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Protocol

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Nano-CUT&Tag for multimodal chromatin profiling at single-cell resolution

In the format provided by the authors and unedited

SUPPLEMENTARY METHODS

Determination of Tn5 (ME-B) activity by qPCR

Additional reagents:

- NEBNext High-Fidelity 2 x PCR Master Mix (NEB, cat. no. M0541S)
- pX458 or similar plasmid containing Cas9 insert (Addgene, cat. no. 48138)
- Unloaded Tn5 transposase protein, in house produced or commercial (e.g., Diagenode, cat. no. C01070010-10)
- 10X SYBR Green (SYBR™ Green I Nucleic Acid Gel Stain – 10,000X concentrate in DMSO, cat. no. S7563)
- LA and PCR primers (Integrated DNA Technologies; Supplementary Methods B, unimodal bulk nano-CT, Steps 5 and 12 respectively). Sequences are listed in Supplementary Table 1.

Additional General Equipment:

- Microamp Optical 384 well Reaction plate with Barcode (qPCR) (Applied Biosystems (FisherScientific, cat. no. 4309849) or alternative qPCR plate
- Microamp Optical 384 Adhesive Film (qPCR) (FisherScientific, cat. no. 4311971)
- qPCR cycler (Thermo Fisher Scientific, model no. Quantstudio 5) (optional)
- Plate centrifuge **(**Axygen Plate Spinner-230EU) (optional).

A. **Determination of Tn5 (ME-B) activity by qPCR ● Timing 4h**

Part 1| Template generation ● Timing 1 h 30 min

1. Prepare a PCR reaction, mixing the components in table below. For each PCR reaction, prepare 1-4 technical replicates to be run in parallel to obtain more product. The amplified reference material will be used as tagmentation template in later steps.

Reagent	Volume
2x NEBNext Mastermix	25 µL

Nuclease-free water	19.8 µL
qPCR_Tn5_forward (10 µM)	2.5 µL
qPCR_Tn5_reverse (10 µM)	2.5 µL
pX458 plasmid (concentration cca 50 ng/µL)	0.2 µL
Total volume	50 µL

2. Place the samples in a PCR cycler. Set the heated lid at 105 °C
3. Run the following PCR program

Step	No. of cycles	Temperature	Time
1	1	98°C	1 min
2	30	98°C	15s
		57°C	15s
		72°C	2 min
3	1	72°C	5 min
4	1	4°C	Hold

4. Perform PCR clean up, using a 0.9x ratio of SPRIselect reagent (40 µL), following manufacturers' instructions. Elute in 20 µL of EB Buffer.
5. In a new PCR tube, combine the 4 eluted products.
6. Measure DNA concentration with Qubit High Sensitivity dsDNA kit, using 1-2 µL of the purified sample following the manufacturer's instructions.
7. Dilute an aliquot of the product to 10 ng/µL (e.g., prepare 100-200 µL of 10 ng/µL). PAUSE POINT Store the undiluted PCR product at -20°C for future use for maximum 3 months.

Part 2| qPCR for Tn5 tagmentation efficiency test and calibration curve ● Timing 2 h 30 min

8. Prepare minimum of two PCR reactions replicates for different volume titration of in-house produced Tn5 (ME-B) transposome (e.g., 0.5, 1.0, 1.5, 2.0 µL) as detailed in the table below. If including titration volumes of Tn5 below 1 µL, dilute the Tn5 10x in

nuclease-free water and use 10x more of the volume. If working with commercial alternative of Tn5, we suggest using their recommended volumes as a starting point and test additional ratios.

Reagent	**Titration (1X)**	**Negative control (1X)**
2Kb diluted PCR product (10 ng/µL) **(Step 7)**	1 µL	1 µL
Nuclease-free water	Up to 50 µL	24 µL
2x TD Buffer	25 µL	25 µL
In-house or commercial Tn5 (loaded only by ME-B)	Volume varies 0.1-4 µL	0 µL
Total volume	50 µL	50 µL

9. Mix the reaction well, by gently pipetting up and down five times.
10. Incubate the tubes in a PCR cycler and run the program indicated in the table below. Set the heated lid at 105°C.

Step	No. of cycles	Temperature	Time
1	1	37°C	30 min
2	1	98°C	10 min
3	1	4°C	Hold

11. Dilute the reaction 1:100, mixing a 2 µL aliquot with 198 µL of nuclease-free water.
12. Set up a qPCR reaction, mixing the components indicated in the table below. Prepare 10x SYBR-green by dilution from 10,000 concentrated SYBR. Prepare 3 technical replicates for each template.

Reagent	Volume
2x NEBNext Mastermix	5 µL
Nuclease-free water	1.5 µL
qPCR_Tn5_forward (10 µM)	0.25 µL
qPCR_Tn5_reverse (10 µM)	0.25 µL
Diluted tagmented template (From Step xi)	2 µL
10X SYBR Green	1 µL
Total volume	10 µL

13. Run the following program in a qPCR cycler:

Step	No. of cycles	Temperature	Time
1	1	95°C	2 min
2	40	95°C	15s
		57°C	15s
		72°C	2 min

14. Calculate the deltaCT values (average CT values from Titration− average CT values from "Negative control"). Plot deltaCT values against the volume of Tn5 used in each reaction. The expected linear curves will have various slopes for every Tn5 batch/commercial alternative (Extended Data Figure 1b).
15. Calculate the slope of the curves obtained in previous step, for example with "SLOPE" calculation function included in Microsoft Office Excel, based on the different Ct values obtained from the same batch of Tn5 transposome.
16. Calculate the volume of Tn5 required to obtain a 99% tagmentation fraction, using the following formula: Tn5 volume = -log2(1 – 0.99)/slope. For example, if the slope for the Tn5 batch is 3.66, the formula will look like:

Tn5 volume (batch A) = -log2(1-0,99)/3,66 = -log2(0.01)/3.66 = 6.64/3,66 ~= 1.8 µL .

Tn5 volume (batch B) = -log2(1-0,99)/6,68 = -log2(0.01)/6,68 = 6.64/6,68 ~= 0.99 µL .

Tn5 volume (batch C) = -log2(1-0,99)/8,10 = -log2(0.01)/8,10 = 6.64/8,10 ~= 0.82 µL .

CRITICAL STEP: The formula provides the amount of Tn5 required per 10 ng of template.

CRITICAL Using a 99% tagmentation efficiency and the recommended volume does not provide information about library size distributions of nano-CT libraries. This will need to be tested empirically, for sample type and chromatin marks. To verify the volume required to obtain a nano-CT library with proper size distribution, we also provide a protocol for unimodal nano-CT in bulk below.

B. Unimodal nano-CT in bulk, for assessing batch variation of Tn5 activity ● Timing 2 d

CRITICAL: The DNA:Tn5 ratio can be optimized in experiments performing nano-CT in bulk and before performing single cell protocol. Alternatively, this protocol can be used to perform multimodal bulk nano-CT.

1. Perform Steps 34-64 (Main procedure nano-CT until Nuclei washing and nanobody-Tn5 (ME-A only) tagmentation) using 200,000 cells as input material, profiling one or two modalities and following in option B in Step 47.
2. Add enough volume of Dig-300 buffer to complete total volume of 100 µL and resuspend gently by pipetting three times until fully resuspended.
3. Add 500 µL of DNA binding buffer (Zymo DNA Clean & Concentrator-5), according to the manufacturer's instructions. Perform the elution step in 25 µL of EB buffer.
4. Measure the concentration of the DNA sample with Qubit High Sensitivity using 1 µL of the purified DNA.
   ? TROUBLESHOOTING
5. Prepare the reaction for linear amplification, as detailed in the table below:

Reagent	Volume (for one reaction)
2x NEBNext Mastermix	25 µL
Nuclease free Water	13 µL
LA_noBCD primer (10µM)	2 µL

Zymo DNA elution (step iii)	10 µL
Total volume	50 µL

6. Run the following program on a PCR cycler:

Step	No. of cycles	Temperature	Time
1	1	72°C	5 min
2	1	98°C	30s
3	12	98°C	10s
		59°C	30s
		72°C	1 min
4	1	4°C	Hold

7. Perform PCR clean up with 1.2x ratio of SPRIselect reagent (60 µL) . Elute in 25 µL of EB Buffer. This elution (Linear Amplification product) will be used later for assessing Tn5 activity.
8. Measure the Linear amplification yield with a dsDNA-specific assay, such as Qubit dsDNA HS following the manufacturer´s instructions. Use 1 µL of sample for the measurement. Keep a small aliquot (1-2 µL) of LA product to load in later steps in Bioanalyzer High Sens if needed.
9. Mix 20 ng of dsDNA with varying amounts of transposome complex Tn5 (ME-B)(e.g., 0.1 µL- 4 µL) in 1X TD buffer following the table below:

Reagent	Volume
Linear Amplification product	X µL (calculate the volume required to load 20 ng)
In-house or commercial Tn5 loaded with ME-B/ME-REV (Step 32 of main procedure)	X µL
2X TD buffer	25 µL

Nuclease-free Water	Up to 50 µL
Total volume	50 µL

CRITICAL STEP: If the library generated with this protocol will be sequenced, use up to 50 ng of the LA product for ME-B tagmentation and library preparation for increased library complexity.

10. Incubate the reaction in a PCR cycler for 30 min at 37°C and hold at 4°C. Set the heated lid at 50°C.
11. Add 250 µL DNA binding buffer (Zymo DNA Clean & Concentrator-5) and follow the purification as outlined in the manufacturer's instructions. Elute in 25 µL of EB buffer. This elution (Second round Tagmented product) will be used for library preparation.
12. Prepare the following master mix for library amplification

Reagent	Volume
2x NEBNext Mastermix	25 µL
PCR_Rev_primer (10 µM)	2 µL
LA_noBCD (10 µM)	2 µL
Second round Tagmented product	10 µL
Nuclease-free Water	11 µL
Total	50 µL

13. Run the following program in a PCR cycler:

Step	No. of cycles	Temperature	Time
1	1	72°C	5 min
2	1	98°C	45s
3	6-10	98°C	20s
		67°C	30s

		72°C	20s
4	1	72°C	1 min
5	1	4°C	Hold

14. Perform PCR clean up with 1.2x ratio of SPRIselect reagent (60 µL). Elute in 20-25 µL of EB Buffer.
15. Measure the library yield with a dsDNA-specific assay, such as Qubit DNA HS using 1-2 µL from previous step following the manufacturer's instructions.
16. Verify the size distribution of the library by capillary electrophoresis (e.g., Agilent Bioanalyzer HighSens), following manufacturer's instructions. Identify the optimal Tn5-(ME-B) ratio to DNA that gives the best library distribution (with a range of 300-700 bp). Refer to Figure 4a and compare to Figure 4b-c.

C. Step-by-step execution of Nanoscope pipeline ● Timing 2 h 30 min

1. Demultiplex the sequencing reads into separate fastq files for individual modalities using `workflow/scripts/debarcode.py` script. For example, to extract three most abundant barcodes use the following command:

```
$ python3 workflow/scripts/debarcode.py -i
Fastq_file_R1.fastq.gz Fastq_file_R2.fastq.gz
Fastq_file_R3.fastq.gz -o $OUT_DIR --single-cell --
Nbarcodes 3
```

Alternatively, to extract reads with a specific barcode ATAGAGGC use the following command:

```
$ python3 debarcode.py -i Fastq_file_R1.fastq.gz
Fastq_file_R2.fastq.gz Fastq_file_R3.fastq.gz -o $OUT_DIR
--single-cell --barcode ATAGAGGC
```

2. For each demultiplexed modality, run `cellranger-atac count` pipeline (10x Genomics) to map the reads with standard parameters. For more details on cellranger-atac refer to https://github.com/10XGenomics/cellranger-atac

```
$ cellranger-atac count --id YOURID --reference
PATH_TO_REFERENCE --fastqs PATH_TO_FASTQ
```

CRITICAL STEP Cellranger typically underestimates the number of identified high-quality cells in nano-CT experiment and therefore custom cell calling based on number of reads per cell before removal of PCR duplicates and fraction of reads in peak regions is recommended.

3. Summarize number of reads per cell (with PCR duplicates) using the following bash script.

```
$ samtools view -f2 possorted_bam.bam| awk 'BEGIN
{FS=OFS="\t"} {for(i=12;i<NF;i++){if($i ~
"CB:Z:"){print $i}}}' | sed "s/CB:Z://g" | sort -T
$TMPDIR | uniq -c > all_barcodes.txt
```

4. Perform peak calling using MACS2[51] (or alternative, e.g. SEACR[52]) for each modality. The default cellranger peak caller is not well suited for broad peaks such as histone modifications.

```
$ macs2 callpeak -t possorted_bam.bam -g mm -f BAMPE -n
$MODALITY --outdir $OUTPUT_DIR --llocal 100000 --keep-dup
1 --broad-cutoff 0.1 --broad --max-gap 1000
```

5. Summarize the number of reads per cell falling into peak regions using bash script.

```
$ bedtools intersect -abam possorted_bam.bam -b
peaks.broadPeak -u | samtools view -f2 | awk 'BEGIN
{FS=OFS="\t"} {for(i=12;i<NF;i++){if($i ~ "CB:Z:"){print
$i}}}' | sed "s/CB:Z://g" | sort -T $TMPDIR | uniq -c >
peak_barcodes.txt
```

? TROUBLESHOOTING

6. Select the cells by using a provided R script `workflow/scripts/pick_cells.R` which uses gaussian mixture model-based clustering (mclust) to cluster and classify cells and empty droplets.

```
$ Rscript pick_cells.R --metadata cellranger/outs/metadata.csv
--fragments cellranger/outs/fragments.tsv.gz --bcd_all
all_barcodes.txt --bcd_peak peak_barcodes.txt --modality
$MODALITY --out_prefix $OUTDIR
```

?TROUBLESHOOTING

7. Remove linear amplification duplicates following the instructions below:
8. sort the possorted_bam.bam output from cellranger by read name, using the samtoools sort tool:

```
$ samtools sort -T $TMP -@ 16 -n -o $BAM_NAMESORTED
possorted_bam.bam;
```

9. Run script `workflow/scripts/find_LA_duplicates`.py

```
$ python3 workflow/scripts/remove_LA_duplicates.py
$BAM_NAMESORTED $BAM_NOLA_NAMESORTED
```

10. Sort the bam output back by read position and index the bam file.

```
$ samtools sort -T $TMP -@ 16 -o $BAM_NOLA_POSSORTED
$BAM_NOLA_NAMESORTED
```

11. Convert the bam file with removed LA duplicates to fragments file without LA duplicates using sinto fragments tool and sort and index the new fragments file.

```
$ sinto fragments -b $BAM_NOLA_POSSORTED -f
$FRAGMENTS_NOLA -p 16

$ sort -k1,1 -k2,2n $FRAGMENTS_NOLA | bgzip >
$FRAGMENTS_NOLA_SORTED;
```

```
$ tabix -p bed $FRAGMENTS_NOLA_SORTED;
```