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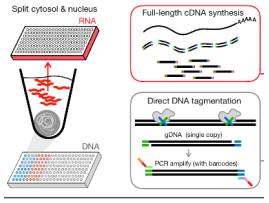
Direct nuclear tagmentation and RNA-sequencing (DNTR-seq) V.1



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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: 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

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.

	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

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.

	Reagent	Reaction conc	µL per reaction	384 w 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

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	384 w 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.

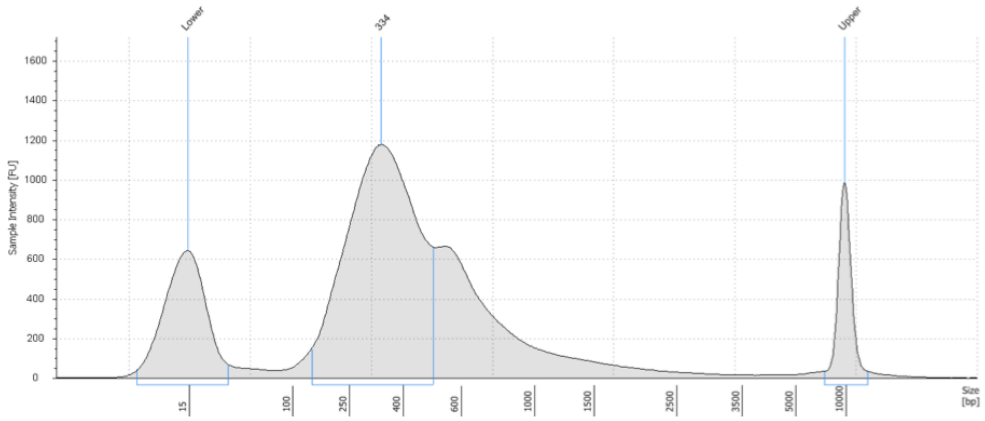
10 **Library cleanup**

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

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	384 w 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)	1.66M	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.

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**	**384 w 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

	Anne aling	67ºC	15 sec	
	Elong ation	72ºC	4 min	
	Final elong ation	72ºC	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*

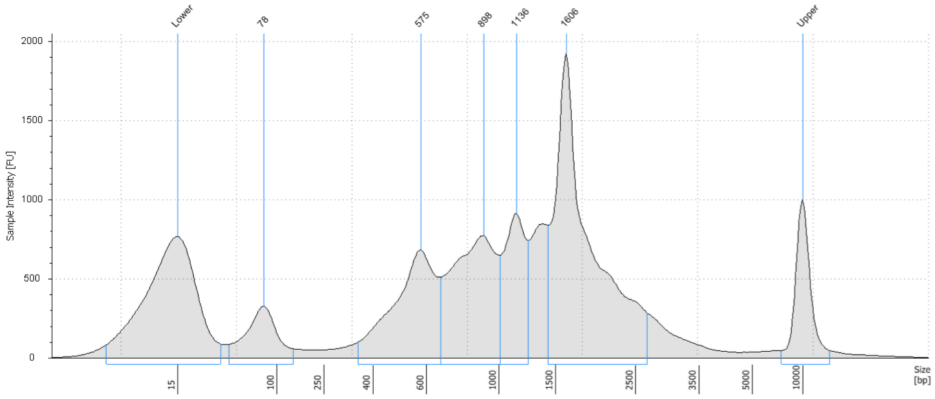
18 **cDNA quantification**

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 37ºC and mix well before use.

	Reagent	Reaction conc.	µl per reaction	384 w 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**	**384 w plate (420 x)**
	H2O	-	13.25	5565
	KAPA HiFi	1X	5	2100

	Buffer (5X)			
	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)

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

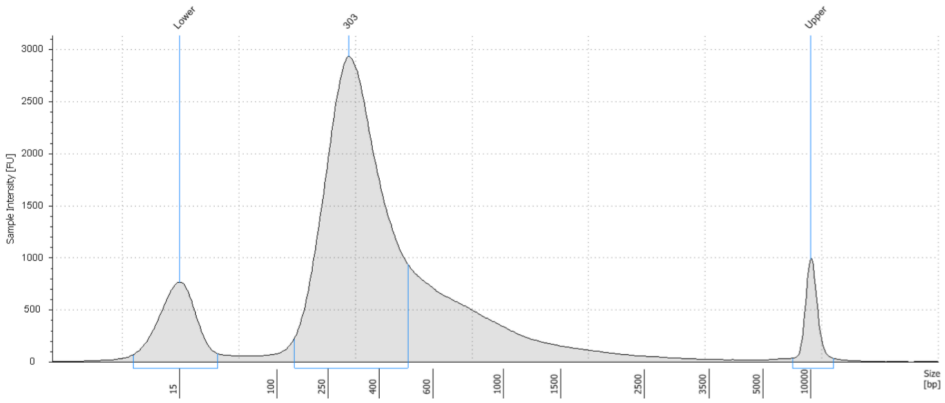
27 Pool 2.5 µL from each well to an 1.5ml Eppendorf tube

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

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