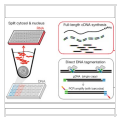

Mar 20, 2020

Direct nuclear tagmentation and RNA-sequencing (DNTR-seq)

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1 Works for me dx.doi.org/10.17504/protocols.io.bdthi6j6

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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.

EXTERNAL LINK

https://www.biorxiv.org/content/10.1101/2020.03.04.976530v1.full

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

A highly scalable method for joint whole genome sequencing and gene expression profiling of single cells bioRxiv 2020.03.04.976530; doi: https://doi.org/10.1101/2020.03.04.976530

GUIDELINES

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

Oligo-dT: AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTT(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.

MATERIALS

NAME	CATALOG #	VENDOR
HotStart ReadyMix (KAPA HiFi PCR kit)	KK2601	Kapa Biosystems
Proteinase K	EO0491	Thermo Fisher Scientific
Tween-20	P-7949	Sigma-aldrich
psfTn5	79107	addgene
10% SDS solution	S0287	Teknova
SMARTScribe Reverse Transcriptase	634888	Takarabio
Magnesium chloride solution for molecular biology (1.00 M)	M1028	Sigma – Aldrich

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NAME	CATALOG #	VENDOR
Ice		
Triton X-100	93426	Sigma
Microseal® 'F' Foil	MSF-1001	BioRad Sciences
dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM)	R0192	Thermo Fisher Scientific
KAPA HiFi PCR kit with dNTPs	NC0142652	Fisher Scientific
Betaine 5M	B0300	Sigma Aldrich
Dry ice	View	
UltraPure™ DNase/RNase-Free Distilled Water	10977035	Thermo Fisher
ERCC RNA Spike-In Mix	4456740	Thermo Fisher
USB Dithiothreitol (DTT), 0.1M Solution	707265ML	Thermo Fisher
Sera-Mag Speed Beads	65152105050250	Ge Healthcare
RNase Inhibitor	2313A	Takara
Hard-Shell® 384-Well PCR Plates thin wall skirted	HSP3801	BioRad Sciences

BEFORE STARTING

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 H2O	-	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**

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Sort single-cells

2 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

3 Thaw plate on ice.

Centrifuge at **500 x g, 4°C 00:05:00** .

Keep on ice.

4 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: We use the Eppendorf EpMotion 5073m benchtop liquid handler. We have succesfully 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: 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**

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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.*

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. 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 MgCl2, 40% PEG-8000)	10mM TAPS 5mM MgCl2 8% PEG-8000	1.6	672
psfTn5, loaded with 50µM MEDS-A/B		0.1	42
Nuclease free H2O		3.3	1386
To dispense		*5*	2100

Add **5 µl** per well. Vortex and spin down plate.

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**

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8 **PCR amplification and barcoding**

1. Prepare PCR master-mix

Reagent	Reaction conc.	µl per reaction	384w plate
Nuclease free H2O	-	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	Cycles
Gap fill	72ºC	3 min	1x
First denature	95ºC	30 sec	1x
Denature	95ºC	15 sec	18x
Anneal	67ºC	30 sec	
Extend	72ºC	45 sec	
Final extension	72ºC	4 min	1x
	4-10ºC	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 µl EB or TE buffer**
9. Repeat cleanup (from step 1-7) and elute in **30 µl EB or TE buffer**

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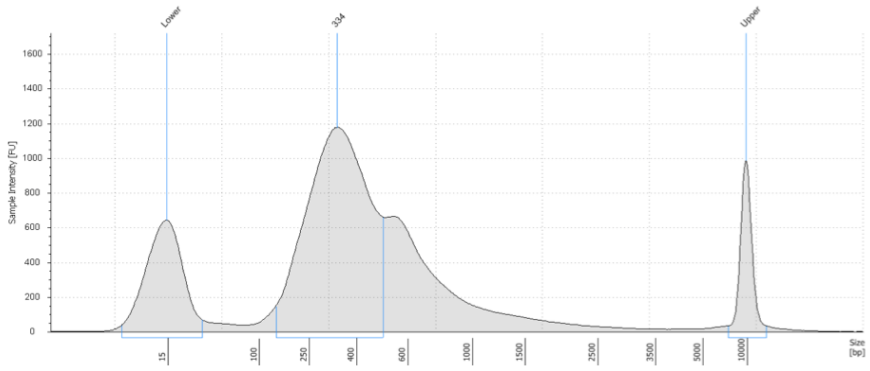
11 **(optional) Quality control of DNA libraries**

Using Agilent HS 5000 DNA chips (or equivalent)



Pooled (and dliuted) 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 2µl 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)	8.33mM	0.25	105
Betaine (5M)	1.66M	1	420
MgCl2 (1M)	10mM	0.03	12.6
TSO (100uM)	1.66µM	0.05	21
Nuclease free H2O	-	0.07	29.4
Total		3	1260

Dispense **2 µl per well**

Cover plate with new film and spin down.

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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 H2O	-	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	0.05625	24.75
Total		7.5000	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	37ºC	30 min	1x
Initial denaturation	95ºC	3 min	1x
Denaturation	98ºC	20 sec	18-24x
Annealing	67ºC	15 sec	
Elongation	72ºC	4 min	
Final elongation	72ºC	5 min	
	4C	Hold	

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)

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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*

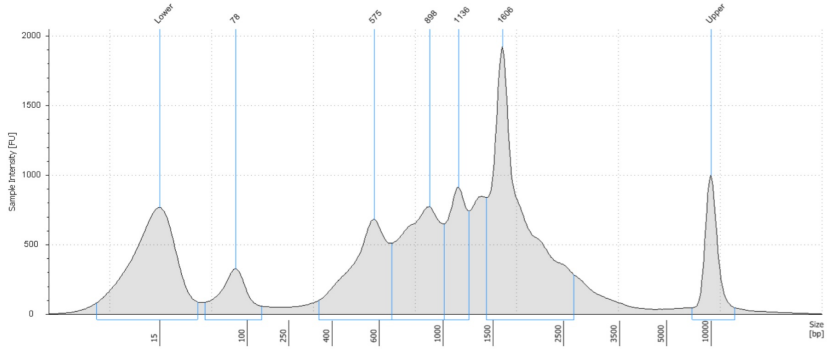
18 cDNA quantification

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)

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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 37ºC and mix well before use.

Reagent	Reaction conc.	µl per reaction	384w plate
Nuclease free H2O	-	1.050	525
TAPS-PEG (50mM TAPS, 25mM MgCl2, 40% PEG-8000)	10mM TAPS 5mM MgCl2 8% PEG-8000	0.500	250
psfTn5, loaded 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**

Reagent	Reaction conc.	µl per reaction	384w plate (420x)
H2O	-	13.25	5565
KAPA HiFi Buffer (5X)	1X	5	2100
dNTP (10mM/each)	0.3mM/each	0.75	315
KAPA enzyme (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)

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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	Cycles
Gap fill	72ºC	3 min	1x
First denature	95ºC	30 sec	1x
Denature	95ºC	15 sec	12x
Anneal	67ºC	30 sec	
Extend	72ºC	45 sec	
Final extension	72ºC	4 min	1x
	4-10ºC	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 µl EB or TE buffer**
9. Repeat cleanup (from step 1-7) and elute in **30 µl EB or TE buffer**

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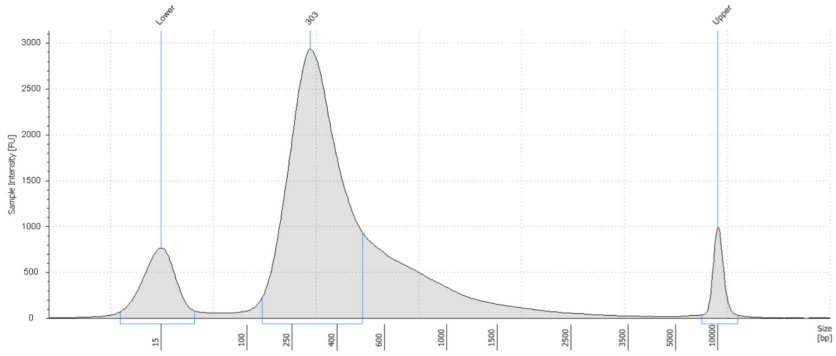
29 Pooled library QC



Pooled cDNA library of 784 cells on HS D5000 Agilent tapestation

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