chromium Next GEM Single Cell ATAC Reagent Kits v1.1.



4.2

Post Sample Index
Double Sided Size
Selection – SPRIselect



- a. Vortex to resuspend SPRIselect reagent. Add **40 µl** SPRIselect reagent to each sample. Pipette mix.
- b. Incubate **5 min** at **room temperature**.
- c. Place on the magnet•**High** until the solution clears.
- d. Transfer **130 µl** supernatant to a new strip tube. **DO NOT** discard the supernatant.
- e. Vortex to resuspend SPRIselect reagent. Add **74 µl** SPRIselect reagent to each sample. Pipette mix.
- f. Incubate **5 min** at **room temperature**.
- g. Place on the magnet•**High** until the solution clears.
- h. Remove the supernatant.
- i. Add **200 µl** 80% ethanol to the pellet. Wait **30 sec**.
- j. Remove the ethanol.
- k. **Repeat** steps i and j for a total of 2 washes.
- l. Centrifuge briefly. Place on the magnet•**Low**.
- m. Remove remaining ethanol.
- n. Remove from the magnet. **Immediately** add **20.5 µl** Buffer EB. Pipette mix.
- o. Incubate **2 min** at **room temperature**.
- p. Centrifuge briefly. Place on the magnet•**Low** until the solution clears.
- q. Transfer **20 µl** sample to a new tube strip.
- r. Store at **4°C** for up to **72 h** or at **-20°C** for **long-term** storage.



Sequencing preparation

- 55 Sequence on Illumina NovaSeq v1.5 platform with read setup : **36-8-48-36 (R1-I1-I2-R2)** using custom sequencing primers:

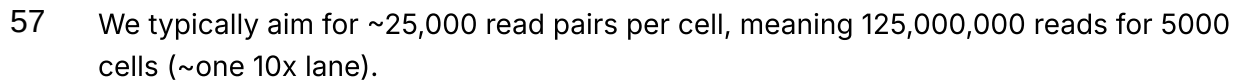
Custom_primer_R1: GCGATCGAGGACGGCAGATGTGTATAAGAGACAG

Custom_primer_I2: CTGTCTCTTATACACATCTGCCGTCTCGATCGC

R2 & I1 standard

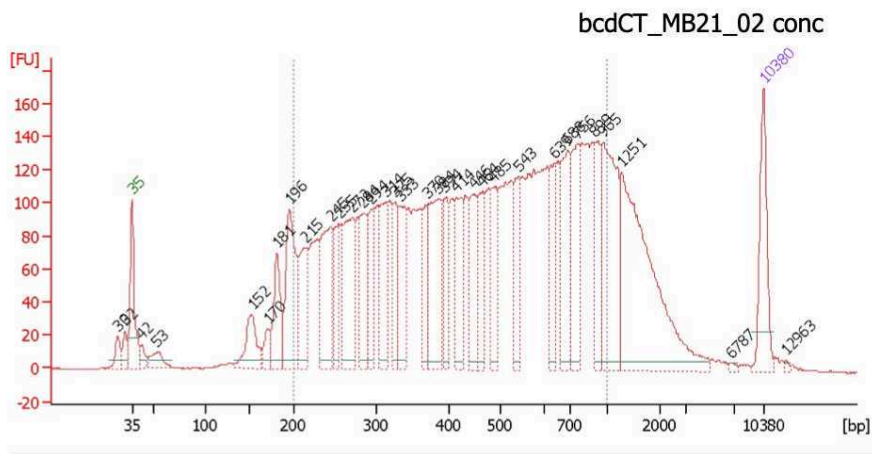
- 56 **Library structure:**

P5 side:

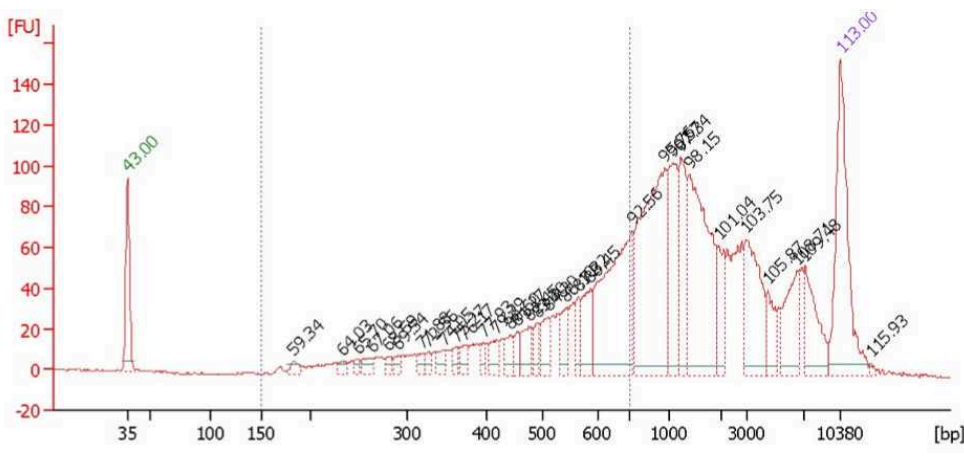


Desired outcome

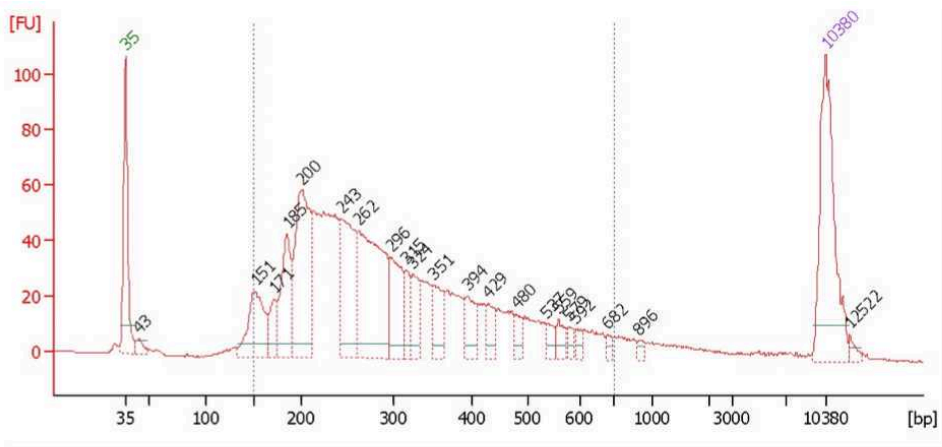
- 58 Typical bioanalyzer trace of a successful experiment shows good and even distribution of fragment sizes and is not overtagmented or undertagmented. Both over- and undertagmentation will lead to reduced complexity of the library.



Bioanalyzer trace showing a successful multimodal nano-CUT&Tag library. Majority of fragments are between 300-1000 bp



Bioanalyzer trace of under-tagmented nano-CUT&Tag library - increase the amount of Tn5-MeB in library prep



Bioanalyzer trace of over-tagmented nano-CUT&Tag library - decrease the amount of Tn5-MeB in library prep