08/07/25 Version 3 – John Blair and Carol Dalgarno

PG-Flex

This protocol will take you through performing oligo hashing, intracellular protein staining and guide capture with a 10x Flex kit v1 kit. The initial processing is identical for the 10x FLEX GEM-X kit where there are only changes in the 10x component of the protocols. The pre-processing steps are contained here with the necessary modifications to the 10x protocol attached at the end.

Materials

16% FA – 30 ul/sample – 500 ul

- 219 µl 36.5% Formaldehyde (Sigma: F8775)
- 281 µl 1X PBS (Cytiva: SH30256)

1M Glycine – 68.5 ul/sample – 10 ml

- 750.7 mg Glycine (BioBasic: GB0235)
- 10 ml H2O

1X PBS

Permeabilization buffer (100 ul/ sample - 1 ml)

- 1M Tris-HCl (Thermo: 15567027)- 10 µl
- 5M NaCl (Thermo: AM9759) - 2 µl
- 1M MgCl2 (Sigma: M1787) - 3.33 µl
- 10% NP40 (Thermo: 28324) - 10 µl
- 10% BSA in H2O (Fisher: BP9706100) - 100 µl
- H2O - 874 µl
- RNAse Inhibitor (Lucigen: 30281-1) – 50 µl
- 0.1M DTT (Invitrogen: y00147) - 10 µl

Wash Buffer (#1) (1 ml/ sample - 10 ml)

- 1M Tris-HCl - 100 µl
- 5M Nacl - 20 µl
- 1M MgCl2 - 33.3 µl
- 10% BSA in H2O - 1 ml
- H2O -8850 µl
- RNAse Inhibitor (Lucigen: 30281-1) – 250 µl
- 0.1M DTT (Invitrogen: y00147) - 100 µl

Staining Buffer (100 ul/ sample - 1 ml)

- PBS w/ 3% BSA - 909 µl

Note: Make a 500 ml bottle of this (15 g BSA in 500 ml PBS), filter and store at 4C

- ssDNA - 1 mg (50 µl at 20 mg/ml)

Note: Use a 30 nt oligo that is blocked at the end to reduce random amplification -

NNNNNNNNNNNNNNNNNNNNNNNNNNNN/3ddC/

- RNAse Inhibitor (Lucigen: 30281-1) – 25 µl
- 0.1M DTT (Invitrogen: y00147) - 10 µl

Wash buffer #2 – 6 ml/ sample - 25 ml

- PBS w/ 3% BSA – 24.75 ml
- 10% Tween (BioVision: 2109-100) - 250 µl

10X NEB Buffer 4 (NEB: B7004S) – 10 µl

E.Coli Single-stranded binding protein (SSB) (Promega: M3011) - 8 ug/ug of ab

9% BSA and 3X PBS – 50 ul/ experiment – 10 ml

- 10X PBS (Thermo: 70011-044) – 3 ml
- BSA – 900 mg
- 7 ml H2O

Harvesting Cells

Note: If using adherent cells, process them as you would if you were passaging them. If using frozen cells, thaw them into a solution with a small amount of FBS or BSA to prevent clumping

1.Count cells in whichever solution they are in
2.take 2 million cells total for experiment (or more, just scale volumes up throughout)
3.Centrifuge 300 rcf for 5 mins
4.Resuspend in 475 µl PBS
5.Run through a 40 µm Flowmi cell strainer (Bel-Art: H13680-0040) into a 1.5 ml tube

Fixation and Permeabilization

1.Add 30 µl 16% FA and pipette to mix
2.Leave at RT for 10 mins, swirl/invert occasionally
3.Quench fixation by adding 68.5 µl of 1M Glycine
4.Fill tube with ice cold 1X PBS
5.Centrifuge for 5 mins at 400 rcf at 4C
6.Remove supernatant and add 1 ml cold 1X PBS
7.Centrifuge for 5 mins at 400 rcf at 4C
8.Discard supernatant and resuspend in 100 µl permeabilization buffer
9.Incubate on ice for 5 mins
10.Add 1ml Wash buffer #1, centrifuge for 5 mins at 500 rcf at 4C

Blocking and Hashing

Note: The hashing within this step is optional but blocking is not. Adjust volumes of premade buffer accordingly for more or less hashes.

1.Remove supernatant, and resuspend in 250 µl staining buffer with 15 µl of 200 µM hashing oligo. If you are using multiple hashes for one sample, split each sample before

adding the hashing oligo.
4.Place on the tube rotator for 30 mins at RT
5.In the meantime prepare the SSB-bound antibodies

Binding SSB to antibodies
1.Add an appropriate amount of pooled antibody to a 1.5 ml tube
Note: Usually I add approximately 0.5 µg of antibody mix per antibody – e.g. in a panel of 30 antibodies I would add 15 µg of antibody pool – but this can go as low as 0.1 ug/ab or as high as 1 ug/ab with expected changes in sensitivity.
2.Add H2O to fill up to 100 µl less 10µl and less the amount of SSB you put in (8ug/ug of antibody) e.g. for 20 µl of antibody pool with 10 ug, you will add roughly 16 µl of SSB, so you would add 100- 20 -10 -16 = 54 µl H2O
3.Add 10 µl 10X NEB buffer 4
4.Add SSB and pipette mix until well distributed
5.incubate at 37C for 30 mins
6.Add 50 µl of 3X PBS + 9% BSA to make a final concentration of 3% BSA in 1X PBS

Staining and washes
1.After hashing, centrifuge at 600 rcf for 5 mins and resuspend in 500 µl wash buffer #2
2.Remove supernatant and repeat centrifugation
3.Remove supernatant and resuspend in wash buffer #2, count each sample and combine into one tube (1 million cells total)
Note: You can scale up this reaction if you have excess cells since you can input up to 2 million cells into the FLEX protocol (v1 - it is less in GEM-X).
4.Centrifuge at 600 rcf for 5 mins, remove supernatant
5.Resuspend in the 150 µl of Staining Buffer + ab
6.Put in the tube rotator at RT for 1 hr
7.Centrifuge at 600 rcf for 5 mins, remove super and add 500 µl wash buffer #2. Repeat this step
8.Remove supernatant
9. From here, follow the 10X Fixed RNA Profiling protocol and the then the 10X FLEX Protocol.

• Fixed RNA protocol: https://www.10xgenomics.com/support/single-cell-gene-expression-flex/documentation/steps/sample-prep/fixation-of-cells-and-nuclei-for-Chromium-single-cell-gene-expression-flex
Note: The fixation for the GEM-X is different and uses a different kit and is available on the 10x website

Adjustments to 10X FLEX protocol
1. If capturing guides, or using other custom probes, spike in 5 ul of custom probe working stock, diluted according to the 10x protocols.
2. At step 2.2 (GEM master mix + sample dilution), adjust the number of cells loaded to superload since sample are hashed

3. At step 3.2 (Pre-Amplification PCR) add a 1 ul of 0.2 µM OligoHash additive primer. Note: You should also be using the Feature Barcoding primer set which contains a TruSeq R2 primer to amplify both the guides and the ADTs. If you are doing GEM-X, you will have to spike in your own additive primer.
4. At step 3.3o (SPRI cleanup), the 100 µl elution will ultimately be split into fractions for GEX, OligoHash, GDO and ADT library preparation.

Note: Current 10x protocols use a dual indexing strategy with 10 bp illumina indices. We choose to only use i7 indices at 8bp with no i5 index in order to fit the libraries on a 75 cycle kit. For this, all PCRs below, including the GEX library use the SI-PCR primer and then an individual P7 primer.

Perform the GEX library preparation as written in the 10x protocol with the exception of the primer choice if sequencing using a 75-cycle kit. The correspond primer in this case for GEX will have the following format:
RPNYxx: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

Hashing (OligoHash) or intracellular protein (TSB) library prep
1. Set up separate indexing PCRs for hashing libraries (TSX) and protein libraries (TSB) using the following protocol:

PCR reaction:
50 µl 2X KAPA master mix (KAPA Biosystems: 07958935001)
2.5 µl 10 uM SI-PCR primer
2.5 µl 10 uM TXNYxx (OligoHash) or D7xx (TSB) primer
20 µl input fragments
25 µl H2O

TXNYxx Primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGGAGATGTGTATAAG
D7xx Primer: CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTGACTGGAGTTCAGACGTGTGC
SI-PCR Primer: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC*T*C
X's denote the i7 barcode in the primers

PCR Program:
1.95 C 3 min
2.95 C 20s Repeat steps 2-4 for 12-16 total cycles
3.60 C 30s
4.72 C 20s
5.72 C 5 min
6.4 C Hold
Note: Depending on the number of cells and antibody input into the reaction, I will remove 2 µl of product after 12 cycles and look for product (188-205 bp) on a 2% e-gel. If no product is visible, I will run 2-4 more cycles.
2. After PCR, clean up the reactions with SPRI beads: Add 160 µl SPRI beads to 100 µl

PCR reaction and let bind for 5 mins. Place on magnet and wash 2X with 80% EtOH. Remove all EtOH and resuspend in 20 µl buffer EB. Let bind for 2 mins at RT and place back on the magnet. Remove elute which is your ADT or HTO sequencing library.

Guides Library Prep

Due to the RNA-level quantification of guides, they need to have additional separate amplification. Also, there are a limited number of native Illumina sequencing probes so the ADT libraries and GDO libraries have the same PCR handles on both ends.

PCR reaction:
50 µl 10x Amplification Mix (can also use KAPA as above)
1 µl 10 uM TruSeq F
1 µl 10 uM TruSeq R2_GDO
20 µl input fragments
28 µl H2O

TruSeq F Primer: `CTACACGACGCTCTTCCGATCT`
TruSeq R2_GDO Primer: `GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTATGCTGT`
X's denote the i7 barcode in the primers

PCR Program:
1.98 C 45s
2.98 C 20s Repeat steps 2-4 for 8 total cycles
3.63 C 30s
4.72 C 20s
5.72 C 60s min
6.4 C Hold

After PCR, clean up the reactions with 1.8x SPRI cleanup and resuspend in 30 µl buffer EB. Let bind for 2 mins at RT and place back on the magnet. Remove elute which is the GDO preamp library.

Indexing PCR reaction:
50 µl 2X KAPA master mix (KAPA Biosystems: 07958935001)
2.5 µl 10 uM SI-PCR primer
2.5 µl 10 uM D7xx (TSB) primer
30 µl input fragments from reaction above
15 µl H2O

Note: Be sure to use a different D7NY primer from the ADT library

PCR Program:
1.95 C 3 min

2.95 C 20s Repeat steps 2-4 for 7 total cycles
3.60 C 30s
4.72 C 20s
5.72 C 5 min
6.4 C Hold
Note: Depending on the number of cells input into the reaction, I will remove 2 µl of product after 12 cycles and look for product (188-205 bp) on a 2% e-gel. If no product is visible, I will run 2-4 more cycles.
2. After PCR, clean up the reactions with SPRI beads: Add 160 µl SPRI beads to 100 µl PCR reaction and let bind for 5 mins. Place on magnet and wash 2X with 80% EtOH. Remove all EtOH and resuspend in 20 µl buffer EB. Let bind for 2 mins at RT and place back on magnet. Remove elute which is your GDO sequencing library.

Sequencing
1. Run all libraries on a Bioanalyzer High Sensitivity DNA chip to determine molarity. For GEX libraries, look for a single product around 250 bp. For GDOs, ADTs and HTOs look for strong, clean products at 188-205 bp.
2.Combine the libraries to aim for 10,000 reads per cell for the GEX fraction, 5,000 reads per cell for the ADT fraction, 2,000 reads per cell for the OligoHash fraction and 3,000 reads per cell for the GDO portion
3.Run on whichever sequencer you have available as long as there is >28 bp Read 1, 8 bp i7 index, and >50 bp Read. A 75 cycle NextSeq high-output kit performs well for 20,000 cells for this with 28/8/0/50 sequencing recipe.
4. As the ADT and GDO libraries have the same PCR handles, there will be significant overlap between the two libraries, even with selective amplification. As the internal structure of these libraries is significantly different, when quantifying, you can just combine those two libraries together and each count will easily be separated by their structure.