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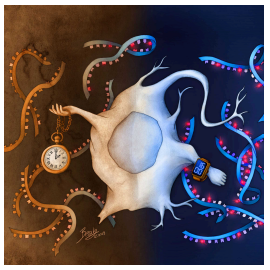
NASC-seq2 Protocol



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We use this protocol and it's working

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Abstract

Insights into transcriptional bursting kinetics and regulation have emerged from real-time nascent RNA imaging and analyses of static RNA counts over cells. Here, we developed sensitive single-cell profiling of newly transcribed (or new) RNA in cells (NASC-seq2) that can easily be applied to tens of thousands of single cells to help shed new light on bursting dynamics and coordination.

Image Attribution

Illustration by Beata Edyta Mierzwa (www.BeataScienceArt.com, social media: @beatascienceart)

Materials

EQUIPMENT

- Single-channel and multichannel pipettes
- Contact liquid handler for sample transfer and Vapor-Lock dispensing (Such as Agilent Bravo)
- Nanodispenser (such as Dispendix I.DOT, I.DOT Mini or Formulatrix Mantis)
- Plate centrifuge
- Tube centrifuge
- Fluorescence plate reader (compatible with Quantifluor dsDNA dye or similar)
- PE200 Sequencing (such as MGI G400 or Illumina NovaSeq)
- Magnetic Rack (384-well plate)
- Magnetic Rack (1.5ml Eppendorf tubes)
- 384-well compatible thermal cyclers

MATERIALS

General Protocol

- Armadillo PCR Plate, 384-well, clear, clear wells **Thermo Fisher Catalog #**AB2384
- Vapor-Lock **Qiagen Catalog #**981611
- Adhesive sealing sheets **Thermo Scientific Catalog #**AB0558
- Filter Tips
- dimethylsulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich)**
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787-50ML
- UltraPure DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- DNA LoBind Tubes **Eppendorf Catalog ##**022431021
- 4-thiouridine (4sU) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T4509
- Tris-HCL (pH 8.4 1M) **Merck MilliporeSigma (Sigma-Aldrich)**
- Iodoacetamide (single-use vial 56mg) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A3221
- Tris-HCl, pH 8.0 (UltraPure) **Thermo Fisher Scientific Catalog #**15568025
- Sodium Chloride (5M) **Invitrogen - Thermo Fisher Catalog #**AM9760G
- GTP (Tris buffered solution 100mM) **Thermo Scientific Catalog #**R1461
- Magnesium Chloride (1M Solution) **Invitrogen - Thermo Fisher Catalog #**AM9530G
- Poly Ethylene Glycol (PEG) 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**89510-250G-F
- Dithiothreitol (DTT) **Thermo Fisher Scientific Catalog #**707265ML

Recombinant RNAse Inhibitor **Takara Bio Inc.** **Catalog #**2313A

Maxima H Minus Reverse Transcriptase (200 U/µL) **Thermo Fisher** **Catalog #**EP0751

KAPA HiFi Hotstart PCR kit **Roche** **Catalog #**KK2502

dNTP Set 100 mM Solutions **Thermo Fisher Scientific** **Catalog #**R0182

QuantiFluor(R) dsDNA System **Promega** **Catalog #**E2670

Agilent High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626

Tris-HCl pH 7.5

Nextera XT sample prep kit, 96 samples **Illumina, Inc.** **Catalog #**FC-131- 1096

NN-Dimethylformamide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D4551

SDS, 10% Solution **Life Technologies** **Catalog #**AM9822

Phusion High-Fidelity DNA Polymerase (2 U/µL) **Thermo Fisher** **Catalog #**F530L

When using AmpureXP beads

Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880

When using DIY beads

Sera-Mag Speed Beads **GE Healthcare** **Catalog #**65152105050250

Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2002-100G

IGEPAL-CA630 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I3021 SIGMA-ALDRICH

EDTA (0.5 M), pH 8.0 **Life Technologies** **Catalog #**AM9260G

PRIMERS

All primers are the same as used in Smart-seq3 and were HPLC purified, with the TSO below being RNase-free HPLC purified.

- Smartseq3_OligodT30VN
  /5Biosg/ACGAGCATCAGCAGCATACGATTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
- Smartseq3_N8_TSO
  /5Biosg/AGAGACAGATTGCGCAATGNNNNNNNNrGrGrG
- Fwd_PCR_primer
  TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTGCGCAA*T*G
- Rev_PCR_primer
  ACGAGCATCAGCAGCATAC*G*A

Citation

Hagemann-Jensen M, Ziegenhain C, Chen P, Ramsköld D, Hendriks GJ, Larsson AJM, Faridani OR, Sandberg R (2020). Single-cell RNA counting at allele and isoform resolution using Smart-seq3.

https://doi.org/10.1038/s41587-020-0497-0 LINK

OPTIONAL

- Molecular Spike synthetic RNAs that were *in vitro* transcribed in the presence of 4sUTP (NU-1156S, Jena Bioscience).

Citation

Christoph Ziegenhain, Gert-Jan Hendriks, Michael Hagemann-Jensen, Rickard Sandberg (2022). Molecular spikes: a gold standard for single-cell RNA counting. Nature Methods.

https://doi.org/10.1038/s41592-022-01446-x LINK

Prepare lysis plates

1 Using an automated pipetting platform or multichannel pipette, dispense 3uL of Vapor-Lock (Qiagen) to each well of a 384-well plate.

Note:
Do not use a non-contact dispenser (such as Dispendix IDOT or Formulatrix Mantis) for this step as the Vapor-Lock may severely damage the dispenser. We do this using an Agilent Bravo platform.

2 Prepare the following lysis buffer on ice.

	Reagent	Final concentration	1 reaction	384-well plate
	Recombinant RNAse Inhibitor (40 U/uL)	2.5 u/uL	0.0188 uL	8.66 uL
	4sU containing spUMI pool (OPTIONAL, 0.01 ng/uL)	0.04 pg/uL	0.0012 uL	0.553 uL
	Triton-X100 (2 %)	0.1%	0.015 uL	6.91 uL
	Nuclease-free water	-	0.265 uL	122.1 uL
	Total		0.3 uL	138.2 uL

On ice

3 Use a nanodispenser to distribute 0.3 uL of freshly prepared lysis buffer to each well of vapor-lock containing 384-well PCR plate.

0.3 µL Lysis buffer

On ice

4 Spin down at >3,000 G for 10 seconds. 10s

3000 x g, 00:00:10

5 Optional: Store lysis plate.

If not immediately continuing with the next step, prepared lysis plates can be sealed with aluminium seals and stored at -80°C.

Cell culture and FACS sorting

6 Grow cells in the presence of 50 µM 4sU. It can be helpful to collect untreated cells as a control.

Note

- 4sU is light-sensitive, and direct light (i.e. light in the cell culture hood, etc) should be reduced to a minimum.
- Do not refreeze leftovers from 4sU aliquot.
- Labeling times can vary for different celltypes and biological applications, but in general, short labeling times (i.e. less than 30 minutes) may result in poor separation of new and old molecules. Depending on your downstream analysis, this may affect your results.

7 Stop the labeling by transferring your cells to 15-ml falcon tubes on ice and wash them with cold PBS.

On ice

8 Distribute single cells into each well of the lysis plate by FACS sorting.

9 Spin down and seal the plates with aluminium seals and store them at -80°C.

Alkylation

10 Prepare the alkylation mix at room temperature.

	Reagent	Final concentration	1 reaction	384-well plate
	Tris-HCL (pH 8.4, 1 M)	50 mM	0.03 uL	13.8 uL

	Reagent	Final concentration	1 reaction	384-well plate
	DMSO (100 %)	45 %	0.24 uL	110.6 uL
	IAA in DMSO (200 mM)	10 mM	0.03 uL	13.8 uL
	Total		0.3 uL	138.2 uL

Please note that the calculations in this mix are calculated to the final volume in the alkylation reaction (i.e. 0.6uL). The DMSO final concentration listed above also accounts for the DMSO that is added with the IAA.

Room temperature

Note

To avoid problems with iodocatamide stability and variability of alkylating potential, we use single-use vials (Sigma A3221-10VL) of iodoacetamide that are dissolved in DMSO at 200mM right before they are used to prepare the alkylation mix above and the remainder is discarded.

Safety information

Iodoacetamide should be handled in a fume hood and in accordence with local environmental health and safety regulations.

11 Using a nanodispenser, distribute 0.3 uL of freshly prepared alkylation mix to all wells of the 384-well plate containing cells and lysis-buffer.

12 Spin the 384-well plate down at >3,000 x G for 10 seconds.

13 Incubate the plate at 50C for 15 minutes. While this is running, prepare the quenching mix (see the next section).

50 °C 15 minutes

Quenching and Denaturation

14 Prepare the quencing mix on ice.

	Reagent	Final concentration	1 reaction	384-well plate
	DTT (1 M in H2O)	35 mM	0.035 uL	16.13 uL
	dNTPs (10 mM each)	0.5 mM each	0.2 uL	92.16 uL
	Oligo-dT (100 uM)	0.6 uM	0.024 uL	11.06 uL
	Recombinant RNAse Inhibitor (40 U/uL)	0.4 u/uL	0.04 uL	18.43 uL
	Nuclease-free water	-	0.101 uL	46.54 uL
	Total		0.4 uL	184.32

Note that the dNTPs, oligo-dT and RRI concentrations above are calculated to the final volume in the Reverse Transcription mix (4uL) and not the Quenching mix (1uL).

15 Using a nanodispenser, distribute 0.4 uL of freshly prepared quenching and denuturation mix to all wells of the 384-well plate.

16 Spin the 384-well plate down at >3,000 x G for 10 seconds

17 Incubate the plate for 5 minutes at room temperature, followed by 10 minutes at 72C and a final hold at 4C. While this is running, prepare the Reverse Transcription mix (see the next section)

Reverse Transcription

18 Prepare the Reverse Transcription mix as below

	Reagent	Final concentration	1 reaction	384-well plate
	Tris-HCL (1 M, pH 8.0)	25 mM	0,1 uL	46.08 uL
	NaCl (1 M)	35 mM	0,14 uL	64.51 uL
	GTP (100 mM)	1 mM	0,04 uL	18.43 uL
	MgCl2 (1 M)	2.5 mM	0.01 uL	4.61 uL
	PEG (40 %)	5 %	0.5 uL	230.4 uL

	Reagent	Final concentration	1 reaction	384-well plate
	DTT (1 M)	2 mM + carry-over from quenching and denaturation mix	0.008 uL	3.69 uL
	Recombinant RNAse Inhibitor (40 U/uL)	0.4 U/uL + carry-over from lysis as well as quenching and denaturation mix	0.04 uL	18.43 uL
	Template Switching Oligo (1 mM)	2 uM	0.008 uL	3.69 uL
	Maxima H-minus RT enzyme (200 U/uL)	2 U/uL	0.04 uL	18.43 uL
	H2O	-	2.11 uL	974.13 uL
	Total		3 uL	1382.4 uL

19 Dispense 3 uL of the freshly prepared Reverse Transcription mix to all wells of the 384-well plate.

20 Spin the 384-well plate down at >3,000 x G for 10 seconds

21 Place the plate in the thermal cycler and start the Reverse Transcription program

Preamplification PCR

22 For the remainer of the protocol we are using standard Smart-seq3 reaction conditions.

Citation

Hagemann-Jensen M, Ziegenhain C, Chen P, Ramsköld D, Hendriks GJ, Larsson AJM, Faridani OR, Sandberg R
(2020). Single-cell RNA counting at allele and isoform resolution using Smart-seq3.
Nature biotechnology.

https://doi.org/10.1038/s41587-020-0497-0 LINK

A detailed description of the Smart-seq3 protocol is also available on protocols.io.

Protocol

NAME

Smart-seq3 Protocol

CREATED BY

Michael Hagemann-Jensen

Preview

For convenience, the preamplification PCR protocol is also included below.

23 Prepare the preamplification PCR mix as below

	Reagent	Final concentration	1 reaction	384-well plate
	Kapa HiFi HotStart buffer (5 X)	1 X	2.0 uL	820 uL
	dNTPs (25 mM/each)	0.3 mM/each	0.12 uL	49.2 uL
	MgCl2 (100 mM)	0.5 mM	0.05 uL	20.5 uL
	Fwd Primer (100 uM)	0.5 uM	0.05 uL	20.5 uL
	Rev Primer (100 uM)	0.1 uM	0.01 uL	4.1 uL
	Polymerase (1 U/uL)	0.02 U/uL	0.2 uL	82 uL
	Nuclease Free Water	-	3.57 uL	1463.7 uL
	Total		6 uL	2460 uL

Preamplification PCR mix from Hagemann-Jensen et al.

24 Dispense 6 uL of the freshly prepared preamplification PCR mix to all wells of the 384-well plate.

25 Spin the 384-well plate down at >3,000 x G for 10 seconds

26 Place the plate in the thermal cycler and start the following PCR program

	A	B	C	D
	Step	Temperature	Time	Cycles
	Initial denaturation	98 °C	3 min	1x
	Denaturation	98 °C	20 sec	20-25x
	Annealing	65°C	30 sec	
	Elongation	72 °C	4 min	
	Final Elongation	72 °C	5 min	1x
	Hold	4 °C	Hold	

Purification, QC and Tagmentation

27 For details on the purification of the amplified cDNA, the quality control and the tagmentation to produce the final sequencing libraries, please refer to the Smart-seq3 protocols.io.

Protocol

NAME

Smart-seq3 Protocol

CREATED BY

Michael Hagemann-Jensen

Preview

Sequencing and data analysis

28 We have chosen to perform relatively long short-read sequencing with most data produced for NASC-seq2 being sequenced PE200. We highly recommend this as it will increase your ability to call individual molecules as either new (labeled) or old (unlabeled).

For details on data processing and analysis, please see the lab github:

https://github.com/sandberg-lab/NASC-seq2

Citations

Christoph Ziegenhain, Gert-Jan Hendriks, Michael Hagemann-Jensen, Rickard Sandberg. Molecular spikes: a gold standard for single-cell RNA counting
https://doi.org/10.1038/s41592-022-01446-x