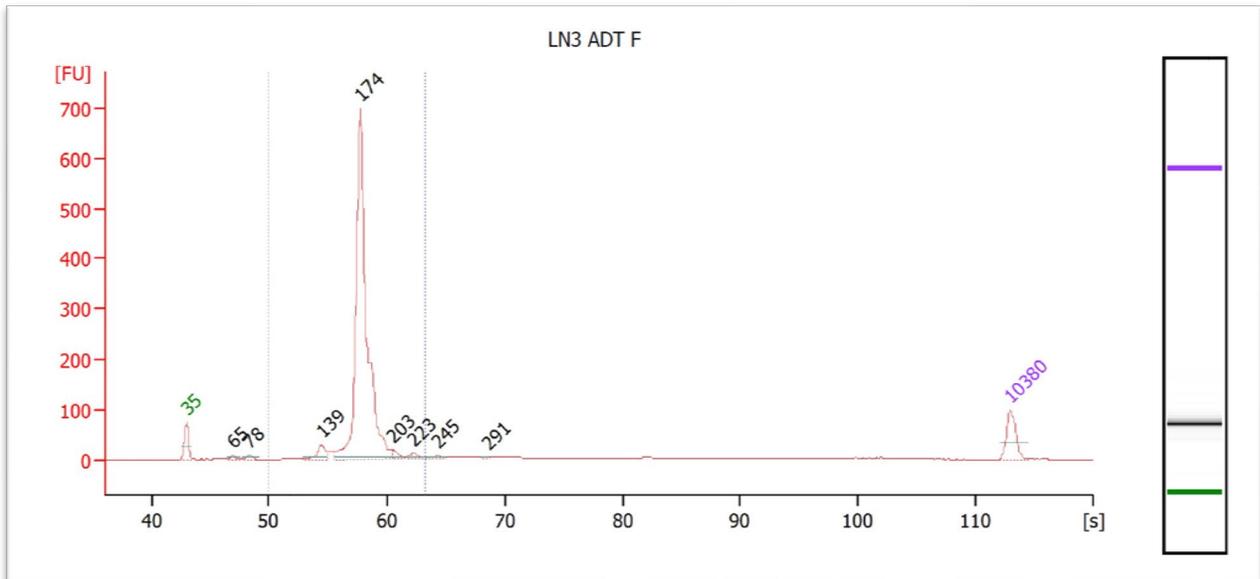


Construction of libraries

This guide will explain the few modifications to the standard 10X workflow necessary for amplification of the zipcode sequences. Many sections are near-identical to the protocols written by (M. Stoeckius and P. Smibert 2018) and (C. McGinnis and D. Patterson 2018) It will also describe sequencing parameters, and analysis approaches we have used. This manual was written to be in-line with the 10X v3 3' Chemistry user guide REV C CG000183, but is generalizable to NEXTGEM 3' as well. Note that we currently are testing compatibility with 5' chemistry but this is not formalized yet.

1. Following encapsulation, carry forth to step 2.2, cDNA Amplification. Here we will spike in an additive primer a la CITE-Seq to amplify off the Read2 handle which is distinct from the Read2 sequence used for the cDNA.
 - a. Add 1 uL of 0.2 uM of the additive primer (#0001) in to the cDNA Amplification Mix. This additional 1 uL into 100 uL will not affect the amplification reaction appreciably we've found.
2. Proceed to the cleanup step after cDNA amp 2.3. **IMPORTANT: RETAIN THE SUPE and transfer it to a DNA Lo-Bind 1.5 mL Eppendorf Tube** There should be 150-160 uL of supernatant at this step. Here is where we deviate a bit from CITE and MULTI-Seq protocols. We've found two sequential 3X SPRI cleanups to be the best balance between capturing as much zipcode as possible and not retaining a bunch of smaller DNA contaminants.
 - a. For this, simply add 240 uL of SPRI to the 160 uL of supe to reach a final volume ~400uL which represents a 3X SPRI.
 - b. Wait 10 min, put on magnet and remove supernatant. Millipore sells a nice magnetic rack to hold 1.5mL eppendorfs. LSKMAGS08
 - c. Wash once with 400 uL of 80% EtOH
 - d. Briefly spin down, remove excess EtOH, resuspend in 50 uL water, incubate 2 min, no need to clear the beads out
 - e. Add 150 uL of SPRI on top of this for another 3X SPRI. 10 min incubation, then remove supernatant, and wash twice with 250 uL of 80% EtOH
 - f. Spin down and remove excess EtOH. Let air dry for 1 min, then resuspend in 40 uL of water, wait 2 min, and transfer to new clean tube.
3. Proceed to amplification of Zipcode sequences with addition of the full-length Read2 sequence and P5/7 clustering adaptors:
 - a. Prepare the following mix:
 - i. 20 µL of the purified Zipcodes from above (carryover half)
 - ii. 2.5 µL of SI-PCR (identical to the one 10X provides) (#0010) @ 10µM
 - iii. 2.5 µL of Illumina Small RNA RPI(n) primer (#0011-N)@ 10µM
 - iv. 25 µL nuclease free water
 - v. 50 µL of KAPA HiFi Hotstart ReadyMix 2X

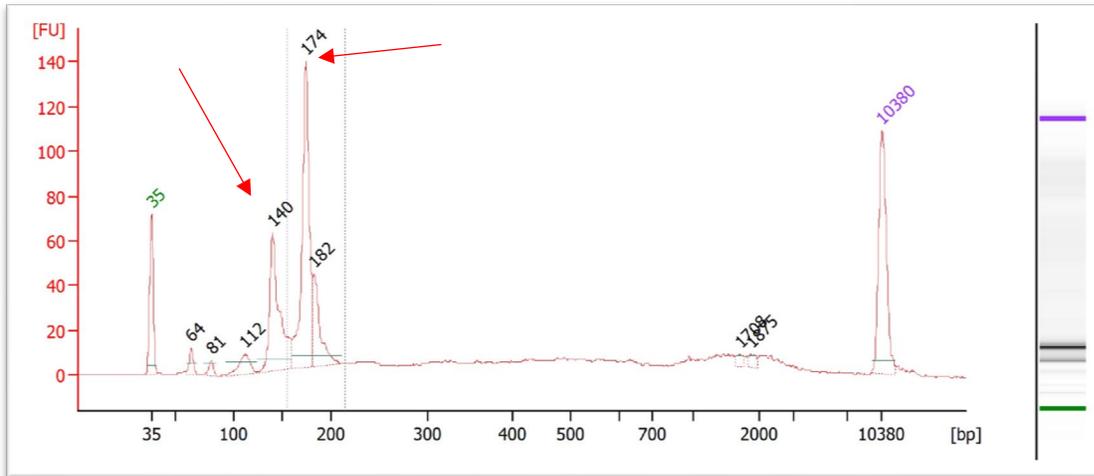
- b. Cycle with the following parameters:
 - i. 95°C – 5 min.
 - ii. 98°C – 5 min.
 - iii. 60°C – 5 min.
 - iv. 72°C – 5 min.
 - v. Repeat ii-iv 11-13x
 - vi. 72°C – 1 min.
 - vii. 4°C – hold
4. Purify amplified barcode products from step 3:
 - a. Add 160 μ L of SPRI select and mix to the amplification mix from step 3
 - b. Wait 10 min, place on magnet, remove supernatant
 - c. Wash twice with 200 μ L of 80% EtOH
 - d. After removing the second wash of EtOH, briefly spin down, place on magnet, pipette any residual liquid and let air dry 1 minute
 - e. Resuspend in 25 μ L of water, let sit for 2min.
 - f. Place on magnet, pipette out your eluted barcode product and store @ -20°C alongside the GEX library.
5. Proceed to quantification of the GEX and Zipcode libraries using your tool of choice. Bioanalyzer, Fragment Analyzer, Tapestation etc. Your traces should resemble these, with a sharp peak @ 172-175 bp:



Experiment: CD45 anchored oligos on mouse lymph node section, encapsulated only labeled, live CD45+ cells. Following 12 rounds of amplification in step 3, diluted 5x before loading in DNA High Sensitivity Chip on the Agilent Bioanalyzer:

Troubleshooting: Occasionally, you may observe a significant peak at around 140 bp. This is likely the 'TSO-RT-oligo product' described by Stoeckius and Smibert in their CITE-Seq protocol. Because our 3X SPRI select is even more permissive than their 2X one, it is possible this is more prevalent in our

protocol. Nevertheless, the solution is the same, reamplify 2-3 cycles with a P5/P7 primer pair (PN#) and repeat the 1.6x SPRI cleanup



In addition, another phenomenon is the appearance of a ~ 400bp broad peak. The etiology is unclear, Mcginnis and Patterson suggest this is due to carryover of cDNA which didn't get stuck on the beads in the post-cDNA amp SPRI select. Another explanation is the formation of daisy chains or bubble products which can form when overamplifying i.e. when primer or dNTP's become limiting and strands denature and reanneal in funny ways. Nevertheless, the solution remains the same, reamplify 2-3 cycles with P5/P7 primer pair (#0012 and #0013) in excess and repeat the 1.6X SPRI cleanup

Example cycling parameters for re-amplification:

- a. 12.5 μ L purified, amplified zipcode product (half of the material from the previous purification)
- b. 5 μ L of 10 μ M P5 FWD primer (#0012)
- c. 5 μ L of 10 μ M P7 REV primer (#0013)
- d. 27.5 μ L of nuclease free water
- e. 50 μ L of 2x KAPA HiFi ReadyMix

Same cycling parameters as above for 2-3 cycles. Repeat the 1.6X SPRI cleanup.

Sequencing:

1. Similar to other protocols, we recommend mixing the ZipCode library to GEX at a 1:10 molar ratio for sequencing. Aim for a target of at least 500 reads per cell.

* Example: Consider a lane of an SP flowcell run on the NovaSeq which is advertised to run 400M reads. Approximately 40M reads will go towards the ADT and 360M towards GEX. With a targeted cell # of 10k, this results in 36k reads of GEX per cell, and 4000 ADT per cell. However a significant % of these ADT reads are not actually cell associated (either floating around and got RT'd or stuck on debris or dead cells). See below as 'Antibody Reads in Cells'. Note that we started with only 26.5 M reads.

Antibody Sequencing [?]		Antibody Application [?]	
Number of Reads	26,567,149	Fraction Antibody Reads	95.4%
Valid Barcodes	98.9%	Fraction Antibody Reads Usable	63.5%
Valid UMIs	100.0%	Antibody Reads Usable per Cell	2,445
Sequencing Saturation	54.9%	Fraction Reads in Barcodes with High UMI Counts	0.0%
Q30 Bases in Barcode	97.1%	Fraction Unrecognized Antibody	4.6%
Q30 Bases in Antibody Read	52.4%	Antibody Reads in Cells	67.3%
Q30 Bases in UMI	97.0%	Median UMIs per Cell (summed over all recognized antibody barcodes)	723

Oligo Parts List for library construction:

Additive Primer (to be added during the cDNA amp step) (PN#0001)
GCACCCGAGAATTCCA
SI-PCR Primer FWD (PN#0010)
AATGATACGGCGACCACCGAGATCTACACTCTTCCCTACACGACGCTC
Illumina Small RNA RPI(n) Primer REV (Bolded represents the 8 bp sample index i7 sequence. Use the reverse complement of this in your sample sheet for demultiplexing. Ensure it does not overlap with other library i7 indices.) (PN#0011-N)
CAAGCAGAAGACGGCATACTGAG ATNNNNNN GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
P5 Primer FWD for re-amp (PN#0012)
AATGATACGGCGACCACCGA
P7 Primer REV for re-amp (PN#0013)
CAAGCAGAAGACGGCATACTGAGAT