USER MANUAL

Single Cell RNA Boost for PacBio MAS-Seq for 10x Single Cell 3’ kit

KIT1018

Contents

Contact Us

If you have any questions, contact Technical Support at support@jumpcodegenomics.com

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Product Overview

Single Cell RNA Boost Kit leverages Cas9 and specifically designed CRISPR guide RNA to target and remove uninformative molecules from next generation sequencing (NGS) libraries. When combined with PacBio® MAS-Seq libraries generated using the MAS-Seq for 10x Single Cell 3' kit, depletion increases informative reads in single cell RNA-seq data and enables discovery of additional isoforms and cell types.

Description	
Assay Time	2.5 Hours
Hands-On Time	45 Minutes
Input	50-100 ng of cDNA • Generated from 10x Genomics Chromium 3' single cell kit (v3.1), standard throughput • 3,000 - 10,000 target cell recovery per sample.
cDNA Fragment Size	500 – 1,500 bp
Method	CRISPR-Cas9 depletion
Designed to deplete	• Reads that do not align to the transcriptome • Ribosomal • Mitochondrial • Non-variable genes Depletion content can be downloaded from Jumpcode website.
Library Prep	PacBio MAS-Seq for 10x Single Cell 3' kit (102-659-600)
Sequencing Platform	PacBio systems

Workflow

Single-Cell RNA Boost for MAS-Seq is a simple 4-step depletion protocol easily inserted at the beginning of the PacBio single-cell MAS-Seq library preparation protocol. The workflow is approximately 2.5 hours in length. After depletion, the user may proceed directly to the MAS-Seq protocol or pause at the safe stopping point at the end of depletion.

The user performs Single Cell RNA Boost depletion using single-cell cDNA material generated from the 10x Chromium Next GEM Single Cell 3' Kit v3.1 (Step 2.4). After the CRISPRclean protocol is complete, the user switches to the first step of the PacBio MAS-Seq protocol [Template Switching Oligo PCR (Step 2.1)] using the depleted cDNA as input material.

Single Cell RNA Boost for PacBio MAS-Seq for 10x Single Cell 3' Kit

1. 10x Chromium Next GEM Single Cell 3' Kit v3.1
2. **Single Cell RNA Boost Kit**
   - **Hands-on time: 45 min | Total time: 2.5 hrs**
   - A. Ribonucleoprotein Complex Formation
   - B. CRISPR Digestion
   - C. Proteinase K Treatment
   - D. Bead Cleanup
   - **Safe stopping point**
3. PacBio MAS-Seq library preparation
4. PacBio sequencing

Kit Contents & Storage

The CRISPRclean Single Cell RNA Boost Kit contains reagents for the depletion of 24 cDNA samples generated using the 10x Chromium Next GEM Single Cell 3' kit (v3.1). The kit contents and storage temperatures are indicated in the tables below.

Compatible Jumpcode products for use in this protocol:

- KIT1018 Single Cell RNA Boost Kit (Human): ASY-1060, ASY-1061

ASY-1060: Single Cell RNA Boost Kit (24 Samples)

Stored at -20°C

Kit contents	Part number	Quantity per box
Nuclease-Free Water	REA1023	2
10x Cas9 Buffer	REA1001	1
RNase Inhibitor	REA1007	1
Cas9	REA1000	1

ASY-1061: Single Cell RNA Boost Kit (24 Samples)

Stored at -80°C

Kit contents	Part number	Quantity per bag
Guide RNA for Single Cell RNA Boost	REA1025	1 tube

Required Materials and Equipment Provided by the User

Type	Item	Supplier
Plastics	0.5 mL and 1.5 mL DNA LoBind Tubes	Eppendorf 022431005, 022431021
	0.2 mL thin wall PCR tubes	General Lab Supplier
	Low-Retention Filtered Sterile Tips (10 µl, 20 µl, and 1000 µl)	General Lab Supplier
Reagents	Chromium Next GEM Single Cell 3' Kit v3.1 *(Single-Cell cDNA Amplified Product)*	10x Genomics 1000268/9
	MAS-Seq for 10X Single Cell 3' Kit	PacBio 102-659-600
	Thermolabile Proteinase K	New England Biolabs P8111S
	Absolute Ethanol, 200 Proof	General Lab Supplier
	Qubit dsDNA HS Assay Kit	ThermoFisher Scientific Q32854
	Agilent High Sensitivity DNA Kit	Agilent Technologies 5067-4627
	Pronex Beads, 10mL	Promega NG2001
	Elution Buffer, 50mL	PacBio 101-633-500
Equipment	Single Channel Pipettes (10 µl, 20 µl, 200 µl, and 1000 µl)	General Lab Supplier
	Multichannel Pipettes (10 µl, 20 µl, and 200 µl)	General Lab Supplier
	Vortex Mixer	General Lab Supplier
	Microcentrifuge	General Lab Supplier
	Magnetic Rack	General Lab Supplier
	Ice Bucket	General Lab Supplier
	Thermal Cycler	General Lab Supplier
	Qubit Fluorometer	ThermoFisher Scientific Q33238
	Agilent 2100 Bioanalyzer System	Agilent Technologies G2939BA

Best Practices

General

- Periodically, optimizations and revisions are made to the kit components and user manual. Therefore, it is important to follow the protocol included with or appropriate for the kit in question. This can be done by comparing the name and version number of the product to the name and version number of the protocol. If further assistance is required in this regard, please contact support@jumpcodegenomics.com
- Room temperature refers to a temperature of 20°–25°C.
- Follow good laboratory practices throughout the protocol, including RNA-safe procedures when handling RNA. Ensure that all pipette tips, microcentrifuge tubes, and other consumables are DNase and RNase-free.
- Use properly calibrated pipettes as low volume transfers are sensitive to pipetting error.
- Use low retention filtered sterile pipette tips throughout the protocol and avoid vortexing to minimize shearing of the DNA.
- Always proceed immediately to the next step when following the protocol. If a pause or stop is necessary, safe stopping points are available during the protocol.

Reagent Handling

- Do not remove Cas9, RNase Inhibitor, and Proteinase K from storage until immediately before use. Maintain the enzymes on ice during reaction setup. Return to -25°C to -15°C immediately after use.
- Do not remove Guide RNA from storage until immediately before use. Maintain on ice during reaction setup. Return to -85°C to -65°C immediately after use.
- Avoid more than 3-4 freeze-thaw cycles when handling the Guide RNA. Prepare small aliquots of the Guide RNA when the kit is first used if it is expected that the user will freeze-thaw the material more than 3-4 times.
- Do not freeze Pronex Beads. Beads should always be stored at 2°C to 8°C. Allow beads to come to room temperature before use. A minimum of 30 minutes at room temperature is recommended before use. Vortex and mix by inversion to ensure homogenous resuspension immediately before each use.

Equipment Handling

- Thermal cycling should be performed with a heated lid except where specified. Thermal cycler(s) must support uniform heating up to a sample volume of 50 µL.
- The magnetic rack used for the protocol should be appropriately designed for the tube(s) in question.

Revision Log

Version	Date	Description
V1.0	December 2022	Release
V1.5	January 2023	Edited for clarity

Input Material

The Single Cell RNA Boost Depletion for PacBio MAS-Seq protocol is optimal with 50 - 100 ng of single-cell cDNA input material. Efficient depletion rates and high-quality single cell data have been generated with single-cell cDNA generated from 3,000 - 10,000 individual cells per sample using the 10x Genomics Chromium Next GEM Single Cell 3' Kit (v3.1) standard throughput workflow. Lower amounts of starting material may result in higher duplication rates, reduced library complexity and other changes to sequencing data quality.

Accurate cDNA quality assessment is recommended to maximize depletion efficiency and the efficiency of downstream steps. Validate input cDNA using a fluorometric based method, such as a Qubit Fluorometer. Evaluate quality using an automated electrophoresis method, such as a Bioanalyzer System with a High Sensitivity DNA reagent kit.

Protocol

Single Cell RNA Boost for MAS-Seq

Hands-on time: 45 min | Total time: 2.5 hrs

Reagents preparation

Item	Storage	Handling
Chromium 3' single cell cDNA (50-100ng)	-25°C to -15°C	If frozen, thaw on ice. Finger-tap mix and spin down. Keep on ice. Return to freezer immediately after use.
10X Cas9 Buffer		
RNase Inhibitor		
Cas9		
Proteinase K		
Single-Cell Boost Guide RNA	-85°C to -65°C	
Nuclease-Free Water	-25°C to -15°C	Bring to room temperature. Mix all components by inversion and vortexing.
PacBio Elution Buffer		
Pronex	2°C to 8° C	
80% Ethanol	Room Temperature	Prepare fresh.

* Program thermal cycler(s) prior to beginning the protocol for the first time.

Step A: Ribonucleoprotein (RNP) Complex Formation

1. Dilute the prepared 10x Chromium 3' single-cell cDNA with Nuclease-Free Water to 50-100ng in 15µL. Set the diluted cDNA aside for use in the "Step B: CRISPR Digestion" section of the protocol (below).
2. Combine the following reagents in the order listed below in a 0.2 mL tube:

"RNP Complex Formation" Reaction Mix	Volume
10X Cas9 Buffer	1.0 µL
RNase Inhibitor	1.0 µL
Cas9	2.3 µL
Single Cell Boost Guide RNA	4.0 µL
Total Volume	**8.3 µL**

3. Mix by pipetting up and down 10 times. Briefly spin the contents in a microcentrifuge to collect the liquid at the bottom of the tube.
4. Incubate the reaction at room temperature for 10 minutes. This is the CRISPR ribonucleoprotein complex (RNP).
5. Proceed immediately to the next section **CRISPR Digestion**.

Step B: CRISPR Digestion

1. Add the following reagents in the order listed below to the 0.2mL tube containing the RNP (Step A.4)

CRISPR digestion reaction mix	Volume
10X Cas9 Buffer	1.5 µL
Single-Cell cDNA (50-100ng) (from Step A.1)	15 µL
Total Volume	**~25 µL**

2. Mix by pipetting up and down 10 times. Briefly spin the contents in a microcentrifuge to collect the liquid at the bottom of the tube.
3. Incubate the reaction for 1 hour at 37ºC in a thermal cycler with the heated lid set to ≥50ºC, then hold at 4ºC.
4. Proceed immediately to the next section **Proteinase K Treatment**.

Step C: Proteinase K Treatment

1. Add 1 µL of Proteinase K to the CRISPR digestion reaction from step B.
2. Mix by pipetting up and down 10 times. Briefly spin the contents in a microcentrifuge to collect the liquid at the bottom of the tube.
3. Place the tube in a thermal cycler with a heated lid set to ≥75ºC. Run the following program:

Temp	Cycle Time
37ºC	15 min
65ºC	5 min
4ºC	HOLD

4. Proceed to the next section **Bead Cleanup**.

Step D: Bead Cleanup

1. Remove the Pronex Beads and Elution Buffer from storage and bring them to room temperature. Prepare fresh 80% ethanol.

> **Note:** Pronex Beads should be placed on the laboratory bench for at least 30 minutes to bring to room temperature.

2. Add 25 µL of Nuclease-Free Water to the reaction.
3. Add 75 µL (1.5X) of thoroughly resuspended Pronex Beads to the reaction. Mix by pipetting up and down 10 times. Briefly spin the sample in a microcentrifuge to collect the liquid at the bottom of the tube.
4. Incubate the sample at room temperature for 10 minutes.
5. Place the tube containing the sample on a magnetic stand until the solution is clear (3-5 minutes). Discard the supernatant without disturbing the beads.
6. Add 200 µL of freshly prepared 80% ethanol to the tube while still on the magnetic stand. Wait 30 seconds, then remove and discard the ethanol. Do not remove the tube from the magnetic stand while performing the ethanol wash.
7. Repeat the previous wash step (Step 6).

8. Briefly spin the tube in a microcentrifuge. Return it to the magnetic stand and remove any traces of ethanol. Do not allow the beads to air dry. Air drying the beads will reduce final yields.
9. Remove the tube from the magnetic stand and immediately add 17 µL of PacBio Elution Buffer 101-633-500 to the beads. Slowly pipette mix 10 times to resuspend. Briefly spin the sample in a microcentrifuge to collect the liquid at the bottom of the tube.
10. Place the tube on the laboratory bench for 5 minutes.
11. Return the tube to the magnetic stand. Allow the solution to clear and transfer the supernatant containing the depleted cDNA to a new 0.2 mL tube.
12. Measure the DNA concentration of the sample using a dsDNA fluorescent dye method, such as a Qubit fluorometer. Example of Bioanalyzer® graphs with expected library size distributions are provided below:

cDNA Validation

Jumpcode Genomics recommends assessing the quality, quantity, and size distribution of the depleted single-cell cDNA on an Agilent Bioanalyzer 2100 instrument or equivalent before proceeding to the PacBio MAS-Seq protocol for best results. CRISPRclean-depleted cDNA samples have a similar fragment profile to those of standard 10x Chromium single-cell derived cDNA (500 – 1,500 bp).

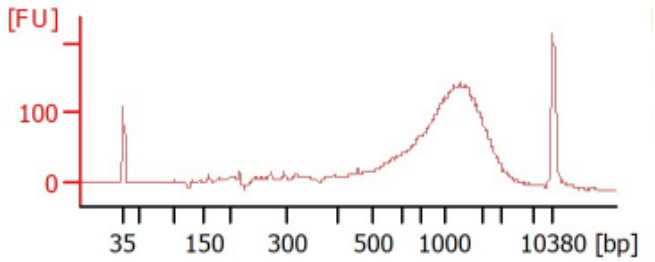

Figure 1. Example of an expected cDNA size distribution using 50-100 ng of PBMC single-cell cDNA for CRISPR-Cas9 depletion. An aliquot of the depleted cDNA was loaded on the Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit.

SAFE STOPPING POINT

If stopping, store the samples at -25°C to -15°C overnight. Alternatively, proceed directly to MAS-Seq library preparation.

Next Steps: MAS-Seq Library Preparation

- Proceed to the beginning of the PacBio "Preparing MAS-Seq Libraries Using MAS-Seq for 10x Single Cell 3' Kit" protocol (PacBio, 102-678-600). The depleted cDNA should be used as input material for the Template Switching Oligo (TSO) PCR (Step 2.1).
- Depletion reduces the amount of amplifiable cDNA in the sample. Thus, 1-2 PCR cycles should be added to the TSO PCR to compensate for CRISPRclean Single Cell RNA Boost depletion. Refer to the table below for recommended TSO PCR cycles.

MAS-Seq Depleted cDNA Input Material	Recommended MAS-Seq TSO PCR Cycles	Target Yield:
50 – 75 ng	4-5*	150ng – 1,000ng
25 – 49 ng	5-6*	
15 – 24 ng	6-7*	

* The number of PCR cycles are guidelines. PCR cycles may have to be adjusted further based on sample type and quality.

- After TSO PCR, follow the MAS-Seq protocol and manufacturer recommendations without any further adjustments.