cDNA synthesis on the Mantis platform with the SMART-Seq® Single Cell Kit

I. Introduction

This protocol describes how to generate cDNA from single cells in a 96-well plate using the SMART-Seq Single Cell Kit (Cat. # 634472) on the Mantis platform.

II. General Considerations

- A 96-reaction kit provides sufficient reagents to perform this protocol. To ensure that reagents are available in sufficient quantities for performing 96 full-volume reactions, please adhere to these priming and predispensing volumes for the LV and HV chips:
  - Priming volume for LV chip = 5.4 µl
  - Predispense volume for LV chip = 1.2 µl
  - Priming volume for HV chip = 12 µl
  - Predispense volume for HV chip = 5 µl
- Use a new chip for every addition. Wash all LV and HV chips at the end of the day by following the corresponding washing protocols.
- Be careful to avoid bubbles throughout the protocol.
- For additional information about the SMART-Seq Single Cell Kit, please refer to the SMART-Seq Single Cell Kit User Manual.

III. Protocol

A. Preparation of a 96-well plate for cell sorting

1. To make CDS Sorting Solution (CSS), begin by manually aliquoting 114 µl of 10X Lysis Buffer into a 1.5-ml nuclease-free tube.
2. Add 6 µl of RNase Inhibitor (40 U/µl), mix by vortexing gently, and quickly spin down the reagents.
3. Add 120 µl of 3′ SMART-Seq CDS Primer II A.
4. Add 1,260 µl of Nuclease-Free Water (total volume = 1,500 µl).
5. Vortex and briefly spin down the mixture.
6. Fill each of two 1,000-µl pipette tips with 750 µl of CSS and load each tip onto an LV Mantis chip (Position 1 and Position 2). For each 1,000-µl pipette tip, program the Mantis to add 6.3 µl of CSS to each well of the plate (such that each well will receive two 6.3 µl aliquots of CSS).

   NOTE: In this protocol, we are assuming that FACS sorting of the cells will not change the volume of liquid in the plate wells. If your sorter dispenses a nonnegligible amount of sheath fluid, adjust the volume of the CSS mix by reducing the amount of Nuclease-Free Water to maintain a total volume of 12.6 µl per well.

SAFE STOPPING POINT: The plate can be stored at –20°C overnight.

B. Cell sorting

1. Sort the cells directly into the plate wells containing CSS.
2. After sorting is complete, seal the plate and spin it down briefly to bring the cells to the bottom of the wells.
3. Immediately place the plate on dry ice for about 5 min before transferring it to a –80°C freezer.

SAFE STOPPING POINT: The plate can be stored at –80°C overnight.

C. Oligo annealing

1. Remove the plate from the –80°C freezer, let it thaw for about 1 min, and vortex briefly. Then spin down the plate to collect the contents at the bottom of the wells.
2. Transfer the plate to a preheated thermal cycler and incubate at 72°C for 3 min.
3. Following the 3-min incubation, place the plate on ice for 2 min.
4. While the plate is on ice, prepare the RT Master Mix (Section D, below).

D. Preparation of RT Master Mix

1. Combine the following reagents in the order shown in a 1.5-ml nuclease-free tube at room temperature to prepare sufficient RT Master Mix for 96 reactions. Be sure to add SMARTScribe™ II Reverse Transcriptase last and just before use. Mix gently by pipetting up and down.

Component	1X (µl)	96X (µl)*
SMART-Seq sc First Strand Buffer	4	424
SMART-Seq sc TSO	1	106
RNase Inhibitor (40 U/µl)	0.5	53
SMARTScribe II Reverse Transcriptase (200 U/µl)	2	212
Total volume	7.5	795

*This column specifies the amount of each component needed for 96 reactions (one plate's worth), including additional volume to account for pipetting inaccuracies.

2. Fill a 1,000-µl pipette tip with 795 µl of RT Master Mix and load the tip onto an LV Mantis chip (Position 3). Program the Mantis to add 7.5 µl of the RT Master Mix to each well from the chosen position.
3. Seal the plate with sealing tape, spin down briefly to collect reagents, and gently vortex the plate 3–5 times. Then, spin the plate at 2,000 rpm for 30–60 sec.

E. First-strand synthesis

1. Transfer the plate from Section D to a preheated thermal cycler and run the following program:

   42°C 180 min
   70°C 10 min
   4°C hold

2. Spin down the plate at 2,000 rpm for 30–60 sec after the program is done.

SAFE STOPPING POINT: The plate can be stored at 4°C overnight.

F. PCR amplification of cDNA

1. Combine the following reagents in the order shown in a 15-ml conical tube on ice to prepare sufficient PCR Master Mix for 96 reactions. Be sure to add SeqAmp™ DNA Polymerase last and just before use. Mix gently by pipetting up and down.

Component	1X (µl)	96X (µl)*
SeqAmp CB PCR Buffer (2X)	25	2,700
PCR Primer	1	108
SeqAmp DNA Polymerase	1	108
Nuclease-Free Water	3	324
Total volume	30	3,240

*This column specifies the amount of each component needed for 96 reactions (one plate's worth), including additional volume to account for pipetting inaccuracies.

2. Fill each of three 1,000-µl pipette tips with 1,080 µl of PCR Master Mix and load each tip onto an HV Mantis chip (Positions 4–6). For each 1,000-µl pipette tip, program the Mantis to add 10 µl of PCR Master Mix to each well of the plate from Section E (such that each well will receive three 10-µl aliquots of PCR Master Mix).
3. Seal the plate with sealing tape, spin down briefly to collect reagents, and gently vortex the plate 3–5 times. Then spin the plate at 2,000 rpm for 30–60 sec.
4. Transfer the plate to a preheated thermal cycler and run the following program:

95°C 1 min
X cycles*:
98°C 10 sec
65°C 30 sec
68°C 3 min
72°C 10 min
4°C hold

*Use the following table as a guideline to help determine the optimal number of PCR cycles for your input:

Cell type	Approximate RNA equivalent (per cell)	Typical # of PCR cycles
K-562 or HEK293 cells	10–15 pg	17
PBMCs	1–5 pg	20
Jurkat cells	5 pg	17
Lymphoblastoid cells	2–15 pg	17–19

5. cDNA can be purified manually using Agencourt AMPure XP beads as indicated in the next section (Section G).

SAFE STOPPING POINT: The plate can be stored at 4°C overnight.

G. Purification of amplified cDNA using the Agencourt AMPure XP Kit

1. Before each use, bring bead aliquots to room temperature for at least 30 min and mix well to disperse.
2. Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
3. You will need a magnetic separation device that accommodates a 96-well plate.
4. Vortex AMPure XP beads until evenly mixed, then add 40 µl of AMPure XP beads to each sample.
5. Mix thoroughly by vortexing or pipetting the entire volume up and down at least 10 times.
6. Incubate at room temperature for 8 min to let the cDNA bind to the beads.
7. Briefly spin the samples to collect the liquid from the sides of the sample wells. Place the samples on the magnetic separation device for ~5 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
8. While the samples are on the magnetic separation device, pipette the supernatant and discard.
9. Keep the samples on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 sec and carefully pipette the supernatant containing contaminants. cDNA will remain bound to the beads during the washing process.
10. Repeat the ethanol wash (Step 9) once.
11. Briefly spin the samples to collect the liquid from the sides of the sample wells. Place the samples on the magnetic separation device for 30 sec, then remove all the remaining ethanol with a pipette.
12. Place the samples at room temperature for approximately 2–2.5 min until the pellet is no longer shiny, but before a crack appears.
13. Once the beads are dry, add 17 µl of Elution Buffer to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads.
14. Incubate at room temperature for 2 min to rehydrate.
15. Briefly spin the samples to collect the liquid from the sides of the sample wells. Place the samples back on the magnetic separation device for 1 min or longer, until the solution is completely clear.
16. Transfer clear supernatant containing purified cDNA from each well to a nuclease-free, low-adhesion tube. Label each tube with sample information and store at–20°C until ready for library preparation.

    SAFE STOPPING POINT: The samples can be stored at –20°C for several months.

17. Refer to the SMART-Seq Single Cell Kit User Manual for quantification and library preparation options.

    NOTE: For information on the Nextera library preparation protocol for the Mantis, please contact technical support at technical_support@takarabio.com.

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