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Version 1

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

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Protocol status: Working

We use this protocol and it's working

Created: December 19, 2018

Last Modified: July 19, 2019

Protocol Integer ID: 18707

Keywords: new transcriptome alkylolation, cell rna, rna, seq



Materials

MATERIALS

✕ Filter Tips

✕ Absolute Ethanol

✕ 15 mL Falcon tubes

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

✕ 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #KK2602**

✕ nuclease free water

✕ Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**

✕ Agencourt AMPure XP SPRI beads **Beckman Coulter Catalog #A63881**

✕ Sodium Phosphate (0.2M) **Catalog #NA**

✕ DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8418**

✕ Aluminium Seal **StarLab Catalog #E2796-9792**

✕ Armadillo High Performance 96-well PCR plate **Thermo Scientific Catalog #AB-2396**

✕ Adhesive sealing sheets **Thermo Scientific Catalog #AB0558**

✕ 1M MgCl₂ **Invitrogen - Thermo Fisher Catalog #AM9530G**

✕ Dynabeads MyOne Streptavidin C1 **Invitrogen - Thermo Fisher Catalog #65001**

✕ DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #43816-10ML**

✕ Iodoacetamide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I1149-5G**

✕ Superscript II **Invitrogen - Thermo Fisher Catalog #18064-014**

✕ Recombinant RNase Inhibitor **Takara Bio Inc. Catalog #2313A**

✕ Eppendorf 1.5ml tube (Amber) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T9786-1000EA**

✕ Dynabeads buffer A **Invitrogen - Thermo Fisher**

✕ Dynabeads buffer B **Invitrogen - Thermo Fisher**

✕ Dynabeads 2x Binding and Washing buffer **Invitrogen - Thermo Fisher**

✕ 1.5ml Eppendorf tubes

✕ 4-Thiouridine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #T4509-25MG**

The following oligonucleotides are required:

- Oligo-dT: biotin-triethyleneglycol-
AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTNN.
- 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 start

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.

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 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.

Note

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).

Note

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 μ l 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 μ l 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 μ l Dynabeads **2x** binding and washing buffer and 60ul of oligo-dT (100 μ M).

Note

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 μ l	188.6 μ l
RNAse inhibitor	0.36 μ l	41.4 μ l
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 μ l Dynabeads **1x** binding and washing buffer.

⇒ go to step #11 Repeat once

- 12 Resuspend the beads in 220 μ l 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

Note

If proceeding directly from FACS sorting the cells to the preparation 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 2µl 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

Note

To avoid problems with iodoacetamide 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.

Safety information

Iodoacetamide should be handled in a fume hood and in accordance 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 alkylation 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 gently 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

Safety information

Iodoacetamide should be handled in a fume hood and in accordance 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

Safety information

Iodoacetamide should be handled in a fume hood and in accordance 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
	MgCl ₂ (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.

Safety information

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

Note

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:

🌡️ 50 °C ⌚ 00:02:00

🌡️ 42 °C ⌚ 00:02:00

🌡️ 4 °C hold

Note

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

Note

This is a good point to interrupt 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 μ L 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 μ L 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 μ L 80% ethanol.

 00:05:00 Immobilize beads

 50 μ L 80% ethanol

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

 00:05:00 Dry beads

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

 17 μ L 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 μ L of supernatant to a new plate.

 00:05:00 Immobilize beads

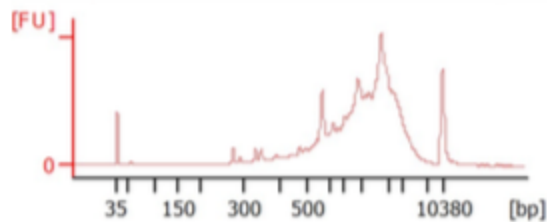
 16 μ L Purified cDNA library

cDNA QC

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

 1 μ L Purified cDNA library

Expected result



cDNA library expected result

- 34 Analyze 1 μ L of each well of the cDNA library plate by QuantiFluor assay

 1 μ L Purified cDNA library

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

Tagmentation (Nextera XT - 1/5 volume)

- 36 Prepare the tagmentation mix

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

Tagmentation mix

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

 4 $^{\circ}$ C Keep tagmentation mix and plate on ice

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

🧪 1 μ L Diluted cDNA library (200 pg/ μ L)

🌡️ 55 °C Tagment for 5 minutes

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

🧪 1 μ L NT buffer

🕒 00:05:00 Remove tagmentation complexes

🌡️ 20 °C Room temperature

- 40 Add 2 μ L of premixed Illumina N7/N5 compatible indexing primers (1 μ M) to each well.

🧪 2 μ L Premixed index primer mix (1 μ M)

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

🧪 3 μ L NPM

🌡️ 72 °C 🕒 00:03:00

🌡️ 95 °C 🕒 00:00:30

10 cycles:

🌡️ 95 °C 🕒 00:00:10

🌡️ 55 °C 🕒 00:00:30

🌡️ 72 °C 🕒 00:00:30

🌡️ 72 °C 🕒 00:05:00

🌡️ 4 °C hold

Two-sided AMPure XP cleanup

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



- 43 Add 100 μ L of AMPure XP beads and allow 5 minutes at room temperature to bind very large fragments and exclude them.

 100 μ L 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 μ L Supernatant (pooled Library + PEG)

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

 60 μ L 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 μ L 80% ethanol. Repeat the ethanol wash 4 times.

 00:05:00 Immobilize beads

 200 μ L 80% ethanol (5x)

- 47 Allow the beads to dry for 5 minutes.

 00:05:00 Dry beads

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

 00:05:00 Release DNA

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

 55 μ L NASC-seq library

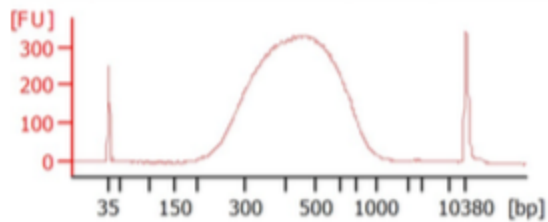
 4 $^{\circ}$ C Store NASC-seq library on ice

Final Library QC

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

 1 μ L NASC-seq library

Expected result



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 Illumina NextSeq or NovaSeq with standard sequencing primers.

Note

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