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HuBMAP UF TMC - Single Cell RNA Seq (10x Genomics) Library Preparation and Sequencing

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1 Works for me dx.doi.org/10.17504/protocols.io.bffvjjn6

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ABSTRACT

This protocol is for the generation of 3' (chemistry version 3) cDNA libraries from individual cells via droplet generation utilizing the 10x Genomics Chromium Controller and associated reagents. Paired end libraries are then sequenced on Illumina instruments.

GUIDELINES

Work quickly, treat cell suspensions gently to avoid damage, and maintain all solutions on ice.

Preparation of cryopreserved cell samples

1 **Preparation of cryopreserved cell samples for 10x Genomics GEM generation:**

a. Prepare 50 ml PBS 0.04% BSA, aliquot 9 ml into a 15 ml conical tube. Maintain PBS 0.04% BSA aliquot and stock tube on ice.
b. Transfer cell vial from liquid nitrogen storage to wet ice and proceed immediately to 37°C water bath.
c. Submerge cryovial in water bath above fill line, agitate vial until sample is liquid.
d. Transfer vial contents to 15 ml conical with PBS 0.04% BSA. Cap conical tube and invert gently to mix. Centrifuge, 350gx5 min at 8 C. Decant supernatant.
e. Gently resuspend cell pellet and add 10 ml cold PBS 0.04% BSA. Centrifuge, 350gx5 min at 8°C.Decant supernatant.
f. Repeat previous step for a total of three washes.
g. Resuspend pellet in 1 ml PBS 0.04% BSA. Strain suspension through a Flowmi tip strainer into a 1.5 ml microcentrifuge tube on ice.
h. Take a 20ul aliquot for counting on a Nexcelom Cellometer.
i. Add 20ul of Viastain AO/PI to aliquot and mix thoroughly with pipette. Add 20ul to counting slide and count with “immune cells low RBC” with dilution factor 2.0. Record total yield and viability in case worksheet.
j. Verify cell viability is >80% with minimal debris. Proceed to encapsulation using the 10x Genomics Chromium Controller and appropriate reagents.

GEM Generation & Barcoding

2 Preparation of Single Cell Master Mix:

a. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.
b. Add 33.4 µl Master Mix into each tube of a PCR 8-tube strip on ice.

3 Load Chromium Chip B:

a. Assemble Chromium Chip B in a 10x Chip Holder.
b. Dispense 50% Glycerol Solution into Unused Chip Wells
i. 75 µl to unused wells in row labeled 1.
ii. 40 µl to unused wells in row labeled 2.
iii.280 µl to unused wells in row labeled 3.
c. Prepare Master Mix + Cell Suspension: Refer to the Cell Suspension Volume Calculator Table and add the appropriate volume of nuclease-free water and corresponding volume of single cell suspension to Master Mix for a total of 80 µl in each tube. Gently pipette mix the single cell suspension before adding to the Master Mix.
d. Load Row Labeled 1: Gently pipette mix the Master Mix + Cell Suspension and using the same pipette tip,

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dispense 75 µl Master Mix + Cell Suspension against the side of each well in row labeled 1.

e. Prepare Gel Beads: Snap the Gel Bead strip into a 10x Vortex Adapter. Vortex 30 sec. Remove the Gel Bead strip and flick in a sharp, downward motion to ensure maximum recovery. Confirm there are no bubbles at the bottom of the tubes and liquid levels look even.

f. Load Row Labeled 2: Puncture the foil seal of the Gel Bead tubes. Slowly aspirate 40 µl Gel Beads. Dispense against the side of each well in row labeled 2.

g. Load Row Labeled 3: Dispense 280 µl Partitioning Oil against the side of each well in row labeled 3 by pipetting two aliquots of 140 µl from a reagent reservoir. Failure to add Partitioning Oil can damage the Chromium Controller.

h. Attach 10x Gasket: Align the notch with the top left-hand corner. Ensure the gasket holes are aligned with the wells. Avoid touching the smooth gasket surface. DO NOT press down on the gasket.

4 Run the Chromium Controller:

a. Press the eject button on the Controller to eject the tray.

b. Place the assembled chip with the gasket in the tray. Press the button to retract the tray.

c. Confirm the Chromium Single Cell B program on screen. Press the play button.

d. At completion of the run (~8.5 min), the Controller will chime. Immediately proceed to the next step.

5 Transfer GEMS:

a. Place a tube strip on ice.

b. Press the eject button of the Controller and remove the chip.

c. Discard the gasket. Open the chip holder. Fold the lid back until it clicks to expose the wells at 45 degrees. Ensure that the partitioning oil from the wells does not spill when exposing the wells.

d. Check the volume in rows 1-3. Abnormally high volume in any well indicates a clog.

e. Slowly aspirate 100 µl GEMs from the lowest points of the Recovery Wells in the top row without creating a seal between the tips and the bottom of the wells.

f. Withdraw pipette tips from the wells. GEMs should appear opaque and uniform across all channels. Excess Partitioning Oil (clear) in the pipette tips indicates a potential clog.

g. Over the course of ~20 sec, dispense GEMs into the tube strip on ice with the pipette tips against the sidewalls of the tubes.

h. If multiple chips are run back-to-back, cap/ cover the GEM-containing tube strip and place on ice for no more than 1 h.

6 GEM-RT Incubation:

a. Incubate in a thermal cycler with the following protocol.

b. Store at 4°C for up to 72 h or at −20°C for up to a week, or proceed to the next step.

Post GEM-RT Cleanup & cDNA Amplification

7 Post GEM-RT Cleanup - Dynabeads

a. Add 125 µl Recovery Agent to each sample at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait 2 min.

b. Slowly remove and discard 125 µl Recovery Agent/Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate any aqueous sample.

c. Prepare Dynabeads Cleanup Mix.

d. Vortex and add 200 µl to each sample. Pipette mix 10x (pipette set to 200 µl).

e. Incubate 10 min at room temperature. Pipette mix again at ~5 min after start of incubation to resuspend settled beads.

f. Prepare Elution Solution I. Vortex and centrifuge briefly.

g. At the end of 10 min incubation, place on a 10x Magnetic Separator•High position (magnet•High) until the solution clears.

h. Remove the supernatant.

i. Add 300 µl 80% ethanol to the pellet while on the magnet. Wait 30 sec.

j. Remove the ethanol.

k. Add 200 µl 80% ethanol to pellet. Wait 30 sec.

l. Remove the ethanol.

m.Centrifuge briefly. Place on the magnet•Low.

n. Remove remaining ethanol. Air dry for 1 min.

o. Remove from the magnet. Immediately add 35.5 µl Elution Solution I.

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p. Pipette mix (pipette set to 30 µl) without introducing bubbles.
q. Incubate 2 min at room temperature.
r. Place on the magnet•Low until the solution clears.
s. Transfer 35 µl sample to a new tube strip.

8 cDNA Amplification:
a. Prepare cDNA Amplification Mix on ice. Vortex and centrifuge briefly.
b. Add 65 µl cDNA Amplification Reaction Mix to 35 µl sample.
c. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.
d. Incubate in a thermal cycler with the following protocol.
e. Store at 4°C for up to 72 h or at −20°C for ≤1 week, or proceed to the next step.

9 cDNA Cleanup - SPRIselect
a. Vortex to resuspend the SPRIselect reagent. Add 60 µl SPRIselect reagent (0.6X) to each sample and pipette mix 15x (pipette set to 150 µl).
b. Incubate 5 min at room temperature.
c. Place on the magnet•High until the solution clears.
d. Remove the supernatant.
e. Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
f. Remove the ethanol.
g. Repeat steps e and f for a total of 2 washes.
h. Centrifuge briefly and place on the magnet•Low.
i. Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.
j. Remove from the magnet. Add 40.5 µl Buffer EB. Pipette mix 15x.
k. Incubate 2 min at room temperature.
l. Place the tube strip on the magnet•High until the solution clears.
m.Transfer 40 µl sample to a new tube strip. STOP
n. Store at 4°C for up to 72 h or at −20°C for up to 4 weeks, or proceed to the next step.

10 cDNA QC & Quantification:
a. Run 1 µl of sample (Dilution Factor 1:10) on an Agilent Bioanalyzer High Sensitivity chip

3' Gene Expression Library Construction

11 Fragmentation, End Repair & A-tailing:
a. Prepare a thermal cycler with the following incubation protocol.
b. Vortex Fragmentation Buffer. Verify there is no precipitate.
c. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly.
d. Transfer ONLY 10 µl purified cDNA sample from cDNA Cleanup (step 2.3n) to a tube strip.
e. Add 25 µl Buffer EB to each sample.
f. Add 15 µl Fragmentation Mix to each sample.
g. Pipette mix 15x (pipette set to 35 µl) on ice. Centrifuge briefly.
h. Transfer into the pre-cooled thermal cycler (4°C) and press “SKIP” to initiate the protocol.

12 Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect:
a. Vortex to resuspend SPRIselect reagent. Add 30 µl SPRIselect (0.6X) reagent to each sample. Pipette mix 15x (pipette set to 75 µl).
b. Incubate 5 min at room temperature.
c. Place on the magnet•High until the solution clears. DO NOT discard supernatant.
d. Transfer 75 µl supernatant to a new tube strip.
e. Vortex to resuspend SPRIselect reagent. Add 10 µl SPRIselect reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 80 µl).
f. Incubate 5 min at room temperature.
g. Place on the magnet•High until the solution clears.
h. Remove 80 µl supernatant. DO NOT discard any beads.
i. Add 125 µl 80% ethanol to the pellet. Wait 30 sec.

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j. Remove the ethanol.
k. Repeat steps i and j for a total of 2 washes.
l. Centrifuge briefly. Place on the magnet•Low until the solution clears. Remove remaining ethanol. DO NOT over dry to ensure maximum elution efficiency.
m. Remove from the magnet. Add 50.5 µl Buffer EB to each sample. Pipette mix 15x.
n. Incubate 2 min at room temperature.
o. Place on the magnet•High until the solution clears.
p. Transfer 50 µl sample to a new tube strip.

13 Adaptor Ligation:
a. Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly.
b. Add 50 µl Adaptor Ligation Mix to 50 µl sample. Pipette mix 15x (pipette set to 90 µl). Centrifuge briefly.
c. Incubate in a thermal cycler with the following protocol.

14 Post Ligation Cleanup – SPRIselect:
a. Vortex to resuspend SPRIselect Reagent. Add 80 µl SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 µl).
b. Incubate 5 min at room temperature.
c. Place on the magnet•High until the solution clears.
d. Remove the supernatant.
e. Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
f. Remove the ethanol.
g. Repeat steps e and f for a total of 2 washes.
h. Centrifuge briefly. Place on the magnet•Low.
i. Remove any remaining ethanol. Air dry for 2 min. DO NOT exceed 2 min as this will decrease elution efficiency.
j. Remove from the magnet. Add 30.5 µl Buffer EB. Pipette mix 15x.
k. Incubate 2 min at room temperature.
l. Place on the magnet•Low until the solution clears.
m. Transfer 30 µl sample to a new tube strip.

15 Sample Index PCR:
a. Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x Sample Index name (PN-220103 Chromium i7 Sample Index Plate well ID) used.
b. Prepare Sample Index PCR Mix.
c. Add 60 µl Sample Index PCR Mix to 30 µl sample.
d. Add 10 µl of an individual Chromium i7 Sample Index to each well and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.
e. Incubate in a thermal cycler with the following protocol.
f. Store at 4°C for up to 72 h or proceed to the next step.

16 Post Sample Index PCR Double Sided Size Selection – SPRIselect:
a. Vortex to resuspend the SPRIselect reagent. Add 60 µl SPRIselect Reagent (0.6X) to each sample. Pipette mix 15x (pipette set to 150 µl).
b. Incubate 5 min at room temperature.
c. Place the magnet•High until the solution clears. DO NOT discard supernatant.
d. Transfer 150 µl supernatant to a new tube strip.
e. Vortex to resuspend the SPRIselect reagent. Add 20 µl SPRIselect Reagent (0.8X) to each sample. Pipette mix 15x (pipette set to 150 µl).
f. Incubate 5 min at room temperature.
g. Place the magnet•High until the solution clears.
h. Remove 165 µl supernatant. DO NOT discard any beads.
i. With the tube still in the magnet, add 200 µl 80% ethanol to the pellet. Wait 30 sec.
j. Remove the ethanol.
k. Repeat steps i and j for a total of 2 washes.
l. Centrifuge briefly. Place on the magnet•Low. Remove remaining ethanol.

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m. Remove from the magnet. Add 35.5 µl Buffer EB. Pipette mix 15x.
n. Incubate 2 min at room temperature.
o. Place on the magnet•Low until the solution clears.
p. Transfer 35 µl to a new tube strip. STOP
q. Store at 4°C for up to 72 h or at −20°C for long-term storage.

17 Post Library Construction QC:
a. Run 1 µl sample at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip. Determine the average fragment size from the Bioanalyzer trace. This will be used as the insert size for library quantification.

18 Preparation for sequencing
Calculate library molarity and dilute all libraries to equimolar concentration. Pool libraries for sequencing with a target of 50,000 reads per cell.

19 Sequencing recommendations
Dilute the final library pool to 5 nM, quantify by qPCR, and sequence on an Illumina instrument (NovaSeq 6000) as per manufacturer's recommendations. (Loading concentration = 170 pM, 1% PhiX spike-in)

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