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Direct nuclear tagmentation and RNA-sequencing (DNTR-seq) V.1

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ABSTRACT

Understanding how genetic variation alters gene expression - how genotype affects phenotype - is a central challenge in biology. To address this question in complex cell mixtures, we developed Direct Nuclear Tagmentation and RNA-sequencing (DNTR-seq), which enables whole genome and mRNA sequencing jointly in single cells.

GUIDELINES

Oligonucleotides (all ordered from IDT using Standard desalting, except barcodes ordered in solution/plates)

Oligo-dT:

AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT(N1:34333300)(N2:25252525)

IS_PCR: 5'-AAGCAGTGGTATCAACGCAGAGT-3'

TSO: 5'-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'

ME-A: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3'

ME-B: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'

ME-Rev: 5'-5Phos/CTGTCTCTTATACACATCT-3'

Illumina-compatible barcodes used (Sxxx/Nxxx series, n=784) are available as a supplementary table in the manuscript.

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MATERIALS

MATERIALS

- ☒ HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**
- ☒ Proteinase K **Thermo Fisher Scientific Catalog #E00491**
- ☒ Tween-20 **Sigma-aldrich Catalog #P-7949**
- ☒ psfTn5 **addgene Catalog #79107**
- ☒ 10% SDS solution **Teknova Catalog #S0287**
- ☒ SMARTScribe Reverse Transcriptase **Takarabio Catalog #634888**
- ☒ Magnesium chloride solution for molecular biology (1.00 M) **Sigma – Aldrich Catalog #M1028**
- ☒ Ice **Contributed by users**
- ☒ Triton X-100 **Sigma Catalog #93426**
- ☒ Microseal® 'F' Foil **BioRad Sciences Catalog #MSF-1001**
- ☒ dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) **Thermo Fisher Scientific Catalog #R0192**
- ☒ KAPA HiFi PCR kit with dNTPs **Fisher Scientific Catalog #NC0142652**
- ☒ Betaine 5M **Sigma Aldrich Catalog #B0300**
- ☒ Dry ice **Contributed by users**
- ☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977035**
- ☒ ERCC RNA Spike-In Mix **Thermo Fisher Catalog #4456740**
- ☒ USB Dithiothreitol (DTT), 0.1M Solution **Thermo Fisher Catalog #707265ML**
- ☒ Sera-Mag Speed Beads **Ge Healthcare Catalog #65152105050250**
- ☒ RNase Inhibitor **Takara Catalog #2313A**
- ☒ Hard-Shell® 384-Well PCR Plates thin wall skirted **BioRad Sciences Catalog #HSP3801**

BEFORE START INSTRUCTIONS

Bleach clean environment - to avoid DNA contamination. And RNase away or similar to avoid degraded RNAs. Prepare solutions in a strictly pre-PCR environment. Keep plates and reagents on ice unless otherwise noted.

Prepare lysis buffer plates for cell sorting

1 Prepare lysis buffer mix

NOTE: Reagents are prepared on ice, working quickly. ERCC is stored in single-use aliquots at -80 °C, thawed on ice and added last.

Reagent	Reaction conc.	μL per reaction	384w plate
Nuclease free H ₂ O	-	1.965	786
RNase Inhibitor (40u/μL)	1 unit/μL	0.075	30
ERCC (1:1 200 000)	-	0.075	30
Triton-X100 (10% solution)	0.2%	0.06	24
dNTP (10mM each)	2.5mM/each	0.75	300
Oligo-dT (100μM)	2.5μM	0.075	30
To dispense		3	1200

Add 3 μL lysis buffer mix to each well. Cover with appropriate lids. Spin down.
Snap freeze on **dry ice**. Store until use at -80 °C

Sort single-cells

- Sort single cells into 3 μL lysis buffer mix
Immediately seal with appropriate seals (approved for -80C > 100C) and centrifuge at 2000 x g, 4°C, 00:05:00
Snap freeze on **dry ice**. Store until use at -80 °C

Separation of nuclear and cytosolic fractions

- Thaw plate on ice.
Centrifuge at 500 x g, 4°C, 00:05:00 .
Keep on ice.
- Transfer 2 μL from each well of the sorted plate into an empty 384-well plate. Use a low flow rate (**2mm/s**) and an aspiration height of **0.9mm** above the bottom.

Note

NOTE: We use the Eppendorf EpMotion 5073m benchtop liquid handler. We have successfully used other solutions, including the Hamilton STARlet, a semi-manual Gilson Platemaster 96-well pipette, and even manual 8-channel pipettes.

- 5 Spin down and freeze nuclear fraction at -20 °C to aid complete lysis.

If proceeding with **cDNA protocol** --> step 12.

If proceeding with **DNA protocol** (step 6): spin down and snap freeze cytosolic fraction on **dry ice** and store at -80 °C

Note

NOTE: We will typically proceed with cDNA synthesis, unless experimental design dictates otherwise, to avoid an additional freeze-thaw cycle for mRNAs in the cytosolic fraction.

Single-cell genomic libraries

- 6 Using plate with nuclear fraction, with remaining volume 1µl/well.

Proteinase K treatment

1. Dilute Proteinase K (stock 20mg/ml) to 0.2mg/ml by 30mM Tris-HCl pH8.0
2. Add 2 µL diluted Proteinase K (0.2mg/ml) to each well. Makes 0.13mg/ml reaction concentration.
3. Incubate in thermocycler at:

50 °C	01:00:00
80 °C	00:30:00
4 °C hold	

- 7 **Tn5 digestion**

Tn5 is produced from psfTn5 (Addgene #79107), purified to ~3mg/ml and assembled with Illumina Tn5 adapters (see **oligos**) as in *Picelli et al, 2014*.

CITATION

Picelli S, Björklund AK, Reinius B, Sagasser S, Winberg G, Sandberg R (2014). Tn5 transposase and tagmentation procedures for massively scaled sequencing projects.. Genome research.

LINK

<https://doi.org/10.1101/gr.177881.114>

- 7.1** Prepare 2X Tn5 Buffer. Keep assembled Tn5 enzyme (Picelli *et al*, 2014) on ice block and add last.

Reagent	Reaction conc	μL per reaction	384w plate
5X TAPS-PEG (50mM TAPS, 25mM MgCl ₂ , 40% PEG-8000)	10mM TAPS 5mM MgCl ₂ 8% PEG-8000	1.6	672
psfTn5, loaded with 50μM MEDS-A/B		0.1	42
Nuclease free H ₂ O		3.3	1386
To dispense		5	2100

Add 5 μL per well. Vortex and spin down plate.

Note

NOTE: Buffer contains PEG, which is viscous. 5X TAPS-PEG buffer should be allowed to assume room temperature before dispensing to allow proper mixing.

- 7.2** Incubate in thermocycler: 55 °C 00:10:00
- Remove immediately and stop reaction by adding 2 μL per well of 0.2% SDS.
- Vortex, spin down and incubate 00:10:00 at 55 °C

8 PCR amplification and barcoding

1. Prepare PCR master-mix

Reagent	Reaction conc.	μl per reaction	384w plate
Nuclease free H ₂ O	-	3.2	1280
KAPA HiFi Buffer (5X)	1X	4	1600
dNTP (10mM/each)	0.3mM/each	0.6	240
KAPA enzyme (1u/μl)	0.02u/μl	0.4	160
Tween-20 (10%)	0.1%	0.2	80
To dispense		8.4	3360

2. Dispense 8.4 μL per well

3. Add primers/barcodes 1.6 μL per well (from 384-well index plates, with 3.75μM/each forward/reverse primers; see **oligos**). Total reaction volume is now 20μl (10μl sample + 10μl PCR mix and primers).

4. Vortex plate, spin down and incubate in thermocycler with the following program:

Step	Temperature	Time	Cycle s
Gap fill	72°C	3 min	1x
First denature	95°C	30 sec	1x
Denature	95°C	15 sec	18x
Anneal	67°C	30 sec	
Extend	72°C	45 sec	
Final extension	72°C	4 min	1x
	4-10°C	hold	

9 Pool 1.5 μL from each well into a 1.5mL Eppendorf tube.

10 Library cleanup

We prepare SPRI-beads in 20% PEG-8000 solution as in:

https://openwetware.org/wiki/SPRI_bead_mix#Ingredients_for_50_mL_2

1. Add 0.9X SPRI-beads in 20% PEG solution. Incubate for 00:05:00 | Room temperature
2. Place on magnetic rack 00:03:00
3. Remove supernatant
4. Add 1 volume 80% EtOH (fresh). Incubate for 00:00:30
5. Remove supernatant
6. Repeat EtOH wash
7. Air dry for 00:10:00 - 00:15:00
8. Re-suspend beads thoroughly in 100 μ L EB or TE buffer
9. Repeat cleanup (from step 1-7) and elute in 30 μ L EB or TE buffer

11 (optional) Quality control of DNA libraries

Using Agilent HS 5000 DNA chips (or equivalent)

Pooled (and diluted) DNA-library from 384-well plate.

This library was sequenced on a NextSeq loading 2.5pM based on a peak of 334bp. Sequencing was paired-end 37bp, 8bp dual index.

Reverse transcription and cDNA amplification

- 12 Following step 4, cytosolic/RNA fraction plate contains 2 μ L solution per well.

Primer annealing

Thaw plate. Spin down. Incubate in thermocycler at 72 °C for 00:03:00 . Remove to ice immediately.

13 Prepare RT master-mix

Reagent	Reaction conc.	µl per reaction	384w plate
SMARTScribe RT (100u/µl)	15u/µl	0.475	199.5
RNase Inhibitor (40u/µl)	1.66u/µl	0.125	52.5
5X First Strand buffer	1X	1	420
DTT (100mM)	8.33 mM	0.25	105
Betaine (5M)	1.66 M	1	420
MgCl ₂ (1M)	10m M	0.03	12.6
TSO (100uM)	1.66µ M	0.05	21
Nuclease free H ₂ O	-	0.07	29.4
Total		3	1260

Dispense 2 µL per well

Cover plate with new film and spin down.

14 Incubate in thermocycler

42 °C 01:30:00
70 °C 00:05:00
4 °C hold

15 cDNA preamplification

	Reaction conc.	µl per reaction	384w plate
Nuclease free H ₂ O	-	1.0688	470.25
Kapa HiFi HotStart ReadyMix (2X)	1X	6.25	2750
IS_PCR primer (10µM)	0.1µM	0.125	55

Lambda Exonuclease (10u/μl)	0,045u/μl	0.056 25	24.75
Total		7.500 0	3300

Dispense 7.5 μL per well . Total reaction volume will be 12.5μl.

16 Spin down. Cover with new lid. Incubate in thermocycler with the following program:

Step	Temperature	Time	Cycles
Lambda exonuclease	37°C	30 min	1x
Initial denaturation	95°C	3 min	1x
Denaturation	98°C	20 sec	18-24x
Annealing	67°C	15 sec	
Elongation	72°C	4 min	
Final elongation	72°C	5 min	
	4C	Hold	

Note

NOTE: The number of cycles of pre-amplification will be different for different cell types. We suggest running a pilot (ideally qPCR-monitored to determine inflection point, for example by using 1X dsGreen to the reaction above)

17 cDNA cleanup

Using 20% SPRI-bead solution (as in step10 for DNA library cleanup).

1. Add 0.7X volume of SPRI beads per well. Mix well by pipetting

2. Incubate 00:05:00 Room temperature
3. Place on magnetic stand for 00:03:00
4. Carefully remove supernatant
5. Add 40 μL 80% EtOH and incubate 00:00:30
6. Remove EtOH (without disturbing the beads)
7. Wash again with EtOH. Make sure to remove well.
8. Allow beads to air-dry for 00:10:00 - 00:15:00
9. Remove plate from magnetic stand
10. Elute beads in 15 μL EB or TE buffer Mix well by pipetting
11. Incubate 00:05:00 Room temperature
12. Place on magnetic plate for 00:03:00
13. *Optional: Carefully remove supernatant to the elution plate*

18 cDNA quantification

We measure concentration of random wells using Qubit HS dsDNA, adapted to a 96-well plate reader.

1. Add 98.5 μL of 1X Qubit HS dsDNA solution (or mix dye and buffer separately) to a flat-bottom, black plate
2. Add 1.5 μL of cDNA sample
3. Add Standards (NOTE: We make a 8-step ladder from 0ng/ μL --> 10ng/ μL Qubit Standard DNA in TE buffer)
3. Read in plate reader using 485nm excitation/528nm emission
4. Calculate cDNA concentration

19 (optional) cDNA quality control

Using Agilent HS 5000 DNA chips (or equivalent)

Example of a single immune (=small) cell cDNA profile (cytosolic fraction from DNTR protocol)

20 Make cDNA dilution plate

Dilute cDNA based on average concentration from Qubit measurements.

Target concentration 150 pg per μL in 15 μL (optionally in same plate)

cDNA tagmentation

21 Prepare Tn5 master mix

Let TAPS-PEG equilibrate at 37°C and mix well before use.

Reagent	Reaction conc.	μL per reaction	384w plate
Nuclease free H ₂ O	-	1.050	525
TAPS-PEG (50mM TAPS, 25mM MgCl ₂ , 40% PEG-8000)	10mM TAPS 5mM MgCl ₂ 8% PEG-8000	0.500	250

psfTn 5, loade d with 50µM MEDS -A/B		0.250	125
Total		1.800	900

Dispense 1.8 µL per well in a new plate (**tagmentation plate**)

- 22** Add 0.7 µL cDNA (normalized to **150pg/µl**)
Mix well by vortexing plate. Cover with new lid and spin down.

- 23** Incubate in thermocycler at 55 °C 00:10:00
Remove immediately and stop reaction by adding 1 µL per well of 0.2% SDS.
Vortex, spin down and incubate 00:07:00 at 55 °C

cDNA library PCR and barcoding

24 Make PCR master-mix

Reag ent	Reacti on conc.	µl per reacti on	384w plate (420x)
H2O	-	13.25	5565
KAPA HiFi Buffer (5X)	1X	5	2100
dNTP (10m M/ea ch)	0.3m M/ea ch	0.75	315
KAPA enzy me (1u/µl)	0.02u /µl	0.5	210
Total		19.5	

Dispense 19.5 µL per well to **tagmentation plate** (containing 3.5µl sample after step 23)

- 25** Add primers/barcodes 2 µL per well (from 384-well index plates, with 3.75µM/each forward/reverse primers; see **oligos**).

Total reaction volume is 25µl (3.5µl sample + 21.5µl PCR mix and primers).

- 26** Vortex. Spin down and cover. Incubate in thermocycler as below:

Step	Temperature	Time	Cycle s
Gap fill	72°C	3 min	1x
First denature	95°C	30 sec	1x
Denature	95°C	15 sec	12x
Anneal	67°C	30 sec	
Extend	72°C	45 sec	
Final extension	72°C	4 min	1x
	4-10°C	hold	

cDNA library pooling and clean-up

- 27** Pool 2.5 µL from each well to an 1.5ml Eppendorf tube

28 Library cleanup (as for DNA libraries)

We prepare SPRI-beads in 20% PEG-8000 solution as in:

https://openwetware.org/wiki/SPRI_bead_mix#Ingredients_for_50_mL_2

1. Add 0.9X SPRI-beads in 20% PEG solution. Incubate for 00:05:00 Room temperature
2. Place on magnetic rack 00:03:00
3. Remove supernatant
4. Add 1 volume 80% EtOH (fresh). Incubate for 00:00:30
5. Remove supernatant

6. Repeat EtOH wash

7. Air dry for 00:10:00 - 00:15:00

8. Re-suspend beads thoroughly in 100 μ L EB or TE buffer

9. Repeat cleanup (from step 1-7) and elute in 30 μ L EB or TE buffer

29 Pooled library QC

Pooled cDNA library of 784 cells on HS D5000 Agilent tapestation