Quarter-volume reaction cDNA synthesis on a 384-well plate on the Mantis Liquid Handler with the SMART-Seq® Single Cell Kit

I. Introduction

This protocol describes how to generate cDNA from single cells in a 384-well plate using the SMART-Seq Single Cell Kit (SSsc kit, Cat. # 634472) on the Mantis automated liquid handler in a quarter-volume reaction.

II. General Considerations

- The reagent volumes specified below were precisely calculated for this protocol using one SSsc kit to perform 384 reactions. To ensure that reagents are available in sufficient quantities for performing 384 quarter-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 384-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 to the 10X reaction buffer (total volume 60 µl).
4. Add 1,260 µl of Nuclease-Free Water (total volume = 1,500 µl).
5. Vortex and briefly spin down the mixture
6. Fill two 1,000-µl pipette tips with 750 µl each of CSS and load the tips onto an LV Mantis chip (Positions 1 and 2). Program the Mantis liquid handler to add 3.2 µl of CSS to each well from the chosen positions.

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 non-negligible 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 3.2 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, put 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 384 reactions. Be sure to add SMARTScribe™ II Reverse Transcriptase last and just before use. Mix gently by pipetting up and down.

Component	1X (µl)	384X (µl)*
SMART-Seq sc First Strand Buffer	1	429
SMART-Seq sc TSO	0.25	107.25
RNase Inhibitor (40 U/µl)	0.125	53.625
SMARTScribe II Reverse Transcriptase (200 U/µl)	0.5	214.5
Total volume	1.875	804.375

*This column specifies the amount of each component needed for 384 reactions (one plate's worth), including additional volume to account for pipetting inaccuracies.

2. Fill a 1,000-µl pipette tip with 804 µl of RT Master Mix and load the tip onto an LV Mantis chip (Position 3). Program the Mantis liquid handler to add 1.9 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.
4. 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. After the program is done, spin down the plate at 2,000 rpm for 30–60 sec.

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 2.0-ml tube on ice to prepare sufficient PCR Master Mix for 384 reactions. Be sure to add SeqAmp™ DNA Polymerase last and just before use. Mix gently by pipetting up and down.

Component	1X (µl)	384X (µl)*
SeqAmp CB PCR Buffer (2X)	10	4,300.8
PCR Primer	0.25	107.5
SeqAmp DNA Polymerase	0.40	172
Nuclease-Free Water	4.35	1,870.8
Total volume	15	6,451.1

*This column specifies the amount of each component needed for 384 reactions (one plate's worth), including additional volume to account for pipetting inaccuracies.

2. Fill six 1,000-µl pipette tips with 1,030 µl of PCR Master Mix and load each tip onto an HV Mantis chip (Positions 2–7). For each 1,000-µl pipette tip, program the Mantis liquid handler to add 15 µl of PCR Master Mix to each well of the plate from Section E.
3. Seal the plate with sealing tape, spin down briefly to collect reagents, and gently vortex the plate 3–5 times.
4. Spin the plate at 2,000 rpm for 30–60 sec.
5. 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 to 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

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

G. Purification of amplified cDNA using NucleoMag NGS Clean-up and Size Select

cDNA can be purified using NucleoMag NGS Clean-up and Size Select (Takara Bio, 744970.5, 744970.50, or 744970.500).

NOTE: NucleoMag NGS Clean-up and Size Select bead suspension may be substituted with an equal volume of Agencourt AMPure XP beads.

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 100 µl per sample.
3. You will need a magnetic separation device that accommodates a 384-well plate.
4. Vortex NucleoMag NGS Clean-up and Size Select beads until evenly mixed, then add 16 µl to each sample.
5. Mix thoroughly by vortexing or gently 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 50 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.

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