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Multiplexed droplet-based single cell RNA Seq (mdroscRNA Seq)

Abstract: Using this technology, we will generate single-cell gene expression data on multiple samples in a single run. First, cells from individual kidney biopsies will be isolated using an optimized method. In the next step, the cells from different individuals will be pooled. The pooled cells will be subjected to RNA Seq. After the RNA Seq run, the data from multiple individuals will be demultiplexed using a tool called "Demuxlet" [1] . This technology will add value to KPMP goals in mapping single cell trancriptome of kidney tissue at a reduced price because of multiplexing ability. In addition to this, using the method multiple samples could be used to cross validate single cell and single nuclear RNA Seq which are integral technologies planned for KPMP.

[A] Cryopreservation for kidney biopsies and Cell Isolation

a. Freezing

1. Wipe HypoThermosol bottle with 70% ethanol prior to use.
2. Add 1 mL HypoThermosol to a 1.8 mL sterile cryovial.
3. Place the cryovial with HypoThermosol on ice.
4. Add the renal biopsy specimen to the cryovial with HypoThermosol as soon as possible to avoid drying out and contamination.
5. Fill the cryovial with HypoThermosol to the rim so that the entire specimen is submerged, and cap.
6. Keep upright and at 4°C (on ice) between the collection step and the cryopreservation step. Cryopreservation should occur as soon as possible following collection. Do not freeze the renal tissue and do not leave at room temperature.
7. Wipe CryoStor CS10 bottle with 70% ethanol prior to use.
8. Add 1 mL CryoStor CS10 to a second sterile 1.8 mL cryovial
9. Place the cryovial with CryoStor on ice.
10. Transfer the renal biopsy specimen to the cryovial with CryoStor CS10 as soon as possible to avoid drying out and contamination.
11. Keep the cryovial upright and on ice for 20-30 minutes. Do not freeze or leave at room temperature.
12. Place the cryovial in a Mr. Frosty freezing containing with isopropanol.
13. Place the Mr. Frosty container in the -80°C freezer and record the time.
14. After 15 minutes, slap the Mr. Frosty container to facilitate nucleation.
15. Transfer the cryovials from the Mr. Frosty to the liquid N2 after 24 hours but no longer than 7 days.

b. Thawing

1. The cryovial was rapidly warmed in 37 water bath until thawed.
2. The sample was then poured into a well of 12-well plate and rinsed in a second well containing RPMI.
3. The tissue was incubated for 10 minutes at RT.
4. Transfer the tissue to the 1.5 ml tube containing Liberase TL 0.25mg/ml and incubate for 30 min. 37 tissue culture incubator. Every 10 min. tap the tube and pipet up and down with p 1000 pipet with cut end.

5. Transfer the liquid into 70microns mesh (put over 50 ml tube).
6. Wash with 5 ml of media (this time with 10% FBS to stop digestion)
7. Spin 470 x g 5 min.
8. Resuspend cells in PBS and count.

   Note: For the downstream analysis cell viability of expected to be >50%. Cells with <50% viability are not considered suitable for downstream use. We require at least 10,000 cells per samples for this.

9. Spin cells 470 x g 5 min and freeze in FBS/10% DMS0.
10. Place them in -80°C in Mr. Frosty, next day transfer into N2.

c. Dissociation and Isolation of Cells

1. Before biopsy arrives in lab, prepare a working concentration of 2.5 mg/mL of Liberase TL enzyme (Millipore Sigma, Cat no. 05401020001).
2. Add 100 µL of enzyme and 900 µL of room temperature serum free DMEM media to 1.5 mL microcentrifuge tube

   Note: Reconstituted enzyme should be aliquoted and stored at -80°C until ready for use

3. Once tissue arrives in lab record sample ID and size of biopsy (if available please take photo of tissue next to a ruler)

   Note: Most biopsy samples average 15 mm x 1 mm x 1 mm

4. Carefully add tissue to enzyme solution (250 µg/mL Liberase TL and 5 µl of DNase I - Roche, #04536282001, 100 U/ml final concentration- in serum free DMEM)
5. Incubate tissue at 37°C for 30 min in a CO2 incubator, gently tapping the tube every 10 min.

   Note: When adding tube to incubator please take caution to clean tube with Ethanol each time to avoid contamination in the incubator.

6. Place a 70 µM cell strainer filter (Corning Falcon, Cat no. 352350) in a 50 mL conical tube and gravity filter the supernatant containing cells through the strainer
7. Wash the 1.5 mL microcentrifuge tube 3 to 5 times with 1 mL of DMEM media with serum, each time adding the media through the cell strainer

   Note: This step helps to bring any additional cells left in the microcentrifuge tube or the cell strainer into the sample.

8. Remove the cell strainer from the conical tube and centrifuge cells at 470 x g for 5 min at room temperature
9. Count cells on ViCell Cell Counter (Beckman Coulter, Cat no. 731050) using a 1:10 dilution
10. Record cell viability and viable cell count

    QA/QC: For a successful downstream application we require minimum of 10,000 cells with >50% viability.

11. Centrifuge the cells at 470 x g for 5 min at room temperature and carefully remove supernatant so as not to disrupt the cells.
12. Freeze cells in 1 mL of freezing media (10% DMSO+FBS) in a cryovial and proceed to 10X genomics partitioning for single cell analysis or store in liquid nitrogen.

[B] Single cell RNA Seq

At the moment, Single cell RNA Seq is performed at a core lab at UCSF following the 10X genomics protocol using ~15,000 cells. A detailed protocol is provided below which is based on online protocol [2].

GEM Generation & Barcoding

a. Preparing Single Cell Master Mix

1. Prepare Master Mix on ice. Add reagents in the order shown below. Pipette mix 15 times and centrifuge briefly. Do not add Single Cell Suspension at this point.
2. Place the Master Mix on a chilled metal block resting on ice.

b. Loading the Single Cell A Chip

1. Place a Single Cell A Chip in a 10x™ Chip Holder. Handle the chip by its edges, taking care to avoid touching its bottom surface. See Practical Tips & Troubleshooting (Section 5) for tips on assembly.
2. If processing fewer than 8 samples per Single Cell A Chip, first add the following volumes of 50% glycerol solution to each unused well:
   a) 90 µl in the row labeled 1
   b) 40 µl in the row labeled 2
   c) 270 µl in the row labeled 3
3. Dispense 66.2 µl Master Mix into each well of an 8-tube strip on a chilled metal block resting on ice.
4. Add the appropriate volume of Nuclease-Free Water (determined from the Cell Suspension Volume Calculator Table) into each well containing Master Mix.
5. Gently pipette mix the tube containing the washed and diluted cells. Add the appropriate volume (µl) of single cell suspension (determined from the Cell Suspension Volume Calculator Table) to each well of the tube strip containing the Master Mix and Nuclease-Free Water.
6. With a pipette set to 90 µl, gently pipette mix the combined cells, Master Mix, and Nuclease-Free Water (from here onwards referred to as Master Mix containing cells) 5 times while keeping the tube strip on a chilled metal block resting on ice.
7. Without discarding the pipette tips, transfer 90 µl Master Mix containing cells to the wells in the row labeled 1, taking care not to introduce bubbles. To do this, place the tips into the bottom center of the wells and raise the tips slightly above the bottom before slowly dispensing the Master Mix containing cells.
8. Snap the Single Cell 3' Gel Bead Strip into a 10x™ Vortex Adapter and vortex for 30 sec.
9. Remove the Single Cell 3' Gel Bead Strip and flick in a sharp, downward motion to ensure maximum recovery. Confirm that there are no bubbles at the bottom of the tube and that liquid levels are uniform.
10. Carefully puncture the foil seal and slowly aspirate 40 µl Single Cell 3' Gel Beads, taking care not to introduce air bubbles.

11. Slowly dispense the Single Cell 3' Gel Bead suspension into the bottom of the wells in the row labeled 2, taking care not to introduce bubbles. To do this, place the tips into the bottom center of the wells and raise the tips slightly above the bottom before slowly dispensing the Gel Beads.
12. Pipette a total volume of 270 µl of Partitioning Oil into the wells in the row labeled 3 by pipetting two aliquots of 135 µl from a reagent reservoir. Do not add Partitioning Oil to any unused input wells that already contain 50% glycerol solution.
13. Attach the 10x™ Gasket. The notched cut should be at the top left corner. Ensure the 10x Gasket holes are aligned with the wells. Avoid touching the smooth side of the 10x Gasket and do not press down on the top of the 10x™ Gasket after attachment. Keep the assembly horizontal to avoid wetting the 10x Gasket with Partitioning Oil.

c. Running the Chromium™ Controller

1. Press the button on the touchscreen of the Chromium Controller to eject the tray.
2. Place the assembled Chip, 10x Chip Holder and 10x Gasket on the tray.
3. Press the button on the touchscreen again to retract the tray. Confirm the Chromium Single Cell A program shows on screen and press the play button to begin the run.
4. At the completion of the run (~6.5 min), the Chromium Controller will chime. Proceed immediately to the next step.

d. Transferring GEMs

1. Maintain an Eppendorf twin.tec® 96-Well PCR plate for GEM transfer on a chilled metal block resting on ice.
2. Press the eject button to eject the tray and remove the Single Cell A Chip. Remove and discard the 10x™ Gasket. Press the button to retract the empty tray (or the tray will automatically close after 40 sec).
3. Open the 10x Chip Holder and fold the lid back until it clicks to expose the wells at a 45-degree angle.
4. Check for volume uniformity in the Gel Bead, Sample, and Partitioning Oil wells remaining in the Single Cell A Chip.
5. Slowly aspirate 100 µl GEMs from the lowest points of the Recovery Wells (row labeled ◀) without creating a seal between the tips and the bottom of the wells. Avoid introducing air bubbles.
6. Withdraw pipette tips from the wells and verify that there is no air in the tips. GEMs should appear opaque and uniform across all channels.
7. Over the course of ~20 sec, dispense the GEMs into the Eppendorf® twin-tec 96-Well PCR plate (on a chilled metal block resting on ice) with the pipette tips against the sidewalls of the wells. (See Practical Tips & Troubleshooting, Section 5). Keep the tips above the liquid level to minimize GEMs lost on the outside of the tips.
8. If multiple Single Cell A Chips are run back-to-back, keep plate containing recovered GEMs on ice and seal the plate wells containing GEMs with Strip Caps before proceeding to load reagents into the next chip. Avoid storing the GEMs on ice for more than 1 h.
9. Discard the used Single Cell A Chip. Push the black sliders on the back of the 10x™ Chip Holder toward the middle to release the lock and close the lid.

e. GEM-RT Incubation

1. If necessary, remove the strip caps from the PCR plate with recovered GEMs. Check that the Plate Sealer plate block is at room temperature.
2. Seal the plate with pierceable foil heat seal at 185°C for 6 sec and promptly remove.
3. Load the sealed PCR plate into a thermal cycler that can accommodate at least 100 µl reaction volume and proceed with the following incubation protocol.
4. Store in the PCR plate at 4°C for up to 72 h or at −20°C for up to a week, or proceed directly to Post GEM-RT Cleanup.

f. Post GEM-RT Cleanup – Silane DynaBeads

1. Remove the foil seal and add 125 µl Recovery Agent to each well containing post incubation GEMs. Do not pipette mix or vortex the biphasic mixture. Wait 60 sec and then transfer the entire volume to an 8-tube strip.
2. The recovered biphasic mixture contains distinct Recovery Agent/Partitioning Oil (pink) and aqueous phases (clear), with no persisting emulsion (opaque).
3. Slowly remove 125 µl Recovery Agent/Partitioning Oil (pink) from the bottom of the tubes and discard. Be careful not to aspirate any of the clear aqueous sample.
4. Vortex DynaBeads MyOne Silane beads until fully resuspended. Prepare DynaBeads Cleanup Mix by adding reagents in the order shown below. Vortex mix thoroughly.
5. Add 200 µl DynaBeads Cleanup Mix to each sample. Pipette mix 5 times (pipette set to 200 µl) and incubate at room temperature for 10 min.
6. Prepare Elution Solution I by adding reagents in the order shown below. Vortex mix thoroughly and centrifuge briefly.
7. After the 10 min incubation step is complete, place the tube strip into a 10x™ Magnetic Separator in the High position until the supernatant is clear.
8. Carefully remove and discard the supernatant.
9. Add 150 µl freshly prepared 80% ethanol twice to the pellet while on the magnet for a total volume of 300 µl and stand for 30 sec.
10. Carefully remove and discard the ethanol wash.
11. Add 200 µl 80% ethanol to the pellet and stand for 30 sec.
12. Carefully remove and discard the ethanol wash.
13. Centrifuge the tube strip briefly and return it to a 10x™ Magnetic Separator in the Low position.
14. Remove and discard any remaining ethanol and allow the samples to air dry for 1 min.
15. Remove the tube strip from the magnet and Pipette mix thoroughly until beads are fully re-suspended (pipette set to 30 µl to avoid introducing air bubbles).
16. Incubate at room temperature for 1 min.
17. Place the tube strip in a 10x Magnetic Separator in the Low position until the solution is clear.
18. Transfer 35 of purified GEM-RT product to a new tube strip.

g. cDNA Amplification Reaction

1. Prepare cDNA Amplification Reaction Mix on ice. Add reagents in the order shown below. Vortex mix and centrifuge briefly. Do not add Purified GEM-RT Product at this point.
2. Add 65 µl cDNA Amplification Reaction Mix to each tube containing 35 µl of purified GEM-RT product.
3. Pipette mix 15 times (pipette setting 90 µl) and centrifuge briefly.

4. Cap and load the tube strip into a thermal cycler that can accommodate at least 100 µl reaction volume and proceed with the following incubation protocol.
5. Store the samples at 4°C in a tube strip for up to 72 h or proceed directly to SPRIselect Cleanup

h. Post cDNA Amplification Reaction Cleanup – SPRIselect

1. Vortex the SPRIselect Reagent until fully resuspended. Add 60 µl SPRIselect Reagent (0.6X) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).
2. Incubate the tube strip at room temperature for 5 min.
3. Place the tube strip in a 10x™ Magnetic Separator in the High position until the solution is clear.
4. Carefully remove and discard the supernatant.
5. Add 200 µl 80% ethanol to the pellet and stand for 30 sec.
6. Carefully remove and discard the ethanol wash.
7. Repeat steps e and f for a total of 2 washes.
8. Centrifuge the tube strip briefly and return it to a 10x Magnetic Separator in the Low position.
9. Remove and discard any remaining ethanol and allow the samples to air dry for 2 min. Do not exceed 2 min as this will lead to decreased elution efficiency.
10. Remove the tube strip from the 10x Magnetic Separator and add 40.5 µl Buffer EB.
11. Pipette mix 15 times and incubate at room temperature for 2 min.
12. Place the tube strip in a 10x Magnetic Separator in the High position until the solution is clear.
13. Transfer 40 µl of sample to a new tube strip and cap the sample wells.
14. Store the samples at 4°C in a tube strip for up to 72 h or at −20°C for up to a week, or proceed directly to Post cDNA Amplification QC & Quantification.

i. Post cDNA Amplification Reaction QC & Quantification

1. Run 1 µl of sample at a dilution of 1 part sample:5 parts Nuclease-Free Water on the Agilent Bioanalyzer High Sensitivity chip for qualitative analysis (trace a). Traces should resemble the overall shape of the sample electropherograms shown below.
2. To determine the cDNA yield per sample, under the “Electropherogram” view choose the “Region Table” tab on the Agilent 2100 Expert Software.
3. Manually select the region encompassing ~200 – ~9000 bp.
4. Multiply the cDNA concentration [pg/µl] reported via the Agilent 2100 Expert Software by the elution volume (40 µl) of the Post cDNA Amplification Reaction Clean Up sample (taking any dilution factors into account) and then divide by 1000 to obtain the total cDNA yield in ng.

j. Library Construction - Fragmentation, End Repair & A-tailing

1. Prepare a thermal cycler with the following incubation protocol and initiate the 4°C pre-cool block step prior to assembling the Fragmentation Mix.
2. Vortex the Fragmentation Buffer. Verify there is no precipitate before proceeding.
3. Prepare the Fragmentation Mix on ice. Add the reagents in the order shown below. Mix thoroughly and centrifuge briefly.
4. Dispense 15 µl Fragmentation Mix into each well of an 8-tube strip on a chilled metal block resting on ice.

5. Add 35 µl purified cDNA to each well of the tube strip containing the Fragmentation Mix. Pipette mix 15 times (pipette set to 35 µl) and centrifuge briefly before returning the tube strip to a chilled metal block resting on ice.
6. Transfer the chilled tube strip into the pre-cooled thermal cycler (4°C) and press “SKIP” to initiate the Fragmentation protocol.
7. After the Fragmentation protocol is complete, proceed directly to step 5.1.4.3.2.

k. Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect

1. Vortex the SPRIselect Reagent until fully resuspended. Add 30 µl SPRIselect Reagent (0.6X) to each sample in the tube strip and pipette mix 15 times (pipette set to 75 µl).
2. Incubate the tube strip at room temperature for 5 min.
3. Place the tube strip in a 10x™ Magnetic Separator in the High position until the solution is clear.
4. Transfer 75 µl supernatant to a new tube strip and discard the previous tube strip.
5. Add 10 µl SPRIselect Reagent (0.8X) to each sample in the tube strip and pipette mix 15 times (pipette set to 75 µl).
6. Incubate the tube strip at room temperature for 5 min.
7. Place the tube strip in a 10x Magnetic Separator in the High position until the solution is clear.
8. Carefully remove and discard 80 µl supernatant.
9. With the tube strip still in a 10x Magnetic Separator, add 125 µl 80% ethanol to the pellet and stand for 30 sec.
10. Carefully remove and discard the ethanol wash.
11. Repeat steps i and j for a total of two washes.
12. Briefly centrifuge the tube strip and return it to the 10x™ Magnetic Separator in the
13. Low position. Carefully remove and discard the remaining ethanol wash.
14. Remove the tube strip from the 10x Magnetic Separator and add 50.5 µl Buffer EB. Pipette mix 15 times.
15. Incubate the tube strip at room temperature for 2 min.
16. Place the tube strip in a 10x Magnetic Separator in the High position until the solution is clear.
17. Transfer 50 µl of sample to a new tube strip and cap the sample wells.

l. Adaptor Ligation

1. Prepare Adaptor Ligation Mix by adding the reagents in the order shown below. Mix thoroughly and centrifuge briefly.
2. Add 50 µl Adaptor Ligation Mix to each tube containing 50 µl sample from the Post Fragmentation, End Repair and A-tailing Size Selection. Pipette mix 15 times (pipette set to 50 µl) and centrifuge briefly.
3. Incubate in a thermal cycler with the following protocol.
4. Proceed immediately to the next step.

m. Post Ligation Cleanup – SPRIselect

1. Vortex the SPRIselect Reagent until fully re-suspended. Add 80 µl SPRIselect Reagent (0.8X) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).
2. Incubate the tube strip at room temperature for 5 min.

3. Place the tube strip in a 10x™ Magnetic Separator in the High position until the solution is clear.
4. Carefully remove and discard the supernatant.
5. Add 200 µl 80% ethanol to the pellet and stand for 30 sec.
6. Carefully remove and discard the ethanol wash.
7. Repeat steps e and f for a total of 2 washes.
8. Centrifuge the tube strip briefly and return it to a 10x Magnetic Separator in the Low position.
9. Remove and discard any remaining ethanol and allow the samples to air dry for 2 min. Do not exceed 2 min as this will lead to decreased elution efficiency.
10. Remove the tube strip from the 10x Magnetic Separator and add 30.5 µl Buffer EB. Pipette mix 15 times.
11. Incubate the tube strip at room temperature for 2 min.
12. Place the tube strip in a 10x™ Magnetic Separator in the Low position until the solution is clear.
13. Transfer 30 µl of sample to a new tube strip.

n. Sample Index PCR

1. Prepare Sample Index PCR Mix by adding the reagents in the order shown below. Mix thoroughly and centrifuge briefly.
2. Add 60 µl Sample Index PCR Mix to each tube containing 30 µl purified Post Ligation sample.
3. Add 10 µl of an individual Chromium i7 Sample Index to each well and record their assignment. Pipette mix 15 times (pipette set to 90 µl) and centrifuge briefly.
4. Index the library DNA in a thermal cycler with the following protocol.
5. Store the tube strip at 4°C for up to 72 h or proceed directly to Post Sample Index PCR Double Sided Size Selection.

o. Post Sample Index PCR Double Sided Size Selection – SPRIselect

1. Vortex the SPRIselect Reagent until fully resuspended. Add 60 µl SPRIselect Reagent (0.6X) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).
2. Incubate the tube strip at room temperature for 5 min.
3. Place the tube strip in a 10x™ Magnetic Separator in the High position until the solution is clear.
4. Transfer 150 µl supernatant to a new tube strip and discard the previous tube strip.
5. Add 20 µl SPRIselect Reagent (0.8X) to each sample in the tube strip and pipette mix 15 times (pipette set to 150 µl).
6. Incubate the tube strip at room temperature for 5 min.
7. Place the tube strip in a 10x Magnetic Separator in the High position until the solution is clear.
8. Carefully remove and discard 165 µl supernatant.
9. With the tube strip still in a 10x Magnetic Separator, add 200 µl 80% ethanol to the pellet and stand for 30 sec.
10. Carefully remove and discard the ethanol wash.
11. Repeat steps i and j for a total of two washes.
12. Briefly centrifuge the tube strip and return it to the 10x Magnetic Separator in the Low position. Carefully remove and discard the remaining ethanol wash.
13. Remove the tube strip from the 10x Magnetic Separator and add 35.5 µl Buffer EB. Pipette mix 15 times.

14. Incubate the tube strip at room temperature for 2 min.
15. Place the tube strip in a 10x Magnetic Separator in the Low position until the solution is clear.
16. Transfer 35 µl of sample to a new tube strip and cap the sample wells.
17. Store the tube strip at 4°C for up to 72 h or at −20°C for long-term storage.

p. Post Library Construction QC

1. EITHER Run 1 µl of sample at 1:10 dilution on the Agilent Bioanalyzer High Sensitivity chip for qualitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below.
2. OR Run 1 µl of sample at 1:10 dilution on the Agilent TapeStation High Sensitivity D1000 ScreenTape for qualitative analysis. Traces should resemble the overall shape of the sample electropherogram shown below.
3. Determine the average fragment size from the Bioanalyzer/TapeStation trace. This value will be used as the insert size for accurate library quantification in qPCR (step 5.1.4.3.8).

q. Post Library Construction Quantification

1. Thaw Kapa DNA Quantification Kit for Illumina platforms.
2. Dilute 1 µl of sample with Nuclease-Free Water to appropriate dilutions that fall within the linear detection range of the Kapa DNA Quantification Kit.
3. Make enough Quantification Master Mix for the DNA dilutions per sample and the DNA Standards (plus 10% excess) using the guidance for 1 reaction volume below.
4. Dispense 16 µl Quantification Master Mix for sample dilutions and DNA Standards into a 96-Well PCR Plate.
5. Add 4 µl of sample dilutions and 4 µl DNA Standards to appropriate wells. Centrifuge the PCR plate briefly.
6. Run DNA Quantification Cycling Protocol with data acquisition at Step 3.
7. Follow the manufacturer's recommendations for qPCR analysis. The average fragment size derived from the Bioanalyzer/TapeStation trace from step 5.1.4.3.7 is used as the insert size for accurate library quantification in qPCR.

r. Sequencing Libraries - Sequencing Depth Recommendations

1. The technical performance of Single Cell 3' libraries is driven by sequencing coverage per cell. 50,000 raw reads per cell is recommended.
2. Adjust loading concentrations according to Illumina specifications.
3. The following are supported sequencing platforms for Single Cell 3' libraries.

s. Sequencing Run Parameters

1. Single Cell 3' libraries use standard Illumina sequencing primers for both sequencing and index reads, and require no custom primers.
2. Single Cell libraries must be run using paired-end sequencing with single indexing. The supported number of cycles for each read is shown below.

t. Sample Indices

1. Sample Indices are a mix of four oligos. The 10x™ Sample Index sequence is not needed for the sample sheet (required for generating FASTQs with cellranger mkfastq),

but the 10x Sample Index name (Chromium™ i7 Sample Index plate well ID) is needed if running more than one sample.

u. Loading Single Cell 3' Libraries

1. Once quantified and normalized, Single Cell 3' Libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Refer to Illumina documentation for Denaturing and Diluting Libraries.
2. Consult the Chromium Single Cell 3' v2 Libraries - Sequencing Metrics for Illumina Sequencers (Document CG000089) for further information.

[C] Exome Sequencing

1. DNA from kidney cells are isolated by QIAmp DNA mini and Blood (page #51) following protocols for cultured cells [2].
   a) Cells in suspension are centrifuged at 300 x g for 5 min at RT in a 1.5mL microcentrifuge tube.
   b) Supernatant is removed and the pellet is re-suspended in 1XPBS to a final volume of 200 µL.
   c) 20 µL of Proteinase K and 2 µL of RNAseA is added to the sample.
   d) 200 µL of Buffer AL (Qiagen) is added to the cell suspension and the sample is vortexed for 15 sec.
   e) The sample is then incubated at 56°C for 10 min.
   f) 200 µL of 200 proof Ethanol is added to the mixture and the sample is again mixed via vortex for 15 sec.
   g) All 600 µL of the mixture were then added to the QIAmp Micro Spin Column (Qiagen).
   h) The sample is centrifuged at 6000 x g for 1 min at RT and flow through is discarded. 500 µL of Buffer AW1 (Qiagen) is added to the column.
   i) The column is centrifuged again at 6000 x g for 1 min at RT and flow through is discarded.
   j) 500 µL of Buffer AW2 (Qiagen) is added to the column.
   k) The column was centrifuged at full speed 20,000 x g for 3 min at RT and flow through was discarded.
   l) The column is placed in a new collection tube and sample is dried via centrifugation at full speed (20,000 x g) for 1 additional min, any remaining flow through is discarded.
   m) Column is placed into a new 1.5 mL microcentrifuge tube and 50µL of Buffer AE (Qiagen) and tube is incubated 5 min at RT.
   n) Cells are eluted via centrifugation at 6000 x g for 1 min at RT.
   o) Concentration is read via nanodrop.

2. Libraries from 500 ng of DNA are pooled into a capture reaction that contains biotinylated DNA oligonucleotides (called 'baits'), from Nimblegen, (SeqCap EZ Human Exome Library v3.0; Roche Nimblegen) for 72 hours. The DNA bait-DNA hybrids are then pulled out of the complex mixture by incubation with streptavidin-labeled magnetic beads and captured onto a strong magnet. After washing, the targeted DNA of interest is eluted and subjected to 18 cycles of DNA amplification.
3. Genomic DNA sheared by Covaris S2 to a target size of 200-300bp and assembled into a library with TruSeq adapters (Kapa Biosystems) containing indexes that differentiates different libraries in a capture reaction as well as a sequencing run.

4. Exome Sequencing was performed on NovaSeq 6000, average insert size was around 300-350bp, paired-end reads on the NovaSeq: 2x150 bp provided, 55 million reads per exome and mean coverage of 80X. Raw data were aligned to the human genome build 37 (hg19) using bwa-mem (0.7.15). Fastqc (0.11.5) was used as a quality control tool for the sequence data.
5. The data is analyzed by following the methods including Demuxlet described in Kang et al Nat Biotechnol [1]. Demultiplexed single cell analysis pipeline involves 10x output files are used for QC. Once they pass the QC the data is used for Clustering followed up by assignment of cluster IDs. The multiplexed single cell analysis pipeline involves 10x outputted BAM files and VCF files from exome seq and use of demuxlet tool, an R package to analyze these files (https://www.nature.com/articles/nbt.4042). Cells with <500 genes or <5000 genes data are excluded.

References:

[1] Kang HM, Subramaniam M, Targ S, Nguyen M, Maliskova L, McCarthy E, Wan E, Wong S, Byrnes L, Lanata CM, Gate RE, Mostafavi S, Marson A, Zaitlen N, Criswell LA, Ye CJ. Multiplexed droplet single-cell RNA-sequencing using natural genetic variation. Nat Biotechnol. 2018 Jan;36(1):89-94. PMID: 29227470

[2] https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/user-guide-chromium-single-cell-3-reagent-kit-v2-chemistry

[3] https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/qiaamp-dnablood-mini-kit/#orderinginformation