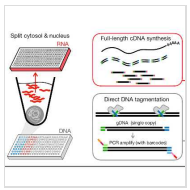

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Jun 21, 2022

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

Vasilios Zachariadis[1], Huaitao Cheng[1], Nathanael Andrews[1], Martin Enge[1]

[1]Karolinska Institutet

1

protocol .

Enge lab

Vasilios Zachariadis

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.

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

Vasilios Zachariadis, Huaitao Cheng, Nathanael Andrews, Martin Enge 2022. Direct nuclear tagmentation and RNA-sequencing (DNTR-seq). **protocols.io** https://protocols.io/view/direct-nuclear-tagmentation-and-rna-sequencing-dnt-b65yrg7w Vasilios Zachariadis

protocol

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

single-cell, scRNA-seq, scWGS

protocol ,

Apr 05, 2022

Jun 21, 2022

60312

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

☒ HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**

☒ Proteinase K **Thermo Fisher Scientific Catalog #EO0491**

☒ 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**

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:** 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**

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.

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: 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

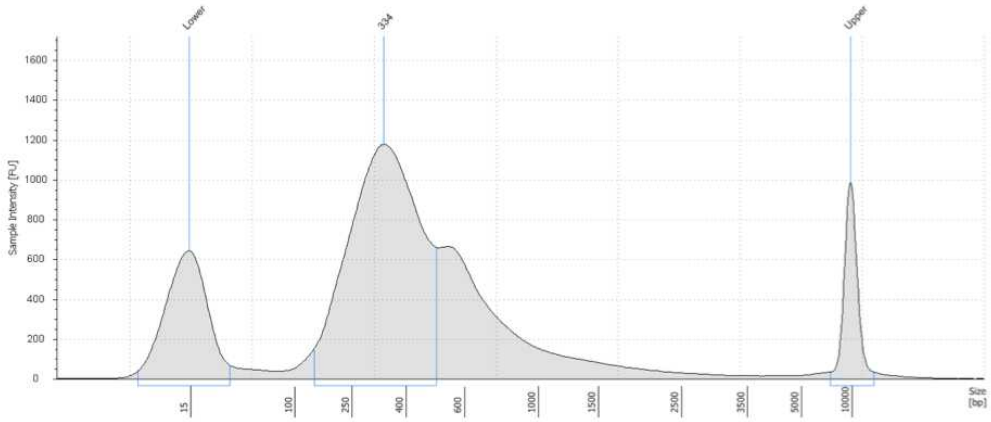
7. Air dry for **00:10:00** - **00:15:00**

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
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	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)

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

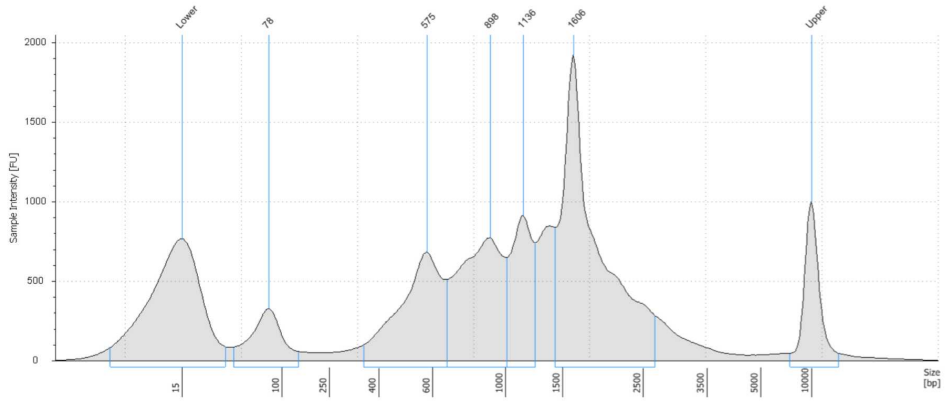
1. Add **20 µL 1X Qubit HS dsDNA solution**

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.

20m

23 Incubate in thermocycler at **55 °C** **00:10:00**
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

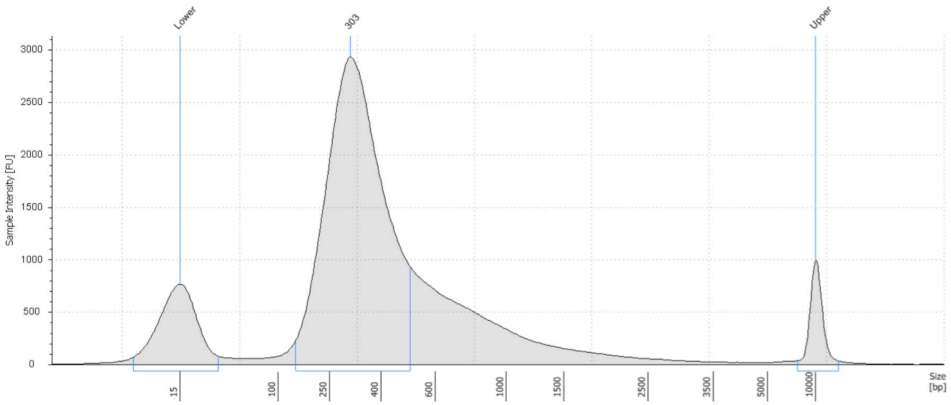
27 Pool **2.5 µL** from each well to an 1.5ml Eppendorf tube

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