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Massively parallel long-read sequencing of single cell RNA isoforms

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ABSTRACT

This protocol describes the subsampling of 10x Chromium generated single cell GEMs after reverse transcription for cDNA amplification. The protocol enables transcriptome sequencing of full-length cDNA from a flexible number of single cells captured on Chromium using long-read sequencers such as Nanopore and PacBio, in addition to preparing short-read Illumina libraries. The protocol is compatible with 10x Single Cell 3' and 5' Gene Expression kits including feature barcoding and also other 10x compatible multiomics methodologies such as CITE-seq.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

This protocol currently has not been linked to any publication.

GUIDELINES

This protocol assumes the operator has access to a 10x Genomics Chromium Controller and expertise in generating single cell 3' or 5' libraries using 10x single cell transcriptomics kits.

MATERIALS TEXT

Name	Catalog#	Vendor
Chromium Chip B Single Cell Kit	1000074	10x Genomics
Or Chromium Single Cell A Chip Kit	1000009	10x Genomics
Chromium Single Cell 3' Library & Gel Bead Kit v3	1000092	10x Genomics
Chromium Single Cell 3' Library Construction Kit v3	1000078	10x Genomics
Or Chromium Single Cell 5' Library & Gel Bead Kit	1000014	10x Genomics
Chromium Single Cell 5' Library Construction Kit	1000020	10x Genomics
PCR Tubes 0.2 ml 8-tube strips	951010022	Eppendorf
DNA LoBind Tubes, 1.5 ml	022431021	Eppendorf
Tips LTS 200UL Filter RT-L200FLR	30389240	Rainin
Tips LTS 20UL Filter RT-L10FLR	30389226	Rainin
Nuclease-free Water	AM9937	Thermo Fisher Scientific
Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA)	12090-015	Thermo Fisher Scientific
Ethanol, Pure (200 Proof, anhydrous)	E7023-500ML	Millipore Sigma
SPRIselect Reagent Kit	B23318	Beckman Coulter
10% Tween 20	1662404	Bio-Rad
Glycerin (glycerol), 50% (v/v) Aqueous Solution	3290-32	Ricca Chemical Company
Qiagen Buffer EB	19086	Qiagen
High Sensitivity D1000 ScreenTape/Reagents	5067-5592/ 5067-5593	Agilent
High Sensitivity D5000 ScreenTape/Reagents	5067-5584/ 5067-5585	Agilent
Qubit dsDNA HS Assay Kit	Q32854	Thermo Fisher Scientific
PrimeSTAR® GXL DNA Polymerase	R050B	Takara

Primer	Sequence	Supplier
FPSfilA	5'-ACTAAAGGCCATTACGGCCTACACGACGCTCTCCGATCT-3'	Oligo vendor
RPSfilBr	5'-TTACAGGCCGTAATGGCCAAGCAGTGGTATCAACGCAGAGTA-3'	Oligo vendor

BEFORE STARTING

It is recommended that the operator reviews the corresponding User Guide for the 10x Genomics kit being used.

☐ [CG000183_ChromiumSingleCell3_v3_UG_RevB.pdf](#)

☐ [CG000204_ChromiumNextGEMSingleCell3_v3.1_Rev_C.pdf](#)

☐ [CG000086_ChromiumSingleCellIV_D_J_ReagentKits_UG_RevL.pdf](#)

☐ [CG000207_ChromiumNextGEMSingleCellIV_D_J_ReagentKits_v1.1_UG_RevD.pdf](#)

GEM Generation & Barcoding

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- 1.1 Follow the 10x Genomics user guide for Single Cell Gene Expression (v3) or Single Cell Immune Profiling (v1 Chemistry) kits for GEM Generation and Barcoding for the required number of cells.

NOTE: This protocol is also compatible with Next Gem single cell 3' (v3.1 Chemistry) and Single Cell Immune Profiling (v1.1 Chemistry) and also feature barcoding.

- 1.2 After transferring GEMs perform GEM-RT Incubation according to the user guide.

NOTE: In some experiments we have extended RT time up to 2 hours to potentially increase the number of longer transcripts and have not observed any adverse effect. However, we have not systematically investigated if increased incubation time results in higher number of longer transcripts.

Sub-Sampling, Post GEM-RT Cleanup and cDNA Amplification

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- 2.1 Prepare Sub-Sampling Cleanup Mix:
Add 2.5 µl Additive A to 97.5 µl EB (v1 chemistry)
Or Add 2 µl Reducing Agent B to 98 µl EB (v3 chemistry).
- 2.2 Transfer 125 µl Recovery Regent into a fresh well of a 8-strip tube.
- 2.3 Dispense 63 µl of Sub-Sampling Cleanup Mix into well containing Recovery Regent.
- 2.4 Transfer 10-20 µl of GEMs from the middle of the opaque emulsion layer into the Recovery Agent containing the Sub-Sampling Cleanup Mix.

NOTE: This proportion can be adjusted to sub-sample required number of cells. For GEM volumes above 20 µl reduce Sub-Sampling Cleanup Mix volume by 0.7 µl for every 1 µl at step 2.3 and add that volume to remaining GEMs at step 2.7.

- 2.5 Using the same pipette, pipette-mix several times to break the emulsions.
- 2.6 Slowly remove 125 µl of Recovery Agent (pink) from the bottom according to the 10x user guide.
- 2.7 Process remaining GEMs (still in emulsion layer) as per the 10x user guide.
- 2.8 The Post GEM-RT Cleanup step with Dynabeads MyOne SILANE is done according to the v1 or v3 user guide for both fractions.
- NOTE:** Each fraction will be treated as a separate sample and will follow the standard 10x protocol for the remaining steps. In addition to standard protocol, the sub-sample will go through long-read template preparation (Step 5).
- 2.9 Set up cDNA Amplification reactions for both fractions according to the user guide.
- NOTE:** Identical reagents from Chromium Single Cell 5' Library Construction Kit can be used for extra cDNA amplification (Kit dependent).
- 2.10 Adjust the PCR cycle numbers for each fraction based on cell number and carry out cleanup as per the 10x user guide.
- NOTE:** In some experiments we have increased extension time to 3 min to allow efficient extension of longer cDNA fragments but we have not quantified the effect comparatively.
- 2.11 After cleanup save 5-10 ng of the sub-sampled cDNA for long-read template preparation according to step 5 of this protocol. The remainder of the sub-sampled cDNA and main cDNA sample are utilised for short-read sequencing in steps 3 and 4 of this protocol.
- NOTE:** Fractions for feature barcoding (if used) will be sequenced by short-read sequencing only.

Gene Expression Library Construction

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- 3.1 Carry out Gene Expression Library Construction for the main sample and the sub-sample separately according to the user guide.
- NOTE:** Identical reagents from Chromium Single Cell 5' Library Construction Kit can be used for extra library prep.

Sequencing short-read Illumina Libraries

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- 4.1 Sequencing is performed according to the 10x user guide recommendations.

Template Preparation for Long-Read Sequencing

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5.1 Optimise cDNA PCR for long-read library preparation.

Reagent	Amount
5x PrimeSTAR GXL Buffer (Mg2+ plus)	10 µl
dNTP Mixture (2.5 mM each)	4 µl
FPSfIIA (10 µM)	1 µl
RPSfIBr (10 µM)	1 µl
Subsample cDNA	0.5-1 ng
Nuclease-free Water	33 µl - volume of subsample cDNA
PrimeSTAR GXL DNA Polymerase	1 µl
Total	50 µl

In a thermocycler with heated lid, incubate as follows:

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	98	30 sec	1
Denaturation	98	10 sec	8-14 (see below)
Annealing	65	15 sec	
Extension	68	8 min	
Final extension	68	5 min	

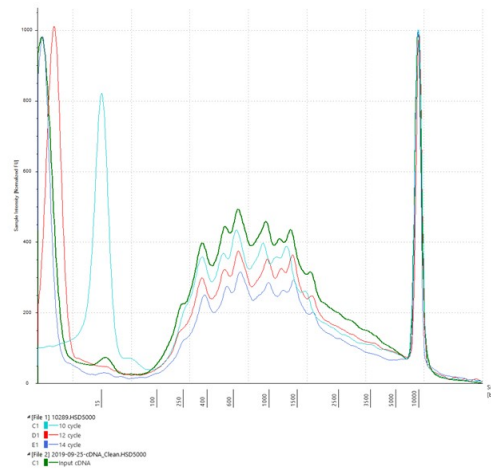
5.2 After 8 cycles take 5 µl out for analysis.

5.3 Repeat from Denaturation for 2 extra cycles and take 5 µl out and continue extra 2 cycles up to 14.

5.4 Quantify 2 µl of each sample taken from different cycles with Qubit dsDNA High Sensitivity assay.

5.5 Dilute each sample to 1 ng/µl and run on HSD5000 Tape.

- 5.6 The optimum number of cycles is that which yields at least 6 ng/μl cDNA without changing the peak distribution in the input cDNA. The following shows a comparison of input cDNA to PCR runs for 10-14 cycles on HSD5000 Tape. In this case all cycles met the peak distribution criteria and we chose 12 cycles for cDNA bulking.



The position of the lower markers were adjusted to overlay peaks that migrate at different speeds due to varying amounts of salts.

Generating a Sufficient Amount of cDNA for Long-Read Library Preparation

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- 6.1 Set up 5 PCR reactions and cycle them to the optimum number obtained from optimisation step.
- 6.2 Pool PCR reactions and add 0.6x SpriSelect beads and mix.
- 6.3 Incubate at room temperature for 5 min.
- 6.4 Place on the magnet until the solution clears.
- 6.5 Remove the supernatant.
- 6.6 To wash add 200 μl freshly prepared 80% ethanol to the pellet. Wait 30 sec.

- 6.7 Remove the ethanol.
- 6.8 Repeat wash step for a total of 2 washes.
- 6.9 Centrifuge briefly and place on the magnet then remove any remaining ethanol.
- 6.10 Air dry for 2 min. Avoid overdrying (beads become cracked).
- 6.11 Remove from the magnet. Add 50 μ l Buffer EB. Pipette mix 15x.
- 6.12 Incubate for 2 min at room temperature.
- 6.13 Place the tube on the magnet until the solution clears.
- 6.14 Transfer 49 μ l of solution to a new tube, without disturbing the beads.
- 6.15 Quantify 1 μ l with Qubit dsDNA HS assay.
- 6.16 QC amplified cDNA by running a 1 ng/ μ l dilution on HSD5000 Tape.

Sequencing Full-Length cDNA

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- 7.1 Prepare library for Nanopore or PacBio sequencing according to the relevant protocol.
- 7.2 Sequencing depth depends on the number of cells captured, sample source, required resolution and sequencing platform. UMI counts from analysis of Illumina reads can be used as a guide. With current chemistry 25% of Nanopore and 65% of PacBio reads can be assigned to cells.



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