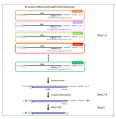

Jan 29, 2020

Low Quantity single strand CAGE protocol

Hazuki Takahashi[1], Hiromi Nishiyori-Sueki[1], Piero Carninci[1]

[1]Laboratory for Transcriptome Technology, RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa 230-0045, Japan

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

Hazuki Takahashi
Laboratory for Transcriptome Technology, RIKEN Center for In...

ABSTRACT

BioLegend MojoSort™ nanobeads work in commonly used separation columns, based on our internal research as well as validation by external testing by academic labs. This simple protocol consists of following the MojoSort™ protocol to label the cells with **pre-diluted** MojoSort™ reagents and using the columns as indicated by the manufacturer.

Note: Due to the properties of our beads, it may be possible to use far fewer beads and less antibody cocktail that with other commercial suppliers. We recommend a titration to find the best dilution factor. However, as a general rule, dilutions ranging from 1:2 to 1:10 for the antibody cocktail can be used. Dilutions ranging from 1:5 to 1:20 for the Streptavidin Nanobeads can be used. Please contact BioLegend Technical Service (tech@biolegend.com) if further assistance is needed.

MATERIALS TEXT

Additional reagents:
-commercially available cell separation columns
-5 mL polypropylene tubes
-70 µm cell strainer

This protocol works with the following MojoSort™ Kits (cat#):
480047, 480048

BEFORE STARTING

RNAs should be treated in the RNAse free solutions, benches and equipments. We highly recommend to perform RNA quality check before starting (see detail at previously published paper RNA stability check).

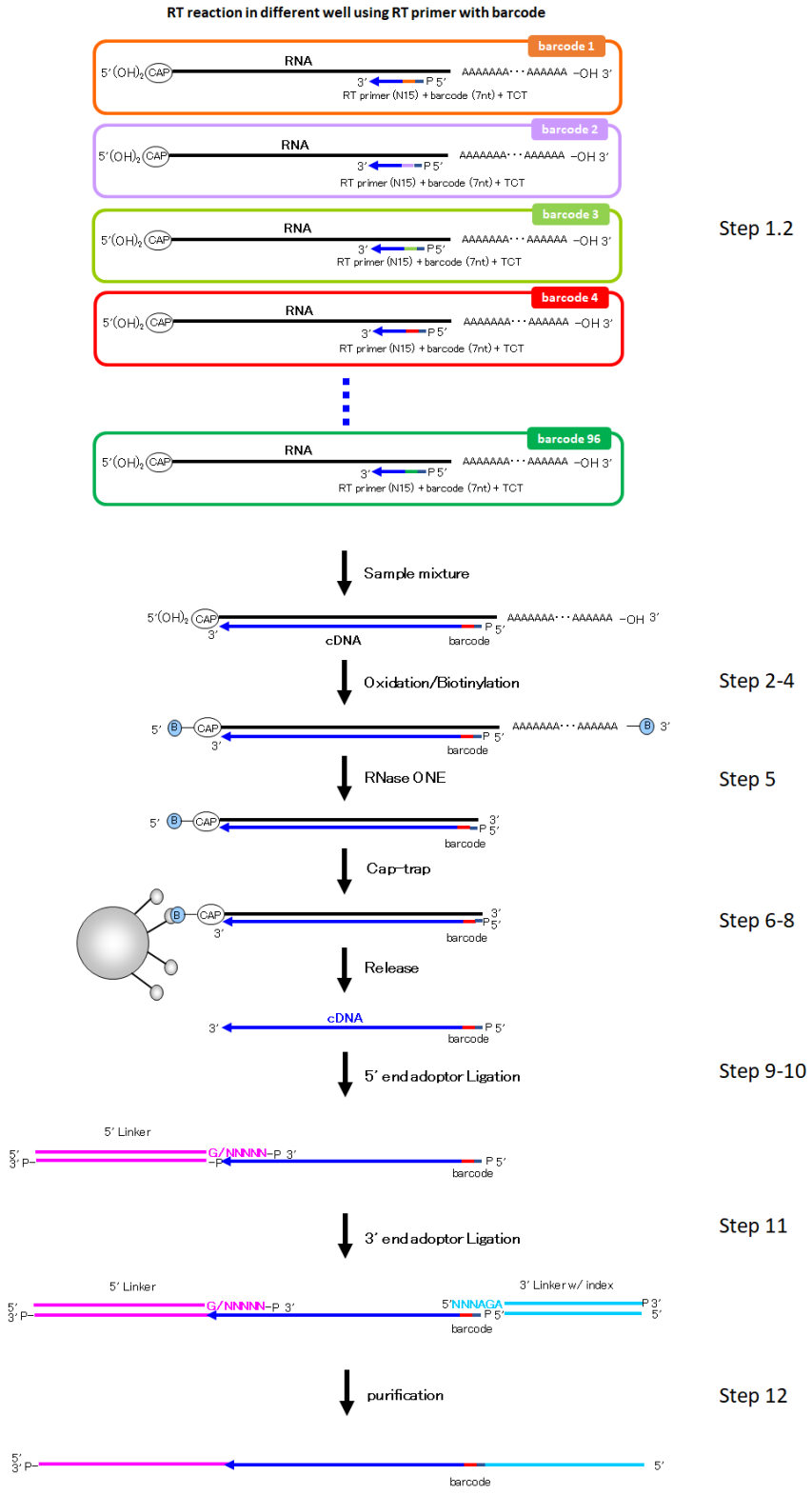

Figure.1 Protocol Flow

1 Reverse Transcription (RT) to generate RNA/cDNA hybrids

1.1

single strand CAGE (ssCAGE)

When the amount of RNAs is more than1 µg, choose this RT protocol. Here is an example of 5 µg starting RNAs.
When the amount of RNAs is less than 1 µg, go to step **1.2.**

a. Mix 5 µL of RNAs (1 µg/µL), 1 µL of RT primer (N6+TCT) and 4 µL of RNase free water in a tube.

NOTE: Use 1.5 mL tube, 8 strip PCR tube or 16-96 wells plate depending on numbers of samples.

b. Incubate the RNA/primer mix from step **a.** at 65 °C for 5 min and immediately put on ice for 2 min.
c. Add the following components to the solution from step **b.** and carefully mix by pipetting on ice.

5 x First-Strand buffer	4 µL
10 mM dNTPs	1 µL
0.1 M DTT	1 µL
RNase free water	2 µL
SuperScript III Reverse Transcriptase	2 µL

d. Incubate the mixed solution (total 20 µL) at 25 °C for 30 sec, 55 °C for 30 min and keep 4 °C to generate RNA/cDNA hybrids.
e. Add 36 µL (1.8-folds) of RNACleanXP beads to 20 µL of RNA/cDNA hybrids from step **d.** and mix well by pipetting.
①. Incubate at room temperature for 5 min. Spin down and set the tube on magnetic bar for 5 min.
②. Discard the supernatant by pipette aspiration.
③. Wash the beads with 200 µL of 70% EtOH.
④. Set the tube on magnetic bar for 5 min.
⑤. Discard the supernatant by pipette aspiration.
⑥. Repeat step ③ ~ ⑤.
⑦. Discard the 70% EtOH completely by pipette aspiration.
⑧. Add 42 µL of RNase free water and mix by pipetting extensively (more than 60 times) to elute RNA/cDNA hybrids.
⑨. Incubate at room temperature for 5 min.
⑩. Spin down and set the tube on magnetic bar for 5min.
⑪. Transfer supernatant (~ 40 µL of the cDNA/RNA hybrids) to new tube.

1.2

Low Quantity single strand CAGE (LQ-ssCAGE)

When the amount of starting RNAs is less than 1 µg, choose this protocol.

NOTE: The amount of RNAs in one tube should be 25 ng ~ 100 ng for the RT reaction. After RT, samples have each barcode and can be mixed up to 5 µg. For instance, if the starting RNA amount is 50 ng, 100 samples can be mixed. The number of mixed samples is depending on how much samples you have and how deep the samples should be sequenced. Here is an example of 48 mix samples from each 50 ng starting RNA.
CRITICAL: The mixed solution should be less than 5 µg. Otherwise the following linkers at step **9** and **11** are not enough for the linker ligation.

a. Mix 4 µL of 50 ng RNAs (12.5 ng/µL) and 1 µL of 1.25 mM RT primer containing barcode in a 96 wells plate by pipetting.
b. Incubate the RNA/primer mix form step **a.** at 65 °C for 5 min and immediately put on ice for 2 min.
c. Mix the following components (enzyme mix) on ice:

5 x First Strand buffer	2.0 µL
0.1 M DTT	0.5 µL
10 mM dNTPs	0.5 µL
RNase free water	1.0 µL
SuperScript III Reverse Transcriptase	1.0 µL

Total volume	5.0 µL

NOTE: Make premix solution for 48 samples x 1.1 samples.

d. Add 5 µL of enzyme mix from premix solution at step **c.** to RNA/primer mix from step **b.** and carefully mix 10 times by pipetting on ice.
e. Incubate at 25 °C for 30 sec, 50 °C for 30 min and keep 4 °C to generate RNA/cDNA hybrids.
f. Mix samples by following steps.
①. Transfer each 10 µL of RNA/cDNA hybrids from step **e.** to one of new 1.5 mL tube (total volume is 480 µL).
②. Add 15 µL of RNase free water to the first 8 wells at the 96 wells plate from step ①, wash wells by pipetting, and transfer the 15 µL of the solutions to the next 8 wells.
③. Wash the 8 wells by pipetting and transfer 15 µL of the solutions to the next 8 wells.
④. Repeat step ②three times until the end of 8 wells and transfer all solutions (total volume is 120 µL (15 µL x 8 wells)) to the 1.5 mL tube from step ①(final volume is 600 µL).
⑤. Mix 600 µL of the solution from step ④by vortex, spin down and aliquot 200 µL in new two tubes of 1.5 mL tube (total 200 µL in 3 tubes of 1.5 mL tube).

NOTE: Step **f.** is for the collection of remaining molecules to avoid losing any RNA/cDNA hybrids in the wells. We recommend to aliquot less than 200 µL per each 1.5 mL tube at step **f.**⑤due to the volume limitations of next RNAClean XP purification step.

g. Add 300 µL (1.8 -folds) of RNAClean XP beads to the 48 mixed RNA/cDNA hybrids in a 1.5 mL tube from step **f.**⑤, mix well by pipetting and then elute the mixed RNA/cDNA hybrids by following steps.
①. Incubate at room temperature for 10 min, spin down and set the 1.5 mL tube on magnetic bar for 5 min.
②. Discard the supernatant by pipette aspiration.
③. Wash the beads with 1.2 mL of 70% EtOH.
④. Set the 1.5 mL tube on magnetic bar for 5 min.
⑤. Discard the supernatant by pipette aspiration.
⑥. Repeat step ③~ ⑤ twice.
⑦. Discard the 70% EtOH completely by pipette aspiration.
⑧. Add 100 µL RNase free water and mix by pipetting extensively (more than 60 times) to elute RNA/cDNA hybrids.
⑨. Incubate at room temperature for 5 min.
⑩. Spin down and set the tube on magnetic bar for 5min.
⓫. Transfer 100 µL of the cDNA/RNA hybrids to new 1.5 mL tube.
⓬. Repeat step ⑧ ~ ⓫ twice (final volume is 200 µL).
⓭. Concentrate 200 µL of the cDNA/RNA hybrids solutions from step ⓬ to around 40 µL by SpeedVac vacuum concentrator at 37 °C, and collect the solutions from 3 of 1.5 mL tubes to 1 of 1.5 mL tube (total volume is around 120 µL) and concentrate to 40 µL in the 1.5 mL tube at 37°C. The timing is around 2 h.

CRITICAL: **DO NOT DRY UP** the cDNA/RNA hybrids solutions by the concentrating step ⓭. Check the sample volume during the concentration several times and adjust the final volume to 40 µL with RNase/DNase-free when the volume become less than 40 µL.

2 **Oxidation to modify diol group of cap structure**

a. Mix 40 µL of RNA/cDNA hybrid from step **1.1e.** or **1.2g.**, 2 µL of 1 M NaOAc (pH 4.5) and 2 µL of 250 mM $NaIO_4$ in a tube by 10 times pipetting.

NOTE: 1.5 mL tube, 8-Strip PCR tube or16 wells plate can be used depending on how many samples should be prepared.

b. Incubate for 5 min on ice in dark by aluminum foil wrapping.
c. Add 16 µL of 1 M Tris-HCl (pH 8.5) to neutralize the solution and mix well by pipetting.

3

Purification

Add 108 µL (1.8 -folds) of RNACleanXP beads to 60 µL of oxidated RNA/cDNA hybrid from step **2**, mix well by pipetting and then elute the 48 samples mixed RNA/cDNA hybrids by following steps.
①. Incubate at room temperature for 5min.
②. Spin down and set the tube on magnetic bar for 5 min.
③. Discard the supernatant by pipette aspiration.
④. Wash the beads with 200 µL of 70% EtOH
⑤. Discard the 70% EtOH.
⑥. Repeat step ④~ ⑤twice and discarded 70% EtOH completely.
⑦. Add 42 µL of RNase free water and mix by pipetting extensively (more than 60 times) to elute supernatant.
⑧. Incubate at room temperature for 5min.
⑨. Spin down and set the tube on magnetic bar for 5 min.
⑩. Collect 40 µL of the supernatant to new tube.

4

Biotinylation by the coupling reaction to the oxidized RNA/cDNA hybrids

a. Mix 40 µL of purified oxidized RNA/cDNA hybrid from step **3**, 4 µL of 1M NaOAc (pH 6.3) and 4 µL of 100 mM Biotin (long arm) hydrazide by 10 times pipetting.
b. Incubate for 30 min at 40 °C.
c. Add 86.4 µL of RNACleanXP (1.8 -folds) to 44 µL of solution from step b, and perform purification by following step **3** ① ~ ⑩.

NOTE: Biotinylation happens both 5'end of capped RNAs and 3'end of RNA (see Figure 2 at published protocol).

5

RNaseONE treatment to digest RNA of RNA/cDNA hybrids

a. Add 4.5 µL of 10 x RNaseONE buffer and 0.5 µL of RNaseONE to 40 µL of purified biotinylated RNA/cDNA hybrids from step **4** and mix 10 times by pipetting.
b. Incubate for 30 min at 37 °C.
c. Add 81 µL of RNACleanXP (1.8 -folds) to the solution from step **b.** and purify by the following step **3** ①~ ⑩.

CRITICAL: This step is critical to digest uncompleted cDNA synthesis to the 5' end of capped RNAs (See Figure 2 at published protocol).

6 **Dynabeads M-270 Streptavidin beads preparation**

a. Add 30 µL of Dynabeads M-270 Streptavidin to new 1.5ml tube, set on the magnetic bar for 5 min, and discard supernatant.
b. Wash the beads with 30 µL of LiCl buffer and discard supernatant.
c. Repeat step **b.** for 2 times.
d. Resuspend the beads in 35 µL of LiCl buffer.

7 **CapTrap reaction**

a. Add 35 µL of beads from step **6** to 40 µL of RNA/cDNA hybrids from step **5** and mix well by pipetting.
b. Incubate for 15 minutes at 37 °C and mix by pipetting at the time of 7 min.
c. Spin down and set the plate on the magnetic bar for 2 min and discard the supernatant by the pipette aspiration.
d. Add 150 µL of TE wash buffer and mix 60 times by pipetting. Spin down and set the plate on the magnetic bar for 2 min and discard the supernatant.
e. Repeat step **d.** for 3 times.
f. Add 35 µL of Release buffer to the beads and mix 60 times by pipetting.
g. Incubate for 5 min at 95°C for 5min and on ice for 1 min.
h. Spin down and set the tube on the magnetic bar for 2 min.
i. Transfer the supernatant containing CapTrapped cDNA from step **h.** to new 16 well plate.
j. Add 30 µL of Release buffer to the beads and mix well by pipetting, spin down, and set the tube on the magnetic stand for 2 min.
k. Transfer 30 µL of supernatant containing CapTrapped cDNA to new tube.

8 ⏸

RNaseONE and RNaseH reaction to remaining RNAs
a. Add 2.9 µL of release buffer, 2 µL of RNaseONE and 0.1 µL of RNase H to 30 µL of CapTrapped cDNA from step **7** and mix 10 times by pipetting.
b. Incubate at 37°C for 30 min.
c. Add 126 µL of AMPureXP (1.8 -folds) to 33 µL of cDNA from step **b.** and perform purification by following **step 3 ① ~ ⑩.**
d. Dry up 40 µL of purified CapTrapped cDNA by the SpeedVac concentrator at 37°C for around 75 min.
e. Add 4 µL of RNase free water to the dried pellet.

9 **5' Single Strand Linker Ligation**

a. Incubate 4 µL of cDNA at 95 °C for 5 min and put on ice for 2 min.
b. Incubate 4 µL of 5' linker at 55 °C for 5 min and put on ice for 2 min.
c. Mix 4 µL of 5' linker, 4 µL of CapTrapped cDNA from step **a.** and 16 µL of Mighty Mix by pipetting (total volume is 24 µL).
d. Incubate at 16 °C for 16 h (overnight).

10

Purification to remove excess 5’ linkers and linker dimers

a. Add 46 µL of RNase free water and 126 µL of AMPureXP (1.8 -folds) to 24 µL of cDNA from step **9**, mix well by pipetting.
b. Purify the solution by following step **3** ①~ ⑩.
c. Incubate at 95 °C for 5 min and immediately put on ice for 2 min.
d. Add 48 µLof AMPureXP beads (1.2 -folds) to 40 µL of cDNA and mix well by pipetting.
e. Repeat purification by following step **3** ① ~ ⑩.
f. Dry up 40 µL of cDNA by SpeedVac concentrator at 80°C for around 35 min.
g. Add 4 µL of RNase free water to the dried pellet.

CRITICAL: It is important to perform heat inactivation and repeat purification step to remove non-ligated excess linkers during the purification step. Otherwise, these excess linkers can disturb library sequencing quality; excess linkers compete with the library to anneal Illumina flow-cells. In addition, 5’linker-3’linker dimers can be created at the next step **11**.

11 **3’ Single Strand Linker Ligation**

a. Incubate 4 µL of cDNA at 95 °C for 5 min and put on ice for 2 min.
b. Incubate 4 µL of 3’ linker at 65 °C for 5 min and put on ice for 2 min.
c. Mix 4 µL of 3’linker, 4 µL of cDNA from step **a**. and 16 µL of Mighty Mix by pipetting.
d. Incubate at 30 °C for 4 h.

NOTE: When you have more than 2 libraries with same barcode at RT primer and plan to mix libraries at the sequencing step **14**, use different Index at the 3'linker.

12

Purification to remove excess 3’ linkers and linker dimers (final library)

a. Purify the solution from step **11** by following step **11a.-f.**
b. Dissolve the pellet with 10 µL of RNase, DNase-free water.

13

Library QC

Quantify the concentration of library by KAPA Library Quantification Kit.

14 **Sequencing**

a. Mix the following components

Library (2250 amol)	X µL
RNase, DNase-free water	(19-X) µL
2N NaOH	1 µL
Total	20 µL

b. Incubate at room temperature for 5 min.

c. Put the tube on ice and add 20 µL of 1 M Tris-HCl (pH 7.0, pre-chilled) to neutralize.

d. Add 110 µL of HT1 buffer.

e. Transfer 120 µL of the denatured and diluted library to HiSeq2500.

Sequencing kit

- ssCAGE: 50 bp Single Read sequencing.
- LQ-ssCAGE: 50 bp Paired-End sequencing (see Figure 1 for the library structure).
  Read 1: to read sequence information of cDNA
  Read 2: to read sequence information of barcode in RT primer
  Index 1: to read sequence information of index at the 3'linker