MULTI-Seq Barcoding and Library Preparation Protocol

Based on McGinnis et. al. 2019. PMID: 31209384 and the 10x Genomics user guide "Chromium Next GEM Single Cell 3' Reagent Kits v3.1 with Feature Barcoding technology for CRISPR Screening"

DPBS – Calcium and Magnesium Free (PBS-CMF)	Corning	MT21031CV
Bovine Serum Albumin	Millipore/Sigma	A3803-50G
SPRIselect Bead-Based Reagent	Beckman Coulter	B23318
Chromium Single Cell 3' Reagent Kits v3 with Feature Barcode technology for CRISPR Screening	10x Genomics	PN-1000075 PN-1000079
Isopropyl alcohol/ Isopropanol(2-propanol)	Fisher Scientific	MFCD00011674
Kapa HiFi HotStart ReadyMix (2X)	Fisher Scientific	NC0295239
Qiagen Buffer EB	Qiagen	19086
SPRIselect Reagent Kit	Beckman Coulter	B23318

Oligonucleotides (store in -20°C for large term storage**):**

- 50 µM Anchor and Co-Anchor
- 10 µM Barcode Oligos
- 10 µM MULTI-seq additive primer
- 10 µM Universal I5 primer
- 10 µM TruSeq RPI primers

Anchor LMO: 5'-TGGAATTCTCGGGTGCCAAGGgtaacgatccagctgtcact-{Lipid}-3'

Co-Anchor LMO: 5'-{Lipid}-AGTGACAGCTGGATCGTTAC-3'

Barcode Oligo: 5'-CCTTGGCACCCGAGAATTCCA**NNNNNNNN**A_{30}-3'

MULTI-seq Primer: 5'-CTTGGCACCCGAGAATTCC-3'

TruSeq RPIX:

5'-CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3'

Universal I5:

5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

1. Make a 10X stock of anchor and barcode strands by mixing at 1:1 molar ratio in PBS-CMF to 2 µM concentration. Pipette to mix.

In total Per sample	22 µL
10 µM unique Barcode Oligo	4.4 µL
50 µM Anchor LMO	0.9 µL
PBS-CMF	16.7 µL

2. Make a 10X solution of the Co-Anchor in in PBS-CMF to a final concentration of 2 µM. Pipette to mix.

In total Per sample	22 µL

50 µM Co-Anchor LMO	0.9 µL
PBS-CMF	21.1 µL

3. Prepare 1% BSA in PBS-CMF and place on ice – at least 3mL per sample
4. Wash cells with PBS-CMF twice.
    a. If adherent – rinse and aspirate on plate.
    b. If suspension cells – centrifuge for 5 min at 200-300g in a 15mL conical tube, carefully aspirate the supernatant, and resuspend in PBS-CMF.
5. Dissociate or lift cells to obtain single cell suspension (will vary depending on sample type).
    a. It is crucial that cells are properly resuspended in buffer at single cell suspension before labeling. For standard cell types, I recommend straining cells through a 40µm mesh before counting and labeling to prevent heterogeneous labeling.
    b. Note: if using Trypsin or similar which is inactivated by the addition of FBS or BSA, rinse the cell suspension with PBS-CMF twice as described in step 4b.
6. Strain single-cell suspension through a 40 µm cell-strainer and count cells.
7. Spin down ~500k cells (or fewer) for 5 min at 200-300g in a 15mL conical tube. Carefully aspirate the supernatant.
8. Barcoding:
    a. Suspend cells in 180 µL of PBS-CMF
    b. Add 20 µL 10X Anchor:Barcode solution and pipette gently to mix.
    c. Incubate on ice for 5 minutes.
    d. Add 20 µL Co-Anchor solution and pipette gently to mix.
    e. Incubate 5 minutes longer.
    f. Add 1 mL of 1% BSA in PBS (ice cold).
9. Transfer each cell sample to a microcentrifuge tube on ice. Keep the labeled cells on ice for the remainder of procedure until starting the 10x workflow to prevent loss of barcodes after washing. The labeling step itself can be done on ice or up to 37 °C.
10. Centrifuge cells for 5 min at 200-300g at 4°C. Remove supernatant and resuspend pellet in additional 1mL of ice cold 1% BSA in PBS-CMF.
11. Repeat step 10 for a total of two wash steps.
12. Filter cell suspension through a 40 µm cell-strainer and count cells again.
13. Combine all samples at desired ratio and continue with scRNA-seq procedure according to 10x Genomics instructions for endogenous transcripts.
14. For each lane of 10X, follow the 10X workflow until cDNA amplification (Step 2.2 in the Chromium Single Cell 3' Reagent Kits v3 with Feature Barcode technology for CRISPR Screening protocol)
    a. During post GEM-RT cleanup the aqueous layer will be cloudy due to the higher BSA concentration we use during our post-barcoding rinses. The BSA helps to quench excess LMO barcodes and limit off-target labeling. This does not cause any issues or negatively affect results.
15. Prepare the following cDNA amplification master mix (volumes per lane):

10X Amp Mix (PN2000047/2000103)	50 µL
Feature cDNA Primers 1 (PN2000096)	15 µL
2.5 µM MULTI-seq primer	1 µL

16. Add 65 µL cDNA Amplification Reaction Mix to 35 µl sample.

17. Perform cDNA amplification according to 10X workflow.
18. To prevent SPRI-bead saturation, add 100 µL water to the 100 µL cDNA amplification mixture. Pipette to mix and split into two tubes of 100 µL volume each.
19. 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).
20. Incubate 5 min at room temperature.
21. Place on the 10x Magnetic Separator in the magnet(High) orientation as described in the 10x workflow until the solution clears.
22. Transfer and save 150 µl supernatant per tube in a new tube strip without disturbing the pellet.
    a. For each sample, you should have two 150 µL supernatant aliquots. One will be used for feature barcode preparation according to the 10X protocol. One will be used for MULTI-seq library preparation
23. Maintain at room temperature. DO NOT discard the transferred supernatant DO NOT discard the pellet.
24. Continue with 2.3A Pellet Cleanup as described in the 10x workflow until step 2.3A.vi.
25. Remove the tube from the magnet. Using 40.5 µL buffer EB, resuspend BOTH SPRI bead pellets per sample by pipetting each pellet up and down 15x to mix thoroughly, combining into a single tube and proceed with protocol for endogenous transcripts without change.
26. Use one 150 µL supernatant aliquot for step 2.3B Transferred Supernatant Cleanup as described in the 10x workflow and proceed to Feature Barcode Library prep without change.
27. Transfer remaining 150 µL supernatant to fresh 1.5 mL microcentrifuge tube. Add 260 µL SPRI beads and 180 µL 100% isopropanol (for a final ratio of 1.8X SPRI). Pipette mix 10 times, incubate at room temperature for 5 minutes.
28. Place tube on magnetic rack and wait for solution to clear.
29. Remove and discard supernatant.
30. Wash beads twice on magnet with 500 µL of 80% ethanol and allow to stand for 30 seconds between washes.
31. After second wash, briefly centrifuge beads and place back on magnetic rack.
32. Remove any remaining ethanol with P10 micropipette.
33. Air-dry beads on magnet for 2 minutes. Do NOT exceed 2 minutes.
34. Remove from magnet, resuspend beads in 50.5 µL buffer EB and pipette mix thoroughly to resuspend.
35. Incubate at room temperature for 2 minutes.
36. Return to magnet and wait for solution to clear.
37. Transfer 50 µL supernatant to PCR strip tube, pipetting carefully to avoid transferring beads.
38. Quantify barcode DNA concentration using Qubit (typical range is 0.5 - 5 ng/µL).
39. For each lane, prepare the following PCR mix:

Kapa HiFi HotStart ReadyMix (2X)	26.25 µL
10 µM Universal I5 primer	2.5 µL
10 µM unique RPI primer *(choose unique RPI for each sample from 10X lane)*	2.5 µL

barcode DNA *(volume based on concentration from Qubit)*	3.5 ng
Nuclease-free water	To 50 µL

40. Perform library preparation PCR:

1	95 °C	5 min
2	98 °C	15 sec
3	60 °C	30 sec
4	72 °C	30 sec
5	Repeat steps 2-5	8-12 times
6	72 °C	1 min
7	4 °C	hold

41. Add 80 µL (1.6X) SPRI to each PCR product, pipette mix thoroughly.
42. Incubate at room temperature for 5 minutes.
43. Place tube on 10x Magnetic Separator in the magnet (HIGH) orientation, wait for solution to clear.
44. Remove and discard supernatant.
45. Wash beads twice on 10x Magnetic Separator in the magnet (HIGH) orientation with 200 µL of 80% ethanol and allow to stand for 30 seconds between washes.
46. After second wash, briefly centrifuge beads, and invert the 10x Magnetic Separator to place the tubes on the 10x Magnetic Separator in the magnet (LOW) orientation.
47. Remove any remaining ethanol with P10 micropipette.
48. Air-dry beads on magnet for 2 minutes. Do NOT exceed 2 minutes.
49. Remove from magnet, resuspend beads in 25 µL buffer EB and pipette mix thoroughly to resuspend.
50. Incubate at room temperature for 2 minutes.
51. Return to 10x Magnetic Separator in the magnet (LOW) orientation, wait for solution to clear, and transfer supernatant to PCR strip tube.
52. Quantify barcode library concentration by running 1ul diluted 1:5 on an Agilent Bioanalyzer High Sensitivity chip . Expected library size is approximately 175bp.

Sequencing:

a. Barcodes can be sequenced independently or as fraction of endogenous cDNA library.
b. Target 3000-5000 barcode reads per cell.

53. Deconvolute barcodes for analysis as described at https://github.com/chris-mcginnis-ucsf/MULTI-seq