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NASC-seq (new transcriptome alkylation-dependent single-cell RNA sequencing) protocol V.1

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1 Works for me dx.doi.org/10.17504/protocols.io.whtfb6n

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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MATERIALS

NAME	CATALOG #	VENDOR
Filter Tips		
Absolute Ethanol		
15 mL Falcon tubes		
QuantiFluor(R) dsDNA System	E2670	Promega
2x Kapa HiFi Hotstart Readymix	KK2602	Kapa Biosystems
nuclease free water		
Nextera XT DNA Library Preparation Kit	FC-131-1096	illumina
Agencourt AMPure XP SPRI beads	A63881	Beckman Coulter
Sodium Phosphate (0.2M)	NA	
DMSO	D8418	Sigma Aldrich
Aluminium Seal	E2796-9792	StarLab
Armadillo High Performance 96-well PCR plate	AB-2396	Thermo Scientific
Adhesive sealing sheets	AB0558	Thermo Scientific
1M MgCl2	AM9530G	Invitrogen - Thermo Fisher
Dynabeads MyOne Streptavidin C1	65001	Invitrogen - Thermo Fisher
DTT	43816-10ML	Sigma Aldrich
Iodoacetamide	I1149-5G	Sigma Aldrich
Superscript II	18064-014	Invitrogen - Thermo Fisher
Recombinant RNAse Inhibitor	2313A	Takarabio
Eppendorf 1.5ml tube (Amber)	T9786-1000EA	Sigma Aldrich
Dynabeads buffer A		Invitrogen - Thermo Fisher
Dynabeads buffer B		Invitrogen - Thermo Fisher

NAME	CATALOG #	VENDOR
Dynabeads 2x Binding and Washing buffer		Invitrogen - Thermo Fisher
1.5ml Eppendorf tubes		
4-Thiouridine	T4509-25MG	Sigma Aldrich

MATERIALS TEXT

The following oligonucleotides are required:

- Oligo-dT: biotin-triethyleneglycol-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN.
- Template switching oligonucleotide: AAGCAGTGGTATCAACGCAGAGTACrGrG+G (rX : RNA base, +X : LNA base).
- ISPCR oligonucleotide: AAGCAGTGGTATCAACGCAGAGT.
- Indexing primers compatible with Illumina's Nextera XT chemistry (These can be ordered from Illumina or ordered .from any other oligonucleotide synthesis service directly).

The following consumables and equipment is required:

- 4sU Spike-in mix. This is prepared as described in Schwalb et. al.
- Multichannels or other high-throughput liquid handling solutions.
- 96-well thermocycler.
- 96-well plate magnetic block (we recommend the Promega V8351 magnet).
- 1.5ml eppendorf tube magnetic rack.

Additionally, the following equipment is recommended:

- Fluorescence plate reader (for Quantifluor measurements).
- Eppendorf thermoshaker C1 (with hot-lid). This helps resuspending the beads without getting drops on the lids etc.

SAFETY WARNINGS

Iodoacetamide (IAA) is an alkylating agent that can be harmful if not handled appropriately, please refer to local health-and-safety guidelines if you are unsure how to handle this chemical safely.

BEFORE STARTING

Dissolve 4-thiouridine in PBS and prepare single-use 50mM or 5mM stock aliquots. Store at -20°C.

Prepare iodoacetamide aliquots before starting by weighing off a small amount of IAA into an amber eppendorf tubes. Write down the weight and store at 4°C until ready to use.

Rinse workbench with ethanol and RNAse-away (or equivalent).

Prepare lysis plate

1 Prepare the following lysis buffer on ice.

Reagent	1 reaction	96-well plate
Sodium Phosphate buffer (200mM)	2.5 µl	264 µl
RNAse inhibitor	0.2 µl	21.12 µl
ERCC + 4sU Spike-in mix	0.1 µl	10.56 µl
Nuclease-free water	0.2 µl	21.12 µl
Total	**3 µl**	**316.8 µl**

NASC-seq lysis buffer

4 °C Keep lysis mix on ice.

2 Distribute 3 µl of freshly prepared lysis buffer to each well of a 96-well PCR plate using a multichannel or high-throughput liquid dispensing system.

3 µl Distribute lysis mix.

4 °C Keep lysis plate on ice.

3 **Optional: Store lysis plate.**

Seal plate with aluminium foil and store at -80°C, if not immediately continuing with **step 4**.

-80 °C Store lysis plate.

Cell culture and FACS sorting

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

- 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 performance of the grandSLAM mixture model. Depending on your downstream analysis, this may affect your results.

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

4 °C Keep cells on ice.

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

Seal the plates with aluminium foil seal and store at -80°C.

4 °C Keep cells and lysis plate cooled before, during and after sorting.

-80 °C Store lysis plate.

Oligo-dT bead preparation

7 Transfer 60 µl Dynabeads to a clean eppendorf tube (for a 96-well plate).

Dynabeads sediment with time and need to be vortexed well before pipetting.

Do not use DNA-low-bind consumables since dynabeads may stick to the tips and tubes

8 Place the beads on a magnet and remove the supernatant, then remove the tube from the magnet and resuspend the beads in 60 µl Dynabeads buffer A.

go to step #8 Repeat once

9 Place the beads on a magnet and remove the supernatant, then remove the tube from the magnet and resuspend the beads in 60 µl Dynabeads buffer B.

go to step #9 Repeat once

10 Place the Dynabeads on a magnet and remove the supernatant, then remove the tube from the magnet and resuspend the beads in 60 µl Dynabeads **2x** binding and washing buffer and 60ul of oligo-dT (100µM).

Mix the beads and oligonucleotides well by pipetting up and down

00:15:00 Incubate beads with oligo-dT at room temperature with agitation

Prepare the Dynabead resuspension mix during the binding reaction:

Reagent	1 reaction	96-well plate
Nuclease-free water	1.64 µl	188.6 µl
RNAse inhibitor	0.36 µl	41.4 µl
Total	**2 µl**	**230 µl**

Dynabead resuspension mix

11 Place the Dynabeads on a magnet and remove the supernatant, then remove the tube from the magnet and resuspend the beads in 60 µl Dynabeads **1x** binding and washing buffer.

go to step #11 Repeat once

12 Resuspend the beads in 220 µl of freshly prepared bead resuspension buffer.

220 µl Dynabead resuspension mix.

4 °C Keep prepared beads on ice until ready to use

Binding of beads to RNA

13 Remove the lysis plate from -80°C and thaw on ice.

4 °C Thaw lysis plate on ice

If proceeding directly from FACS sorting the cells to the prepration of the NASC-seq libraries, it is recommended to freeze the cells at -80°C for 5 minutes and continue with step 5 to ensure that the cells are completely lysed.

14 Place the lysis plate in a thermocycler and denature the RNA at 80°C for 3 minutes.

80 °C Lyse cells and denature RNA

00:03:00

4 °C Cool plate after denaturing RNA

15 Distribute 2ul of beads to each well of the lysis plate.

2 µl Oligo-dT beads

16 Incubate the beads for 20 minutes at room temperature with agitation.

20 °C Room temperature

00:20:00 Bind mRNA to oligo-dT beads

Alkylation

17 Prepare the alkylation mix at room temperature.

Reagent	1 reaction	96-well plate
DMSO	4.5 µl	517.5 µl
Iodoacetamide (200mM, in DMSO)	0.5 µl	57.5 µl
Total	**5 µl**	**575 µl**

Alkylation mix

20 °C Keep at room temperature

To avoid problems with iodocatamide stability and variability of alkylating potential, we use single-use aliquots 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.

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

18 Add 5 µl of the alkylation mix to each well of the sample plate, spin gently, and incubate with agitation at 50°C for 15 minutes.

5 µl Alkylation mix

50 °C

00:15:00

While the alklylation reaction is running, prepare the STOP mix:

Reagent	1 reaction	96-well plate
Superscript II RT buffer	4.0 µl	460 µl
Nuclease-free water	5.10 µl	586.50 µl
Tween 20 (10%)	0.3 µl	34.50 µl
DTT (1M)	0.6 µl	69.00 µl
Total	**10 µl**	**1150 µl**

STOP mix

While the alkylation reaction is running, prepare the reverse transcription mix (*see step 14*)

4 °C Keep STOP mix on ice

19 Spin the plate down genlty and place on the magnet.
Pipette 10 µl of STOP mix to each well of the plate while the plate is on the magnet.

10 µl **STOP mix**

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

20 Spin the plate down gently and place back on the magnet and incubate for 5 minutes at room temperature.

20 °C **Room temperature**

00:05:00

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

Reverse Transcription

21 Prepare the reverse transcription mix:

Reagent	1 reaction	96-well plate
Superscript II Reverse Transcriptase	0.5 µl	55.2 µl
Superscript II RT buffer	2 µl	220.8 µl
RNAse inhibitor	0.2 µl	22.08 µl
DTT (0.1M)	0.5 µl	55.2 µl
Betaine (5M)	2 µl	220.8 µl
MgCl2 (1M)	0.1 µl	11.04 µl
Template Switching Oligo (100µM)	0.2 µl	22.08 µl
dNTPs (25 mM each)	0.4 µl	44.16 µl
Nuclease-free water	4.1 µl	452.64 µl
Total	**10 µl**	**1104 µl**

Reverse transcription mix

22 Remove the supernatant from each well carefully while the plate is on the magnet. Avoid touching the beads. Immediately after pipetting off the supernatant in a column of wells, add 10 ul of Reverse Transcription mix to those wells.

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

Avoid touching the beads while pipetting the supernatant off, and pipette the Reverse Transcription mix to the side where the beads are immobilized to avoid drying out the beads.

23 Spin the beads down gently and resuspend by careful vortexing.

24 Perform the RT according to the following program:

42 °C 01:30:00

10 cycles:

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50 °C 00:02:00

42 °C 00:02:00

---

4 °C hold

To optimize cDNA yield it is recommended to vortex the plate every 20 minutes during the RT program to avoid settling of the beads.

When the RT program is almost done, continue with **step 25**

Pre-amplification PCR

25 Prepare the PCR mix:

Reagent	1 reaction	6-well plate
KAPA HiFi HotStart Readymix (2x)	11 µl	1214 µl
ISPCR primer (10µM)	0.3 µl	33.12 µl
H2O	0.7 µl	77.28 µl
Total	**12 µl**	**1324.8 µl**

PCR mix

26 Add 12 µl of the PCR mix to each well of the plate with the reverse transcribed cDNA.

12 µl PCR mix

27 Perform PCR according to the following program:

98 °C 00:03:00

22 cycles:

---

98 °C 00:00:20

67 °C 00:00:15

72 °C 00:06:00

---

72 °C 00:05:00

4 °C hold

This is a good point to interupt the protocol and store the unpurified, amplified cDNA library at -20°C for up to 3 days.

Two-sided AMPure XP cleanup

28 Add 18 µl AMPure XP beads to each well of the amplified cDNA libraries and mix by pipetting up and down. Incubate for 15 minutes at room temperature.

18 µl AMPure XP beads

00:15:00 Bind DNA to beads

29 Move plate to magnet and wait up to 5 minutes to immobilize the beads. Remove the supernatant and wash the beads with 50 µl 80% ethanol.

00:05:00 Immobilize beads

50 µl 80% ethanol

30 Remove the supernatant and dry the beads for 5 minutes.

00:05:00 Dry beads

31 Resuspend beads into 17 µl of nuclease free water and incubate at room temperature for 3 minutes to release DNA.

17 µl Nuclease-free water

00:03:00 Release DNA

32 Move the plate onto the magnet and allow the beads to immobilize for 5 minutes before moving 16 µl of supernatant to a new plate.

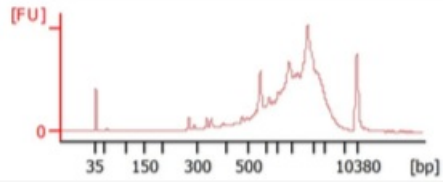
00:05:00 Immobilize beads

16 µl Purified cDNA library

cDNA QC

33 Analyze 1 µl of 11 selected libraries with a Agilent bioanalyzer on a Agilent DNA high-sensitivity chip.

1 µl Purified cDNA library



cDNA library expected result

34 Analyze 1 µl of each well of the cDNA library plate by QuantiFluor assay

1 µl Purified cDNA library

35 Use the concentrations measured in step 32 to prepare a plate with cDNA from all libraries diluted to 200 pg/µl.

Tagmentation (Nextera XT - 1/5 volume)

36 Prepare the tagmentation mix

Reagent	1 reaction	96-well plate
Tagment DNA buffer (TD)	2 µl	211.2 µl
Amplicon Tagmentation mix (ATM)	1 µl	105.6 µl
Total	**3 µl**	**316.8 µl**

Tagmentation mix

37 Add 3 µl of Tagmentation mix to each well of a new 96-well plate. Keep this plate on ice after dispensing the amplicon tagmentation mix.

4 °C Keep tagmentation mix and plate on ice

38 Pipette 1 µl of each well of the diluted cDNA library (200 pg/µl) plate to the plate with the tagmentation mix and incubate the plate at 55C for 5 minutes.

1 µl Diluted cDNA library (200 pg/µl)

55 °C Tagment for 5 minutes

39 Stop the tagmentation reaction and remove the tagmentation complexes from the DNA by adding 1 µl of NT buffer to each well and incubating for 5 minutes at room temperature.

1 µl NT buffer

00:05:00 Remove tagmentation complexes

20 °C Room temperature

40 Add 2 ul of premixed Illumina N7/N5 compatible indexing primers (1 µM) to each well.

2 µl Premixed index primer mix (1 µM)

41 Add 3 µl of Illumina PCR mix (NPM) to each well of the plate and perform PCR according to the following conditions:

3 µl NPM

72 °C 00:03:00

95 °C 00:00:30

10 cycles:

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95 °C 00:00:10

55 °C 00:00:30

72 °C 00:00:30

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72 °C 00:05:00

4 °C hold

Two-sided AMPure XP cleanup

42 Pool 2.5 µl from each well into a new 1.5ml eppendorf tube and transfer 200 µl of the mixed pool to another new eppendorf tube.

43 Add 100 µl of AMPure XP beads and allow 5 minutes at room temperature to bind very large framgents and exclude them.

100 µl AMPure XP beads

00:05:00 Bind largest fragments to beads

44 Place the beads on a magnet and allow beads to immobilize for 5 minutes. Then transfer the supernatant to a new 1.5ml eppendorf tube.

00:05:00 Immobilize beads

300 µl Supernatant (pooled Library + PEG)

45 Add 60 µl of AMPure XP beads to the 300 µl of library and PEG mixture (supernatant from step 44). Mix by pipetting up and down and allow DNA to bind for 15 minutes.

60 µl AMPure XP beads

00:15:00 Bind DNA

46 Place the beads on the magnet and allow 5 minutes to immobilize the beads. Remove the supernatant and wash the immobilized beads with 200 µl 80% ethanol. Repeat the ethanol wash 4 times.

00:05:00 Immobilize beads

200 µl 80% ethanol (5x)

47 Allow the beads to dry for 5 minutes.

00:05:00 Dry beads

48 Resuspend the beads in 60 µl of nuclease-free water and allow 5 minutes to release the DNA.

00:05:00 Release DNA

49 Transfer 55 µl of the final resuspended NASC-seq library to a new 1.5ml eppendorf tube and keep it on ice.

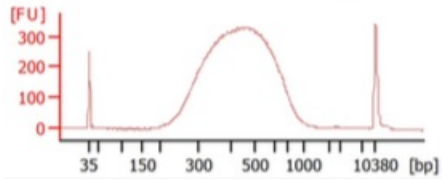
55 µl NASC-seq library

4 °C Store NASC-seq library on ice

Final Library QC

50 Analyze 1 µl of the final NASC-seq library on the Agilent Bioanalyzer using a Agilent DNA high sensitivity chip.

1 µl NASC-seq library



Final NASC-seq library expected results

51 Measure the concentration of the final NASC-seq library using the Qubit DNA HS kit and prepare a 4nM final library for sequencing.

Sequencing

52 Sequence the NASC-seq library on an Illlumina NextSeq or NovaSeq with standard sequencing primers.

We recommend sequencing paired-end (2x) 150 cycles to increase the number of conversions that are detected in each read.