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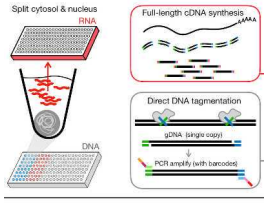
Direct nuclear tagmentation and RNA-sequencing (DNTR-seq) V.3



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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

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We use this protocol and it's working

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Keywords: single-cell, scRNA-seq, scWGS, direct nuclear tagmentation, whole genome, gene expression, rna, complex cell mixture, single cell, mrna, question in complex cell mixture, central challenge in biology, genetic variation, genotype, cell, dntr,

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: 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

MATERIALS

- HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems** **Catalog #**KK2601
- Proteinase K **Thermo Fisher Scientific** **Catalog #**EO0491
- Tween-20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P-7949
- psfTn5 **addgene** **Catalog #**79107
- 10% SDS solution **Teknova** **Catalog #**S0287
- SMARTScribe Reverse Transcriptase **Takara Bio Inc.** **Catalog #**634888
- Magnesium chloride solution for molecular biology (1.00 M) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M1028
- Ice
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**93426
- Microseal® 'F' Foil **Bio-Rad Laboratories** **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 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**B0300
- Dry ice
- 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 Bio Inc.** **Catalog #**2313A
- Hard-Shell® 384-Well PCR Plates thin wall skirted **Bio-Rad Laboratories** **Catalog #**HSP3801

Troubleshooting

Before start

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.

	A	B	C	D
	Reagent	**Reaction conc.**	**µL per reaction**	**384w plate (400x)**
	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

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

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

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.

https://doi.org/10.1101/gr.177881.114 LINK

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

	A	B	C	D
	Reagent	**Reaction conc**	**µL per reaction**	**384w plate (420x)**
	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

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

	A	B	C	D
	Reagent	**Reaction conc.**	**µl per reaction**	**384w plate**
	Nuclease free H2O	-	2.9	1218
	KAPA HiFi Buffer (5X)	1X	3.9	1638
	dNTP (10mM/each)	0.3mM/each	0.6	252
	KAPA enzyme (1u/µl)	0.02u/µl	0.4	168
	Tween-20 (10%)	0.1%	0.2	84
	To dispense		8	3360

2. Dispense 8 µL per well

3. Add primers/barcodes 1.5 µL per well (from 384-well index plates, with 3.75µM/each forward/reverse primers; see **oligos**). Total reaction volume is now 19.5µl (10µl sample + 9.5µl 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 3 µL from each well into a 1.5mL Eppendorf tube.

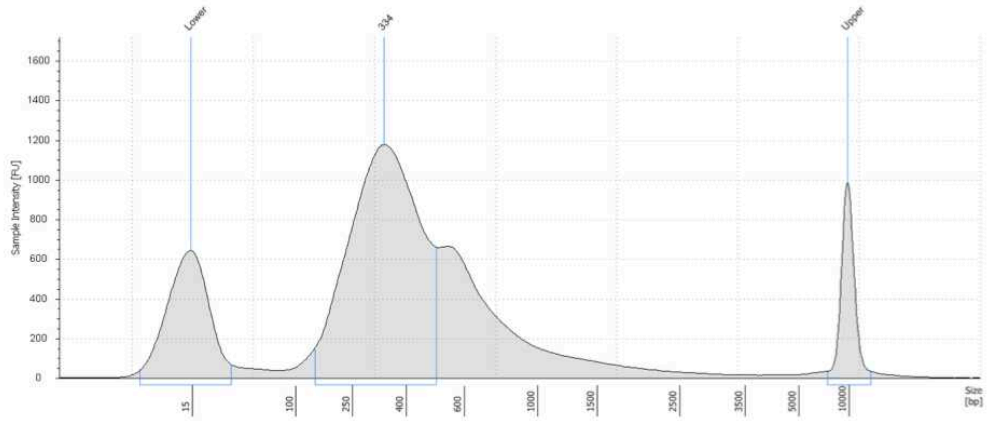
10 **Library cleanup**

We prepare SPRI-beads in 20% PEG-8000 solution as in:
https://openwetware.org/wiki/SPRI_bead_mix#Ingredients_for_50_mL_2

(optional) Take an aliquot of your pool (300µl)
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

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 550 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**

	A	B	C	D
	Reagent	**Reaction conc.**	**µl per reaction**	**384w plate (420x)**
	Maxima H Minus RT (200/µl)	2u/µl	0.05	21
	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

	A	B	C	D
	TSO (100uM)	1.66µM	0.05	21
	Nuclease free H2O	-	0.495	207.9
	Total		3	1260

Dispense 3 µL per well . Total reaction volume will be 5µl.

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**

	A	B	C	D
		Reaction conc.	**µl per reaction**	**384w plate (420x)**
	Nuclease free H2O	-	0.82	345
	Kapa HiFi HotStart ReadyMix (2X)	1X	6	2520
	IS_PCR primer (10µM)	0.1µM	0.12	50.4
	Lambda Exonuclease (10u/µl)	0.05 units	0.06	25.2
	Total		7	2940

Dispense 7 µL per well . Total reaction volume will be 12µl.

16 Vortex, spin down. Cover with new lid. Incubate in thermocycler with the following program:

	A	B	C	D
	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** 31m 30s

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 00:05:00 - 00:10:00 . Take care not to over-dry the beads.

9. Remove plate from magnetic stand
10. Elute beads in 12 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. cDNA plates can also be stored at -20C with beads.*

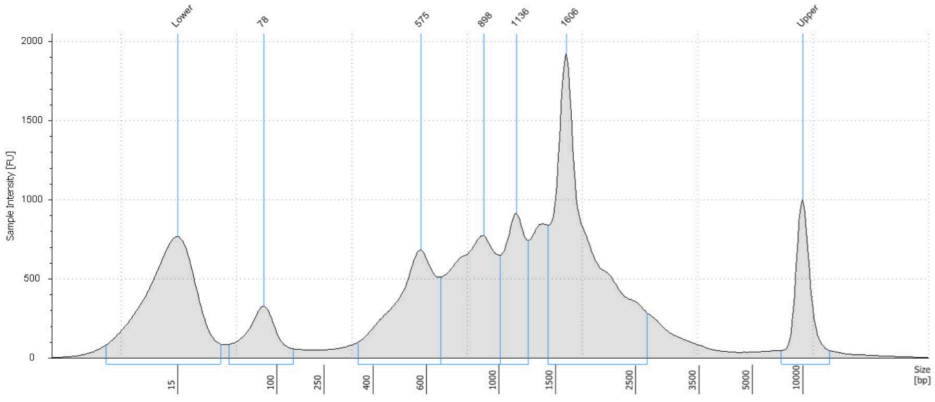
18 **cDNA quantification**

Option 1: 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)
4. Read in plate reader using 485nM excitation/528nm emission
5. Calculate cDNA concentration from linear model of Standards ladder

Option 2: Measure full plate using Qubit HS dsDNA in black, flat-bottom 384-well plate
1. Add 20 µL 1X Qubit HS dsDNA solution
2. Add 1 µL cDNA sample
3-5 as above

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)

Optional: if using a 384w-plate reader, one can normalize each well to 150pg/µl with variable water addition.

cDNA tagmentation

21 **Prepare Tn5 master mix**

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

	A	B	C	D
	Reagent	**Reaction conc.**	**µl per reaction**	**384w plate (420x)**
	Nuclease free H2O	-	0.750	315
	TAPS-PEG (50mM TAPS, 25mM MgCl2, 40% PEG-8000)	10mM TAPS 5mM MgCl2 8% PEG-8000	0.500	210
	psfTn5, loaded with 50µM MEDS-A/B		0.250	105
	Total		1.5	630

Dispense 1.5 µL per well in a new plate (**tagmentation plate**)

22 Add 1 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 20m
Remove immediately and stop reaction by adding 1 µL per well of 0.1% SDS.

Vortex, spin down and incubate 00:10:00 at 55 °C

cDNA library PCR and barcoding

24 **Make PCR master-mix**

	A	B	C	D
	Reagent	**Reaction conc.**	**µl per reaction**	**384w plate (420x)**
	H2O	-	4.85	2037
	KAPA HiFi Buffer (5X)	1X	2.5	1050
	dNTP (10mM/each)	0.3mM/each	0.3	126
	KAPA enzyme (1u/µl)	0.02u/µl	0.2	84
	Tween-20 (10%)	0.12%	0.15	63
	Total		8	

Dispense 8 µL per well to **tagmentation plate** (containing 3.5µl sample after step 23)

25 Add primers/barcodes 1 µL per well (from 384-well index plates, with 3.75µM/each forward/reverse primers; see **oligos**; final primer concentration 0.3µM per primer and reaction).

Total reaction volume is 12.5µl (3.5µl sample + 9µl 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

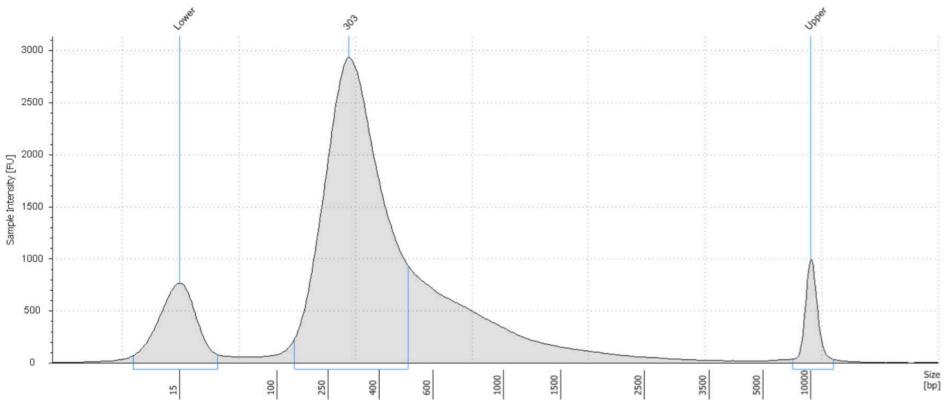
27 Pool 2.5 µL from each well to an 1.5ml Eppendorf tube

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

29 Pooled library QC



Pooled cDNA library of 784 cells on HS D5000 Agilent tapestation

Citations

Step 7

Picelli S, Björklund AK, Reinius B, Sagasser S, Winberg G, Sandberg R. Tn5 transposase and tagmentation procedures for massively scaled sequencing projects.
https://doi.org/10.1101/gr.177881.114