

Aug 11, 2024

scNT-seq2: single-cell metabolically labelled new RNA tagging sequencing for time-resolved analysis of gene expression in single cells

DOI

<https://dx.doi.org/10.17504/protocols.io.j8nlk8811l5r/v1>

Qi Qiu¹, Fan Li¹, Dongming Liang¹, William Gao¹, Hao Wu¹

¹Department of Genetics, Epigenetics Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA 19104, USA

Wu Lab @ UPenn

Tech. support email: haowu2@upenn.edu



Fan Li

University of Pennsylvania

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.j8nlk8811l5r/v1>

Protocol Citation: Qi Qiu, Fan Li, Dongming Liang, William Gao, Hao Wu 2024. scNT-seq2: single-cell metabolically labelled new RNA tagging sequencing for time-resolved analysis of gene expression in single cells. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.j8nlk8811l5r/v1>



License: This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: June 05, 2024

Last Modified: August 11, 2024

Protocol Integer ID: 101225

Keywords: new rna tagging, new rna, resolved rna, metabolic labeling, rna, rnas from the same cell, seq2 library preparation for illumina, seq2 library preparation, sequencing, analysis of gene expression, marking new transcript, gene expression, seq method for joint profiling, single cell, seq2 method, seq2, strand synthesis reaction

Abstract

Single-cell metabolically labeled new RNA tagging sequencing (scNT-Seq) is a droplet microfluidics-based, time-resolved RNA-seq method for joint profiling of newly synthesized ("new") and pre-existing ("old") RNAs from the same cell by marking new transcripts with T-to-C substitutions via chemical conversion of 4-thiouridine (4sU) in new RNAs to cytidine analogs (Qiu et al., Nature Methods 17.10 (2020): 991-1001. PMID: 32868927). Here, we described the updated scNT-Seq2 method in which an optimized second-strand synthesis reaction is employed to increase the mapping rate and library complexity. This step-by-step protocol describes metabolic labeling, sample preparation, and scNT-Seq2 library preparation for Illumina sequencing.

Materials

A	B	C
Chemicals, Peptides, and Recombinant Proteins	Source	Identifier
DPBS, no calcium, no magnesium	Invitrogen	14190136
Barcoded beads	ChemGenes	MACOSKO-2011-10(V+)
1M Tris-HCl, pH 8.0	Invitrogen	15568-025
1 M Tris-HCl, pH 7.5	Invitrogen	15567-027
0.5 M EDTA, pH 8.0	Invitrogen	15575-020
NxGen RNase Inhibitor	Lucigen	30281-2
Bovine Serum Albumin	Sigma-Aldrich	A8806-5G
Ficoll PM-400	GE Healthcare/Fisher Scientific	45-001-745
Sarkosyl	Sigma-Aldrich	L7414-50mL
NaIO ₄	Sigma-Aldrich	7790-28-5
sodium acetate	Invitrogen	AM9740
Bst 3.0 DNA Polymerase	NEB	M0374
Exonuclease I	NEB	M0293L
TFEA	Sigma-Aldrich	269042-1G
SPRISelect beads	Beckman Coulter	B23318
DTT	Fermentas	R0862
QX200 Droplet Generation Oil for EvaGreen	Bio-Rad	186-4006
Perfluoro-1-octanol	Sigma-Aldrich	370533-25G
dNTPs	Clontech	639125
Critical Commercial Assays		
Maxima H Minus Reverse Transcriptase	ThermoFisher	EP0753
Nextera XT DNA sample preparation kit	Illumina	FC-131-1096
KAPA HiFi hotstart readymix	KAPA Biosystems	KK2602

	A	B	C
	Oligonucleotides		
	Template Switch Oligo	AAGCAGTGGTATCAACGCAGAG TGAATrGrGrG	
	TSO-PCR primer	AAGCAGTGGTATCAACGCAGAG T	
	Custom Read 1 Primer	GCCTGTCCGCGGAAGCAGTGG TATCAACGCAGAGTAC	
	TSO-N3G2N4B primer	AAGCAGTGGTATCAACGCAGAG TGA (N1:25252525)(N1)(N1)GG(N1) (N1)(N1)(N1)(N2: 00333433); N1 represents a mixture of A, C, G and T at a 25:25:25:25 ratio, N2 represents a mixture of A, C, G and T at a 0:33:34:33 ratio	
	P5-TSO hybrid primer	AATGATACGGCGACCAACCGAGA TCTACACGCCTGTCCGCGGAA GCAGTGGTATCAACGCAGAGT* A*C	

Metabolic labeling

- 1 Prepare a 1 M stock solution of 4-thiouridine (4sU) by dissolving the powder in DMSO.

Note

Note: the stock is stable for a few months at -20 C. Protect the solution from light.

- 2 For metabolic labeling, the medium was replaced with fresh medium supplemented with nontoxic concentrations of 4sU (i.e. 100 or 200 μ M).

Note

If the labeling time is longer than 4 hours, regular exchange of fresh 4sU-containing media (i.e. every three hours) can enhance 4sU incorporation.

Prepare cell suspension

- 3 After metabolic labeling, cells were rinsed once with DPBS.
- 4 Add 0.5 mL Accutase to each well of the 6-well plate and incubate at 37 C for about 5 min.
- 5 Add 1 mL culture medium to neutralize the Accutase.
- 6 Collect cell suspension in the 15 mL tube, spin down cells at 350 g, 3min.
- 7 Resuspend the cell pellet with 1 mL of DPBS (containing 0.01% BSA + 0.5%RNase-Inhibitor).
- 8 Count the cell number with Countess II.
- 9 Dilute the cell with DPBS (containing 0.01% BSA + 0.5%RNase-Inhibitor) to 100 cells/ μ L (1×10^5 cells/mL).



Cell and beads co-encapsulation

10 Prepare lysis buffer:

	A	B	C
	Reagents	Vol.(μ L)	Final Concentration
	H ₂ O	400	
	20% Ficoll PM-400	300	6%
	20% Sarkosyl	10	0.2%
	0.5 M EDTA	40	20 mM
	1.0 M Tris-HCl, pH 7.5	200	200 mM
	1.0 M DTT (add freshly)	50	50 mM

Lysis buffer master mix

11 Prepare barcoded beads:

Wash beads once with 30 mL of 100% ethanol and twice with 30 mL of TE-TW (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.01% Tween-20). Pass the beads through a 100 μ m cell strainer and count the number of beads. Resuspend the beads at 120 beads/ μ L concentration in 1.5 mL lysis buffer for each run (to profile 1,000~2,000 cells). Transfer 1.5 mL of bead suspension into a 3 mL Luer lock syringe. Make one more mL of lysis buffer to wash the syringe and magnetic disc.

12 Draw up 7 mL of droplet generation oil (Bio-Rad) into a 10-mL Luer-lock syringe.

13 Connect 3 syringes (containing cells, beads, and oil, respectively) to the Aquapel-coated PDMS Microfluidic device (μ Fluidix) with the following flow rate setting:

	A	B
	Syringe Content	Flow Rate (μ L/hr)
	Oil	15000



	A	B
	Cells	4000
	Beads	4000

- 14 Start the run in the following order: cells → beads → oil.
- 15 When the flow of droplets stabilizes, collect ~20 µL of aqueous flow to examine the droplet quality. Check whether the droplet size is uniform and estimate the percentage of bead doublets (the doublet rate should be less than 5%).
- 16 Once confirming the droplet quality, collect 1.2-1.3 mL of droplets into a 50 mL conical tube (target 1,000~2,000 cells for each sample).

Droplet breakage

- 17 Remove the oil layer from the bottom of the 50 mL tube.
- 18 Add 30 mL of room temperature 6X SSC into the tube.
- 19 Add 1 mL of Perfluorooctanol (PFO) into the tube in a fume hood. Shake by hand to break the droplets (3-4 forceful vertical shakes, the shakes should be long distance -- from head to leg) Spin at 1,000x g for 1 min.
- 20 Carefully remove the supernatant on top and then add 25 mL of 6X SSC to kick up the beads into the solution. Wait a few seconds to allow the majority of the oil to sink to the bottom. Transfer the supernatant to a new 50 mL tube.
- 21 Add 25 mL of 6X SSC to kick up the beads into the solution again. Transfer and combine the supernatant.
- 22 Spin at 1,000x g for 1 min to pellet the beads.
- 23 The beads are now pelleted to the bottom of the tube. Carefully remove all but ~1 mL of liquid. Resuspend the beads with the remaining liquid and transfer them to a 1.5 mL Low Binding tube.



- 24 Spin at 1,000x g for 1 min. Remove the supernatant. Wash beads once with 1 mL of 6X SSC.

Chemical conversion of mRNAs on beads

- 25 Wash beads once with 450 μ L reaction buffer (without TFEA and NaIO₄).
- 26 Incubate beads in 474 μ L Reaction-mix + 26 μ L NaIO₄ at 45 C for 1 hr with rotation.

A	B
Reagents	Volume (μ L)
3 M sodium acetate (pH 5.2)	8
0.5 M EDTA (pH 8.0)	2
H ₂ O	214
TFEA	13
Sub-total	237
192 mM NaIO ₄	13

Master mix for chemical conversion

- 27 Wash the beads once with 0.7 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).
- 28 Incubate the beads in 0.7 mL Reducing Buffer at 37 C for 30 min with rotation. Add 2% RNase inhibitor (20 μ L/1 mL) to the reducing buffer before use.

A	B
Reagents	For 1mL (μ L)
1 M Tris-HCl (pH7.5)	10
1M DTT	10
5M NaCl	20
0.5M EDTA	2



	A	B
	RNase Inhibitor	20
	DEPC-H ₂ O	958

Reducing Buffer

Reverse transcription

29 Prepare RT mix:

	A	B
	Reagents	Vol. (μL)
	H ₂ O	80
	Maxima 5X RT buffer	40
	20% Ficoll PM-400	40
	10 mM dNTPs	20
	100 μM Template Switch Oligo	5
	RNase Inhibitor	5
	Maxima H Minus Reverse Transcriptase	10

30 Wash the beads once with 1 mL Tris-HCl buffer (10 mM, pH 7.5).

31 Wash the beads once with 0.3 mL 2X RT buffer.

32 Add 220 μL of RT mix to the beads.

33 Incubate beads at room temperature for 30 min with rotation, then 120 min at 42 C with rotation.



- 34 Wash beads once with 1 mL TE-SDS (10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 0.5% SDS), twice with 1 mL TE-TW, then store the beads at 4°C over-night (in TE-TW buffer). [This is a Stop point. Store at 4C overnight (in TE-Tw buffer).]

Exonuclease I treatment

- 35 Prepare Exonuclease mix (makes 200 µL):

A	B
Reagents	Vol. (µL)
10X Exonuclease I buffer	20
H ₂ O	170
Exonuclease I	10

- 36 Wash beads once with 1 mL 10mM Tris-HCl pH 8.0, re-suspend in 190 µL of exonuclease mix.

- 37 *Incubate beads at 37 C for 45 min with rotation.*

- 38 Wash beads once with 1 mL TE-SDS, and twice with 1 mL TE-TW. [This is a Stop point. Store at 4C overnight (in TE-Tw buffer).]

Run 1st round of TSO-PCR using 6,000-beads as templates

- 39 Wash beads once with 1 mL H₂O. Spin at 1,000x g for 1 min.
- 40 Remove supernatant and re-suspend the beads with 1 mL of H₂O. Quickly transfer 2 µL of beads into a well of a 96-well plate (containing 198 µL of H₂O) and count the number of beads. Repeat bead counting three times and take the average.
- 41 Transfer an aliquot of 6,000 beads (corresponding to ~100 cells) into a PCR tube. Spin down and remove the supernatant, then re-suspend the beads with 50 µL PCR mix:

A	B
Reagents	Vol. (µL)



	A	B
	KAPA HiFi HS Readymix	25
	100 μ M TSO-PCR primer	0.4
	H2O	24.6

TSO-PCR master mix

- 42 Run 1st round of TSO-PCR. PCR program:
Very important: run 1st round of TSO-PCR to determine the exact number of amplification cycles. Over-amplification of the cDNA library will lead to fewer detected nuclei in the end).

95 C for 3 minutes

4 cycles of:

98 C for 20 seconds

65 C for 45 s

72C for 3 min

9 cycles of:

98 C for 20 s

67 C for 20 s

72 C for 3 min

Then:

72 C for 5 min

4 C forever

- 43 Purify PCR products once with 0.7X (35 μ L) SPRI beads once and elute in 15 μ L Elution buffer.

- 44 Measure the concentration of PCR products by Qubit.

- 45 Perform real-time PCR to determine the additional number of PCR cycles needed for optimal cDNA amplification.

	A	B
	Reagents	Vol. (μ L)
	Purified cDNA	1



A	B
25 μ M TSO-PCR primer	0.2
2X KAPA FAST qPCR Readymix	5
H ₂ O	3.8

Real-time PCR master mix

- 46 Run real-time PCR with the following program, and determine the optimal PCR cycle number

95 C for 3 min

25 cycles of:

95 C for 15 s

63 C for 30 s

72 C for 30 s

Second strand synthesis

- 47 Prepare second strand synthesis mix:

A	B
Reagents	Vol. (μ L)
10X Isothermal Amplification Buffer II	10
20% Ficoll PM-400	20
10 mM dNTPs	14
Bst 3.0 DNA Polymerase (NEB)	5
TSO-N3G2N4B primer (100 μ M)	10
MgSO ₄ (100 mM)	6
H ₂ O	35

Second strand synthesis master mix

- 48 After aspiration of TE-TW buffer, resuspend beads in 500 μ L 0.1 M NaOH.



- 49 Incubate beads at room temperature for 5 min with rotation.
- 50 Add 500 μ L 0.2M Tris-HCl (pH 7.5) to neutralize the solution.
- 51 Wash beads once with TE-TW and once with 10 mM Tris-HCl (pH 8.0).
- 52 Add 200 μ L second strand synthesis mix to the beads. [Add Bst3 enzyme right before the reaction.]
- 53 Incubate beads at 15 min at 60 C with rotation.
- 54 Wash beads once with 1 mL TE-SDS, and twice with 1 mL TE-TW and twice with H₂O.

Large-scale TSO-PCR

- 55 After determining the optimal PCR cycle number (usually an additional 0-4 cycles), perform large-scale TSO-PCR with the remaining beads. Wash the remaining beads twice with 1 mL H₂O. Apportion 6,000 beads for each PCR reaction. Spin down and remove the supernatant, then resuspend the beads with 50 μ L PCR mix. PCR program:

95 C for 3 min
4 cycles of:
98 C for 20 s
65 C for 45 s
72 C for 3 min
X plus additional cycles of:
98 C for 20 s
67 C for 20 s
72 C for 3 min
Then:
72 C for 5 min
4 C forever
- 56 Combine the PCR product for a given sample into a 1.5 mL Low Binding tube and purify **twice** with SPRI-select beads (0.6X Volume for the 1st & 0.7X Volume for the 2nd purification).



- 57 Elute the cDNA with 40 μ L Low-EDTA TE.
- 58 Quantify the cDNA library by Qubit and run the bioanalyzer to check the average fragment size of the purified cDNA library (the expected average size of cDNA library is 800-1,500 bp).

Tagmentation (Nextera® XT DNA Sample Preparation kit)

- 59 Preheat the thermocycler to 55 C. For each sample, take out 1 ng of purified cDNA with H₂O in a total volume of 5 μ L to a PCR tube.
- 60 Add 10 μ L of Nextera TD buffer and 5 μ L of Amplicon Tagmentation enzyme to each reaction. Mix by pipetting ~5 times.
- 61 Incubate at 55 C for 5 min.
- 62 Add 5 μ L of Neutralization Buffer to each reaction. Mix by pipetting ~5 times. Spin down and incubate at room temperature for 5 min.
- 63 Add to each PCR tube in the following order:

	A	B
	Reagents	Vol. (μ L)
	Nextera PCR mix	15
	2 μ M P5-TSO hybrid primer	5
	2 μ M Nextera N70X oligo	5

- 64 *PCR program:*
95 C for 30 s
12 cycles of:
95 C for 10 s
55 C for 30 s
72 C for 30 s
Then:
72 C for 5 min
4 C forever



- 65 Purify PCR product twice with 0.6X SPRI beads. Elute the cDNA in 12 μ L H₂O.
- 66 Quantify the concentration of cDNA library by Qubit and check the average fragment size of the purified cDNA library by Bioanalyzer (the expected fragment size is 500-700 bp).

Sequencing

- 67 Dilute the library to 2 nM and pool the libraries according to the estimated cell numbers (100 cells/6000 beads).

Protocol references

1. Hu, Peng, et al. "Dissecting cell-type composition and activity-dependent transcriptional state in mammalian brains by massively parallel single-nucleus RNA-seq." *Molecular cell* 68.5 (2017): 1006-1015.
2. Qiu, Qi, et al. "Massively parallel and time-resolved RNA sequencing in single cells with scNT-seq." *Nature methods* 17.10 (2020): 991-1001.