Dec 19, 2022

UPitt TriState SenNet TMC Single Cell RNA Seq (10X Genomics) Library Preparation and Sequencing 5' with Cell Hashings

DOI
dx.doi.org/10.17504/protocols.io.n2bvj89exgk5/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.n2bvj89exgk5/v1

Protocol Citation: Tracy Tabib, Oliver Eickelberg, Melanie Königshoff, Iafyatis Lafyatis 2022. UPitt TriState SenNet TMC Single Cell RNA Seq (10X Genomics) Library Preparation and Sequencing 5' with Cell Hashings. **protocols.io**
https://dx.doi.org/10.17504/protocols.io.n2bvj89exgk5/v1

Protocol status: Working
We use this protocol and it's working

Created: December 16, 2022

Last Modified: December 19, 2022

Protocol Integer ID: 74115

Keywords: upitt tristate sennet tmc cell hashing, upitt tristate sennet tmc single cell rna seq, cdna libraries from individual cell, cell hashings this protocol, cell hashing protocol, chromium next gem single cell, 10x genomic, 10x genomics chromium controller, cell hashing, upitt tristate sennet tmc, cdna library, cell rna, single cell suspension, scrnaseq, cell suspension, sequencing, rna, cell, droplet generation, individual cell, reagent kits v2

Funders Acknowledgements:
TriState SenNET (Lung and Heart) Tissue Map and Atlas consortium – NIA
Grant ID: U54AG075931

Abstract

This protocol is for the generation of 5' 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.
A modification of the "Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual Index)".

For Cell Hashing protocol:
"UPitt TriState SenNet TMC Cell Hashing of single cell suspension for scRNAseq in 5'; workflow (10x Genomics)"
dx.doi.org/10.17504/protocols.io.36wgqjdy5vk5/v1

Troubleshooting

GEM Generation & Barcoding

1 Preparation of Single Cell Master Mix:

a. Prepare Master Mix on ice. Pipette mix 15x and centrifuge briefly.

	A	B	C	D	E
	Master Mix ***Add reagents in the order listed***	**PN**	**1X (µl)**	**4X + 10% (µl)**	**8X + 10% (µl)**
	RT Reagent B	2000165	18.8	82.7	165.4
	Poly-dT RT Primer	2000007	7.3	32.1	64.2
	Reducing Agent B	2000087	1.9	8.4	16.7
	RT Enzyme C	2000085/ 2000102	8.3	36.5	73.0
	Total	-	36.3	159.7	319.3

b. Add 36.3 µl Master Mix into each tube of a PCR 8-tube strip on ice.

2 Load Chromium Next GEM Chip K:

a. Assemble Chromium Next GEM Chip

Close the holder lid.

Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder.

Gently pull the gasket toward the right and hook it on the two right-hand tabs.

Remove the chip from the sealed bag. Use the chip within ≤ 24 h

b. Dispense 50% Glycerol Solution into each unused wells

(if processing <8 samples/chip)

i. **70 µl** in each unused well in row labeled **1**

ii. **50 µl** in each unused well in row labeled **2**

iii.**45 µl** in each unused well in row labeled **3**

c. Prepare Master Mix + Cell Suspension

Refer to the Cell Suspension Volume Calculator Table

Add the appropriate volume of nuclease-free water to Master Mix.

Add the appropriate volume of single cell suspension to Master Mix

(Total of ***75 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

Using the same pipette tip, dispense **70 µl** Master Mix + Cell Suspension into the bottom center of each well in **row labeled 1** without introducing bubbles.

e. Prepare Gel Beads

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**.
- Centrifuge the Gel Bead strip for **~5 sec**.
- Confirm there are no bubbles at the bottom of the tubes and the liquid levels are even.
- Place the Gel Bead strip back in the holder. Secure the holder lid

f. Load Row Labeled 2

- Puncture the foil seal of the Gel Bead tubes.
- Slowly aspirate **50 µl** Gel Beads.
- Dispense into the wells in **row labeled 2** without introducing bubbles.
- Wait **30 sec**.

g. Load Row Labeled 3

- Dispense **45 µl** Partitioning Oil into the wells in **row labeled 3** from a reagent reservoir. *Failure to add Partitioning Oil to the top row labeled 3 will prevent GEM generation and can damage the Chromium Controller*

h. Prepare for Run

Close the lid (gasket already attached). DO NOT touch the smooth side of the gasket. DO NOT press down on the top of the gasket.

Run the chip in the Chromium Controller or X/iX immediately after loading the Partitioning Oil.

3 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, ensuring that the chip stays horizontal. Press the button to retract the tray.

c. Press the play button.

d. At the completion of the run (**~18 min**), the Controller will chime. **Immediately** proceed to the next step.

4 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.

d. Check the volume in rows labeled 1-2. Abnormally high volume in one well relative to other wells may indicate a clog.

e. Slowly aspirate **100 µl** GEMs from the lowest points of the recovery wells in the top row labeled 3 without creating a seal between the pipette 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.**

5 GEM-RT Incubation:
Use a thermal cycler that can accommodate at least 100 µl volume. A volume of 125 µl is the preferred setting on Bio-Rad C1000 Touch. In alternate thermal cyclers, use highest reaction volume setting.

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

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	53°C	125 µl	~55 min
	Step	Temperature	Time
	1	53°C	00:45:00
	2	85°C	00:05:00
	3	4°C	Hold

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

6 Post GEM-RT Cleanup & cDNA Amplification:

a. Add **125 µl** Recovery Agent to each sample (post GEM-RT incubation) at room temperature. DO NOT pipette mix or vortex the biphasic mixture. Wait **2 min**.

The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and aqueous phase (clear), with no persisting emulsion (opaque).

If biphasic separation is incomplete: Firmly secure the cap on the tube strip, ensuring that no liquid is trapped between the cap and the tube rim. Mix by inverting the capped tube strip 5x, centrifuge briefly, and proceed to step b. DO NOT invert without firmly securing the caps.

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.

	A	B	C	D	E
	Dynabeads Cleanup Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
	Nuclease-free Water		5	22	44
	Cleanup Buffer	2000088	182	801	1602
	Dynabeads MyOne SILANE	2000048	8	35	70
	Reducing Agent B	2000087	5	22	44
	Total		200	880	1760

d. Vortex and add **200 µl** to each sample. Pipette mix 10x (pipette set to 200 µl).
e. Incubate **10 min** at **room temperature** (keep caps open).
f. Prepare Elution Solution I. Vortex and centrifuge briefly

	A	B	C	D
	Elution Solution I Add reagents in the order listed	PN	1X (µl)	10X (µl)
	Qiagen Buffer EB	-	98	980
	10% Tween 20	-	1	10
	Reducing Agent B	2000087	1	10
	Total		100	1000

g. At the end of **10 min** incubation, place on a 10x Magnetic Separator•**High position** (magnet•High) until the solution clears. A white interface between the aqueous phase and Recovery Agent is normal.
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 10x Magnetic Separator•**Low position** (magnet•Low).

n. Remove remaining ethanol. Air dry for **2 min**.

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

p. Pipette mix (pipette set to 30 µl) without introducing bubbles. Pipette mix 15x. If beads still appear clumpy, continue pipette mixing until fully resuspended.

q. Incubate **1 min** at **room temperature.**

r. Place on the magnet•**Low** until the solution clears.

s. Transfer **35 µl** sample to a new tube strip.

7 cDNA Amplification:

a. Prepare cDNA Amplification Mix on ice. Vortex and centrifuge briefly.

	A	B	C	D	E
	cDNA Amplification Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
	Amp Mix Retrieve from Single Cell 5' GEM Kit	2000047/ 2000103	50	220	440
	Feature cDNA Primers 4	2000277	15	66	132
	Total		65	286	572

b. Add **65 µl** cDNA Amplification Reaction Mix to **35 µl** sample (Post GEM-RT Cleanup).

c. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

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

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	105°C	100 µl	~25-50 min
	Step	Temperature	Time
	1	98°C	00:00:45
	2	98°C	00:00:20
	3	63°C	00:00:30
	4	72°C	00:01:00

	A	B	C
	5	Go to Step 2, see table below for total # of cycles	
	6	72°C	00:01:00
	7	4°C	Hold

	A	B	C
	Recommended starting point for cycle number optimization		
	Targeted Cell Recovery	Low RNA Content Cells e.g., Primary Cells Total Cycles	High RNA Content Cells e.g., Cell Lines Total Cycles
	500-2,000	16	14
	2,001-6,000	14	12
	6,001-10,000	13	11

e. Store at **4°C** for up to **72 h** or at **−20°C** for **≤1 week**, or proceed to the next step.

8 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 140 µl).

b. Incubate **5 min** at **room temperature**.

c. Place on the magnet•**High** until the solution clears.

d. Transfer and save **80 µl** supernatant in a new tube strip without disturbing the pellet. Maintain at **room temperature**. DO NOT discard the transferred supernatant (cleanup for Cell Surface Protein library construction).

e. Remove the remaining supernatant from the pellet without disturbing the pellet. DO NOT discard the pellet (cleanup for V(D)J and 5'Gene Expression library construction). **Immediately** proceed to Pellet Cleanup.

8.1 Pellet Cleanup (for V(D)J & 5' Gene Expression library)

i. Add **200 µl** 80% ethanol to the pellet while still on magnet•**High**. Wait **30 sec**.

ii. Remove the ethanol.

iii. **Repeat** steps i and ii for a total of 2 washes.

iv. Centrifuge briefly and place on the magnet•**Low**.

v. Remove any remaining ethanol. Air dry for **2 min**. DO NOT exceed **2 min** as this will decrease elution efficiency.

vi. Remove from the magnet. Add **45.5 µl** Buffer EB. Pipette mix 15x.
vii. Incubate **2 min** at **room temperature**.
viii. Place the tube strip on the magnet•**High** until the solution clears.
ix. Transfer **45 µl** sample to a new tube strip.
x. Store at **4°C** for up to **72 h** or at **−20°C** for up to **4 weeks**, or **proceed to cDNA QC & Quantification**.

9 cDNA QC & Quantification

a. Run **1 µl** undiluted sample from the Pellet Cleanup step 2.3A-x (Dilution Factor 1) on an Agilent Bioanalyzer High Sensitivity chip.

Run 1 µl undiluted product for input cells with low RNA content (<1pg total RNA/cell), and 1 µl of 1:10 diluted product for input cells with high RNA content

b. If proceeding to 5' GEX Library Construction (step 5), determine cDNA yield for each sample.

5' Gene Expression (GEX) Library Construction

10 5' Gene Expression (GEX) Library Construction

a. Determine the volume for **50 ng** mass of sample. Dispense the sample volume in a tube strip **on ice**. If the volume required for **50 ng** is less than **20 µl**, adjust the total volume of each sample to **20 µl** with nuclease-free water. If the volume for **50 ng** exceeds **20 µl**, carry ONLY 20 µl sample into library construction.

b. Prepare a thermal cycler with the following incubation protocol.

	A	B	C
	Lid Temperature	Reaction Volume	Reaction Volume
	65°C	50 µl	~35 min
	Step	Temperature	Time
	Pre-cool block	4°C	Hold
	Fragmentation	32°C	00:05:00
	End Repair & A-tailing	65°C	00:30:00
	Hold	4°C	Hold

c. Vortex Fragmentation Buffer. Verify there is no precipitate.

d. Prepare Fragmentation Mix on ice. Pipette mix and centrifuge briefly

	A	B	C	D	E
	Fragmentation Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)

	A	B	C	D	E
	Nuclease-free Water		15	66	132
	Fragmentation Buffer	2000091	5	22	44
	Fragmentation Enzyme	2000090 / 2000104	10	44	88
	Total		30	132	264

e. Add **30 µl** Fragmentation Mix into each tube containing **20 µl** sample.
f. Pipette mix 15x (pipette set to 30 µl) on ice. Centrifuge briefly.
g. Transfer into the pre-cooled thermal cycler (**4°C**) and press "SKIP" to initiate the protocol.

11 GEX Post Fragmentation, End Repair & A-tailing Double Sided Size Selection – SPRIselect
a. Vortex to resuspend SPRIselect Reagent. Add **30 µl** SPRIselect Reagent (**0.6X**) 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. Add **10 µl** SPRIselect reagent (**0.8X**) to each sample. Pipette mix 15x (pipette set to 75 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. With the tube strip still on the magnet, add **125 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**.
m. Remove the ethanol. DO NOT over-dry beads to ensure maximum elution efficiency.
n. Remove from the magnet. Add **50.5 µl** Buffer EB. Pipette mix 15x.
o. Incubate **2 min** at **room temperature**.
p. Place on the magnet•**High** until the solution clears.
q. Transfer **50 µl** sample to a new tube strip.

12 GEX Adaptor Ligation
a. Prepare Adaptor Ligation Mix. Pipette mix and centrifuge briefly

	A	B	C	D	E
	Adaptor Ligation Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
	Ligation Buffer	2000092	20	88	176
	DNA Ligase	220110/ 220131	10	44	88
	Adaptor Oligos	2000094	20	88	176
	Total		50	220	440

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

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	30°C	100 µl	15 min
	Step	Temperature	Time
	1	20°C	00:15:00
	2	4°C	Hold

13 GEX 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**.

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.

14 GEX 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-3000431 Dual Index Plate TT Set A well ID) used.

b. Add **50 µl** Amp Mix (PN-2000047/2000103) to **30 µl** sample.

c. Add **20 µl** of an individual Dual Index TT Set A to each well and record the well ID used. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.

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

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	105°C	100 µl	~30 min
	Step	Temperature	Time
	1	98°C	00:00:45
	2	98°C	00:00:20
	3	54°C	00:00:30
	4	72°C	00:00:20
	5	Go to step 2, see below for # of cycles	
	6	72°C	00:01:00
	7	4°C	Hold

	A	B
	Recommended cycle numbers	
	cDNA Input	Total Cycles
	1-25 ng	16
	26-50 ng	14

e. Store at **4°C** for up to **72 h** or proceed to the next step.

15 GEX Post Sample Index PCR Double Sided Size Selection – SPRIselect

a. Vortex to resuspend 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 on 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 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 on the magnet•**High** until the solution clears.

h. Remove **165 µl** supernatant. DO NOT discard any beads.

i. With the tube strip still on 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**.

m. Remove the remaining ethanol. DO NOT over-dry beads to ensure maximum elution efficiency.

n. Remove the tube strip from the magnet. Add **35.5 µl** Buffer EB. Pipette mix 15x.

o. Incubate **2 min** at **room temperature**.

p. Place on the magnet•**Low** until the solution clears.

q. Transfer **35 µl** sample to a new tube strip. STOP

r. Store at **4°C** for up to **72 h** or at **−20°C** for **long-term** storage

16 GEX Post Library Construction QC

a. Run **1 µl** sample at **1:10 dilution** on an Agilent Bioanalyzer High Sensitivity chip

b. Determine the average fragment size from the trace. This will be used as the insert size for library quantification.

Cell Surface Protein Library Construction

17 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-3000510 Dual Index Plate TN Set A well ID; verify name and part number) used.

b. Prepare Sample Index PCR Mix.

	A	B	C	D	E
	Sample Index PCR Mix Add reagents in the order listed	PN	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
	Nuclease-free Water		25	110	220
	Amp Mix Retrieve from 5' Feature Barcode Kit	2000047	50	220	440
	Total		75	330	660

c. Transfer **ONLY 5 µl** sample from the Transferred Supernatant Cleanup to a new tube strip.
Note that only 5 µl of the DNA sample is adequate for generating Cell Surface Protein library. The remaining DNA sample can be stored at 4°C for up to 72 h or at −20°C for up to 4 weeks for generating additional Cell Surface Protein libraries.
d. Add **75 µl** Sample Index PCR Mix to the **5 µl** Transferred Supernatant Cleanup sample.
e. Add **20 µl** of an individual sample index (Dual Index Plate TN Set A) to each well and record the well ID. Pipette mix 5x (pipette set to 90 µl). Centrifuge briefly.
f. Incubate in a thermal cycler with the following protocol.

	A	B	C
	Lid Temperature	Reaction Volume	Run Time
	105°C	100 µl	~30 min
	Step	Temperature	Time
	1	98°C	00:00:45
	2	98°C	00:00:20
	3	54°C	00:00:30
	4	72°C	00:00:20
	5	Go to step 2, repeat 7X (total 8 cycles)*	
	6	72°C	00:01:00
	7	4°C	Hold
	*Optimization of cycle number may be needed based on target protein expression levels and number of antibodies used for labeling.		

18 Post Sample Index PCR Size Selection – SPRIselect
a. Vortex to resuspend the SPRIselect reagent. Add **120 µl** SPRIselect Reagent (**1.2X**) 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. Remove the supernatant.
d. Add **300 µl** 80% ethanol to the pellet. Wait **30 sec**.
e. Remove the ethanol.
f. Add **200 µl** 80% ethanol to the pellet. Wait **30 se**c.
g. Remove the ethanol.
h. Centrifuge briefly. Place on the magnet•**Low**. Remove remaining ethanol.

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

19 Post Library Construction QC
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.

Sequencing

20 Sequencing Libraries:
Chromium Single Cell 5' Gene Expression, and Cell Surface Protein Dual Index libraries comprise standard Illumina paired-end constructs which begin with P5 and end with P7. These libraries include 16 bp 10x Barcodes encoded at the start of TruSeq Read 1. Sample index sequences are incorporated as the i5 and i7 index read for 5' Gene Expression libraries; as i5 and i7 index read N for Cell Surface Protein library.

21 Sample Indices
Each well of the Dual Index Kit TT Set A (PN-1000215) and Dual Index Kit TN Set A (PN-1000250) contains a mix of one unique i7 and one unique i5 sample index. If multiple samples are pooled in a sequence lane, the sample index name (i.e. the Dual Index plate well ID) is needed in the sample sheet used for generating FASTQs with “cellranger mkfastq”. If multiple libraries are pooled in a sequence lane, a separate sample index is needed with each library

22 Library Sequencing Depth & Run Parameters
Sequencing Depth
Minimum 20,000 read pairs per cell for 5' Gene Expression Dual Index library
Minimum 5,000 read pairs per cell for Cell Surface Protein Dual Index library
Sequencing Type
Paired-end, Dual indexing
Sequencing Read
Read 1: 26 cycles
i7 Index: 10 cycles
i5 Index: 10 cycles
Read 2: 90 cycles

23 Library Loading
Once quantified and normalized, 5' Gene Expression, and Cell Surface Protein libraries should be denatured and diluted as recommended for Illumina sequencing platforms. Refer to Illumina documentation for denaturing and diluting libraries.

	A	B	C
	Instrument	Loading Concentration (pM)	PhiX (%)
	MiSeq	10	1
	NextSeq 500	1.5	1
	HiSeq 2500 (RR)	10	1
	HiSeq 4000	180	1
	NovaSeq	150*/300	1
	NextSeq 2000	650	1

** Use 150 pM loading concentration for Illumina XP workflow*