CITE-seq and Cell Hashing

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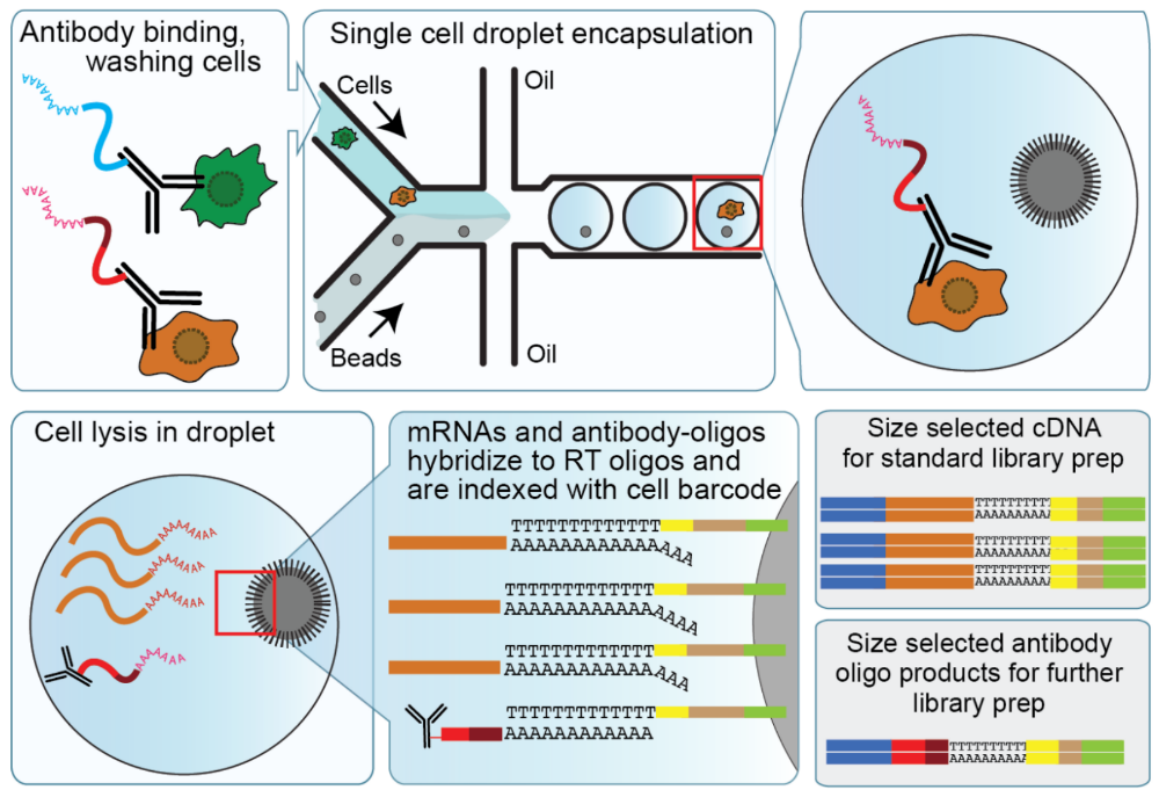
Abstract

This protocol is for performing CITE-seq and Cell Hashing in parallel.

CITE-seq:

Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) is a multimodal single cell phenotyping method developed in the Technology Innovation lab at the New York Genome Center in collaboration with the Satija lab.

CITE-seq uses DNA-barcoded antibodies to convert detection of proteins into a quantitative, sequenceable readout. Antibody-bound oligos act as synthetic transcripts that are captured during most large-scale oligodT-based scRNA-seq library preparation protocols (e.g. 10x Genomics, Drop-seq, ddSeq).

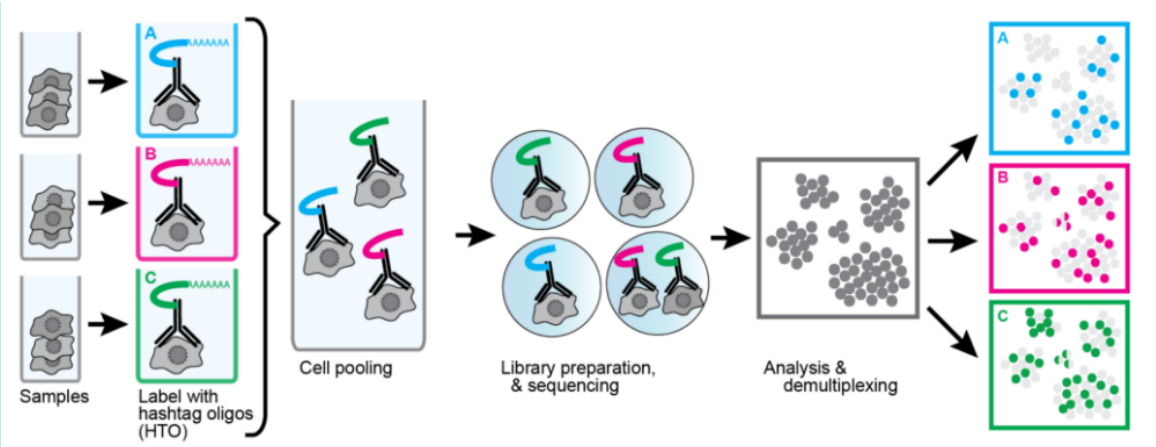


This allows for immunophenotyping of cells with a potentially limitless number of markers and unbiased transcriptome analysis using existing single-cell sequencing approaches.

Cell Hashing:

Sample multiplexing and super-loading on single cell RNA-sequencing platforms.

Cell Hashing uses a series of oligo-tagged antibodies against ubiquitously expressed surface proteins with different barcodes to uniquely label cells from distinct samples, which can be subsequently pooled in one scRNA-seq run. By sequencing these tags alongside the cellular transcriptome, we can assign each cell to its sample of origin, and robustly identify doublets originating from multiple samples.



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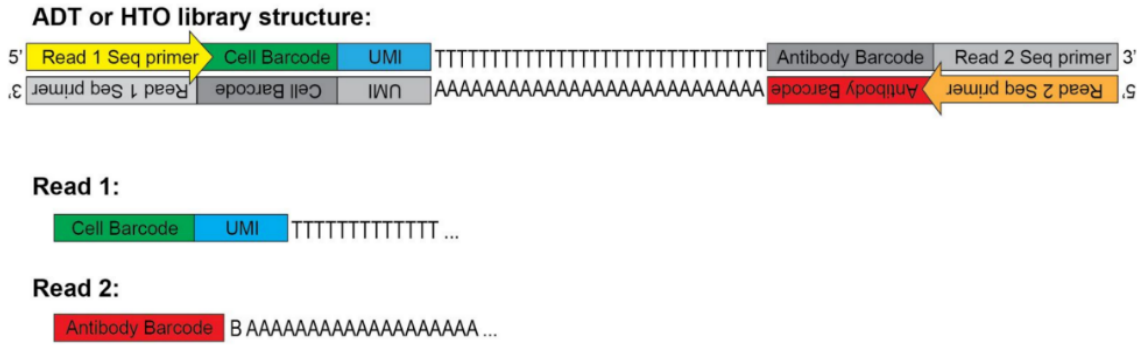
Guidelines

For experiments involving cell hashing, we recommend using the cost per cell calculator from the Satija Lab to plan experiments, determine number of hashes, number of cells to load, expected doublet rates (detected and undetected) and cost considerations.

Sequencing CITE-seq and Hashing libraries:

We estimate that an average of 100 molecules per ADT or HTO per cell is sufficient to achieve useful information, we typically sequence our ADT / HTO libraries to obtain significantly more reads than this per cell. The number of reads required to obtain 100 molecules depends on the complexity of the sequencing library (e.g. duplication rate). ADT, HTO and cDNA sequencing libraries can be pooled at desired proportions. We typically sequence ADT at 10% and HTO libraries at 5% of a lane and cDNA library fraction at 85% of a lane (HiSeq2500 Rapid Run Mode Flowcell).



Oligonucleotide sequences:

CITE-seq antibody-oligos (ADTs):

CITE-seq antibody-oligos contain standard small TruSeq RNA read 2 sequences and can be amplified using Illumina's Truseq Small RNA primer sets (RPIx – primers, see example RPI1 below). See example below with a 12nt barcode:

5'CCTTGGCACCCGAGAATTCCACATGATTGGCTCBAAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Hashtag barcoding antibody-oligos (HTOs):

Cell Hashing antibody-oligos contain standard TruSeq DNA read 2 sequences and can be amplified using truncated versions of Illumina's TruSeq DNA primer sets (see example D701_s below). See example below with a 12nt barcode:

5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Oligos required for ADT and HTO library amplification:

- Drop-seq P5-SMART-PCR hybrid primer (for Drop-seq only)
  5'AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGT*A*C
- 10x Genomics SI-PCR primer (for 10x Single Cell Version 2 only)
  5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC*T*C
- ADT cDNA PCR additive primer
  5'CCTTGGCACCCGAGAATT*C*C
- HTO cDNA PCR additive primer
  5'GTGACTGGAGTTCAGACGTGTGC*T*C
- Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences © 2015 Illumina, Inc)
  5'CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A

- Illumina TruSeq D701_s primer (for HTO amplification; i7 index 1, shorter than the original Illumina sequence)
  5′ CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGT*G*C

  * Phosphorothioate bond
  B C or G or T; not A nucleotide

Before start

Prepare Staining buffer (2%BSA/0.02%Tween, PBS).

Materials

FC blocking reagent (FcX) by BioLegend

8-strip PCR tubes, emulsion safe (!) (e.g. TempAssure PCR 8-strips) by USA Scientific

Bioanalyzer chips and reagents (DNA High Sensitivity kit) by Agilent Technologies

SPRIselect reagent B23317 by Ge Healthcare

E-gel 4% by Invitrogen - Thermo Fisher

✓ Low-bind 1.5 mL tubes by Contributed by users

PCR Thermocycler (e.g. T100) by BioRad Sciences

Magnetic tube rack by Invitrogen - Thermo Fisher

Qubit by Invitrogen - Thermo Fisher

✓ Hemocytometer (e.g. Fuchs Rosenthal) by Contributed by users

✓ DMSO by Contributed by users

✓ PBS by Contributed by users

✓ Tween20 by Contributed by users

✓ TE pH 8.0 by Contributed by users

BSA (DNAse, RNAse and protease free) 0332-25G by Vwr

Dead Cell Removal Kit by Miltenyi Biotec

✓ 80% Ethanol by Contributed by users

Protocol

Cell staining for Drop-seq or 10x Genomics

Step 1.

Obtain all single cell suspensions from different samples/conditions that will be multiplexed in the run. Keep samples in separate tubes until after cell hashing and shortly before loading cells into the single cell RNA-seq instrument. When aiming to super-load the same sample into one run, divide the sample up into equal proportions before staining with distinct cell hashing antibodies. Keep cell suspensions

on ice (unless otherwise stated) at all times.

Cell staining for Drop-seq or 10x Genomics

Step 2.

Carefully count all cells to ensure accurate quantitation.

● Make note of cell viability (>95%) and also include dead cells in the total cell count.

● If you observe many dead cells, live cell enrichment (e.g. Dead Cell Removal kit) is recommended.

Cell staining for Drop-seq or 10x Genomics

Step 3.

Resuspend 1-2 million cells in 100 µl Staining buffer (2%BSA/0.02%Tween, PBS).

AMOUNT

100 µl Additional info: Staining buffer

Cell staining for Drop-seq or 10x Genomics

Step 4.

Add 10 µl Fc Blocking reagent (FcX, BioLegend).

AMOUNT

10 µl Additional info: Fc Blocking reagent

Cell staining for Drop-seq or 10x Genomics

Step 5.

Incubate for 10 minutes at 4˚C.

TEMPERATURE

4 °C Additional info: Incubation

Cell staining for Drop-seq or 10x Genomics

Step 6.

While cells are incubating in Fc Block, prepare antibody-pool using 0.5 - 1 µg (or titrated amounts) of each CITE-seq antibody and 1 µg of single cell hashing antibody (pool).

Cell staining for Drop-seq or 10x Genomics

Step 7.

Add **antibody-oligo pool** to cells.

Cell staining for Drop-seq or 10x Genomics

Step 8.

Incubate for 30 minutes at 4˚C.

TEMPERATURE

4 °C Additional info: Incubation

Cell staining for Drop-seq or 10x Genomics

Step 9.

Wash cells with 1 mL Staining buffer. (wash 1/3)

AMOUNT

1 ml Additional info: Staining buffer

Cell staining for Drop-seq or 10x Genomics

Step 10.

Spin 5 minutes 400g at 4˚C. (wash 1/3)

TEMPERATURE

4 °C Additional info: Spinning

Cell staining for Drop-seq or 10x Genomics

Step 11.

Wash cells with 1 mL Staining buffer. (wash 2/3)

AMOUNT

1 ml Additional info: Staining buffer

Cell staining for Drop-seq or 10x Genomics

Step 12.

Spin 5 minutes 400g at 4˚C. (wash 2/3)

TEMPERATURE

4 °C Additional info: Spinning

Cell staining for Drop-seq or 10x Genomics

Step 13.

Wash cells with 1 mL Staining buffer. (wash 3/3)

AMOUNT

1 ml Additional info: Staining buffer

Cell staining for Drop-seq or 10x Genomics

Step 14.

Spin 5 minutes 400g at 4˚C. (wash 3/3)

TEMPERATURE

4 °C Additional info: Spinning

Cell staining for Drop-seq or 10x Genomics

Step 15.

Resuspend cells in PBS at appropriate concentration for downstream application.

NOTES

Peter Smibert 27 Feb 2018

E.g. for **10x** ~500 cells/µl; for **Drop-seq** [~200 cells/µl]; for **super-loading** ~1,500 cells/µl or higher.

Cell staining for Drop-seq or 10x Genomics

Step 16.

Filter cells through 40 µm strainers (e.g. Flowmi cell strainer).

Cell staining for Drop-seq or 10x Genomics

Step 17.

Verify cell concentration by counting on hemocytometer after filtration.

Cell staining for Drop-seq or 10x Genomics

Step 18.

Pool all different samples/conditions at desired proportions and immediately proceed to next step.

Step 19.

Run Drop-seq (Macosko et al., 2015) or 10x Genomics single cell 3' v2 assay as described until before cDNA amplification.

cDNA amplification

Step 20.

Add "additive" primers to cDNA PCR to increase yield of ADT and/or HTO products:

ADT PCR additive primer (2 µM): 1 µl (for 10x Genomics) or 0.4 µl (for Drop-seq)

HTO PCR additive primer (1 µM): 1 µl (for 10x Genomics) or 0.4 µl (for Drop-seq)

Subtract the total volume of additive primer from the water added to the PCR reaction.

Separating ADT / HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)

Step 21.

Perform SPRI selection to separate mRNA-derived and antibody-oligo-derived cDNAs.

Separating ADT / HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)

Step 22.

DO NOT DISCARD SUPERNATANT FROM 0.6X SPRI. THIS CONTAINS THE ADTs and hashtags!

Separating ADT / HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
Step 23.

Add 0.6X SPRI to cDNA reaction as described in 10x Genomics or Drop-seq protocol.

Separating ADT / HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
Step 24.

Incubate 5 minutes and place on magnet.

Separating ADT / HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
Step 25.

● **Supernatant** contains ADTs and hashtags.

● **Beads** contain full length mRNA-derived cDNAs.

mRNA-derived cDNA >300bp (beads fraction)
Step 26.

Proceed with **standard 10x or Drop-seq protocol** for cDNA sequencing library preparation.

For ADTs and Hashtags <180bp (supernatant fraction), follow the sections below.

Purifying ADTs using two 2X SPRI purifications
Step 27.

To purify ADTs using two 2X SPRI purifications per manufacturer protocol, first, add 1.4X SPRI to supernatant to obtain a final SPRI volume of 2X SPRI.

Purifying ADTs using two 2X SPRI purifications
Step 28.

Transfer entire volume into a low-bind 1.5 mL tube.

Purifying ADTs using two 2X SPRI purifications
Step 29.

Incubate 10 minutes at room temperature.

Purifying ADTs using two 2X SPRI purifications
Step 30.

Place tube on magnet and wait 2 minutes until solution is clear

Purifying ADTs using two 2X SPRI purifications

Step 31.

Carefully remove and discard the supernatant.

Purifying ADTs using two 2X SPRI purifications

Step 32.

Add 400 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (only one Ethanol wash).

AMOUNT

400 µl Additional info: 80% Ethanol

Purifying ADTs using two 2X SPRI purifications

Step 33.

Carefully remove and discard the ethanol wash.

Purifying ADTs using two 2X SPRI purifications

Step 34.

Centrifuge tube briefly and return it to magnet.

Purifying ADTs using two 2X SPRI purifications

Step 35.

Remove and discard any remaining ethanol.

Purifying ADTs using two 2X SPRI purifications

Step 36.

Resuspend in beads in 50 µl water.

AMOUNT

50 µl Additional info: Water

Purifying ADTs using two 2X SPRI purifications

Step 37.

Perform another round of 2X SPRI purification by adding 100 µl SPRI reagent directly onto resuspended beads.

AMOUNT

100 µl Additional info: SPRI reagent

Purifying ADTs using two 2X SPRI purifications

Step 38.

Mix by pipetting.

Purifying ADTs using two 2X SPRI purifications

Step 39.

Incubate 10 minutes at room temperature.

Purifying ADTs using two 2X SPRI purifications

Step 40.

Place tube on magnet and wait 2 minutes until solution is clear.

Purifying ADTs using two 2X SPRI purifications

Step 41.

Carefully remove and discard the supernatant.

Purifying ADTs using two 2X SPRI purifications

Step 42.

Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (1st Ethanol wash).

AMOUNT

200 µl Additional info: 80% Ethanol

Purifying ADTs using two 2X SPRI purifications

Step 43.

Carefully remove and discard the ethanol wash.

Purifying ADTs using two 2X SPRI purifications

Step 44.

Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (2nd Ethanol wash).

AMOUNT

200 µl Additional info: 80% Ethanol

Purifying ADTs using two 2X SPRI purifications

Step 45.

Carefully remove and discard the ethanol wash.

Purifying ADTs using two 2X SPRI purifications

Step 46.

Centrifuge tube briefly and return it to magnet.

Purifying ADTs using two 2X SPRI purifications

Step 47.

Remove and discard any remaining ethanol.

Purifying ADTs using two 2X SPRI purifications

Step 48.

Allow the beads to air dry for 2 minutes (do not over dry beads).

Purifying ADTs using two 2X SPRI purifications

Step 49.

Resuspend beads in 90 µl water.

AMOUNT

90 µl Additional info: Water

Purifying ADTs using two 2X SPRI purifications

Step 50.

Pipette mix vigorously.

Purifying ADTs using two 2X SPRI purifications

Step 51.

Incubate mix at room temperature for 5 minutes.

Purifying ADTs using two 2X SPRI purifications

Step 52.

Place tube on magnet and transfer clear supernatant into two PCR tubes.

Amplifying ADT sequencing library

Step 53.

Prepare 100 µl PCR reaction with purified ADTs as follows:

First, add 45 µl purified ADT/Hashtag fraction.

Reagent	Amount
purified ADT/Hashtag fraction	45 µl
2x KAPA Hifi PCR Master Mix	50 µl
TruSeq Small RNA RPIx primer (containing i7 index) 10 µM	2.5 µl
P5 oligo at 10 µM depending on application*	2.5 µl

* For Drop-seq use P5-SMART-PCR hybrid oligo. For 10x use SI PCR oligo.

AMOUNT

45 µl Additional info: Purified ADT/Hashtag fraction

Amplifying ADT sequencing library

Step 54.

Add 50 µl 2x KAPA Hifi PCR Master Mix.

AMOUNT
50 µl Additional info: 2x KAPA Hifi PCR Master Mix

Amplifying ADT sequencing library

Step 55.

Add 2.5 µl TruSeq Small RNA RPIx primer (containing i7 index) 10 µM.

AMOUNT
2.5 µl Additional info: TruSeq Small RNA RPIx primer (containing i7 index) 10 µM

Amplifying ADT sequencing library

Step 56.

2.5 µl P5 oligo at 10 µM depending on application:

■ For Drop-seq use **P5-SMART-PCR hybrid** oligo.

■ For 10x use **SI PCR oligo**.

Amplifying ADT sequencing library

Step 57.

Cycling conditions:

95˚C	3 min	
95˚C	20 sec	|
60˚C	30 sec	| 6-10 cycles
72˚C	20 sec	|
72˚C	5 min	

Amplifying HTO sequencing library

Step 58.

To prepare 100 µl of PCR reaction with purified small fraction, first, add 45 µl purified ADT/Hashtag fraction.

Reagent	Amount
purified ADT/Hashtag fraction	45 µl
2x KAPA Hifi PCR Master Mix	50 µl
TruSeq DNA D7xx_s primer (containing i7 index) 10 µM	2.5 µl
P5 oligo at 10 µM depending on application*	2.5 µl

* For Drop-seq use P5-SMART-PCR hybrid oligo. For 10x use SI PCR oligo.

AMOUNT

45 µl Additional info: Purified ADT/Hashtag fraction

Amplifying HTO sequencing library

Step 59.

Add 50 µl 2x KAPA Hifi PCR Master Mix.

AMOUNT

50 µl Additional info: 2x KAPA Hifi PCR Master Mix

Amplifying HTO sequencing library

Step 60.

Add 2.5 µl TruSeq DNA D7xx_s primer (containing i7 index) 10 µM.

AMOUNT

2.5 µl Additional info: TruSeq DNA D7xx_s primer (containing i7 index) 10 µM

Amplifying HTO sequencing library

Step 61.

Add 2.5 µl P5 oligo at 10 µM depending on application:

■ For Drop-seq use **P5-SMART-PCR hybrid** oligo.

■ For 10x use **SI PCR** oligo.

Amplifying HTO sequencing library

Step 62.

Cycling conditions:

95˚C 3 min 95˚C 20 sec 64˚C 30 sec 72˚C 20 sec 72˚C 5 min	8-12 cycles

Purifying PCR product

Step 63.

Purify PCR product using 1.6X SPRI purification by adding 160 µl SPRI reagent.

AMOUNT

160 µl Additional info: SPRI reagent

Purifying PCR product

Step 64.

Incubate 5 minutes at room temperature.

Purifying PCR product

Step 65.

Place tube on magnet and wait 1 minute until solution is clear.

Purifying PCR product

Step 66.

Carefully remove and discard the supernatant.

Purifying PCR product

Step 67.

Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (1st Ethanol wash).

AMOUNT

200 µl Additional info: 80% Ethanol

Purifying PCR product

Step 68.

Carefully remove and discard the ethanol wash.

Purifying PCR product

Step 69.

Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (2nd Ethanol wash).

AMOUNT

200 µl Additional info: 80% Ethanol

Purifying PCR product

Step 70.

Carefully remove and discard the ethanol wash.

Purifying PCR product

Step 71.

Centrifuge tube briefly and return it to magnet.

Purifying PCR product

Step 72.

Remove and discard any remaining ethanol.

Purifying PCR product

Step 73.

Allow the beads to air dry for 2 minutes.

Purifying PCR product

Step 74.

Resuspend beads in 20 µl water.

AMOUNT

20 µl Additional info: Water

Purifying PCR product

Step 75.

Pipette mix vigorously.

Purifying PCR product

Step 76.

Incubate mix at room temperature for 5 minutes.

Purifying PCR product

Step 77.

Place tube on magnet and transfer clear supernatant to PCR tube.

Step 78.

ADT and Hashtag libraries are now ready to be sequenced.

Quantify libraries by standard methods (QuBit, BioAnalyzer, qPCR).

EXPECTED RESULTS

ADT and Hashtag libraries will be around 180 bp (Figure 1 and 2).

Figure 1.

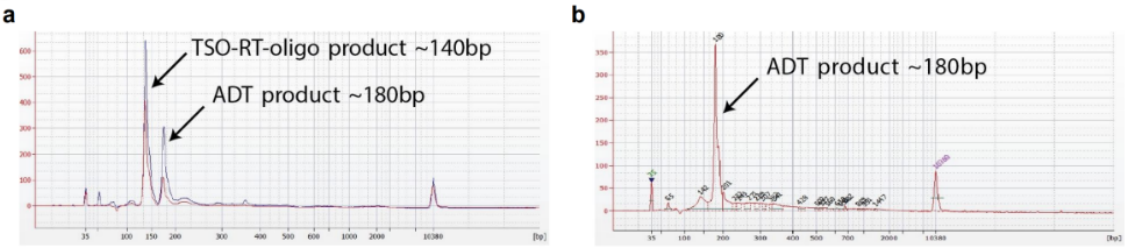


Figure 1. ADT (or Hashtag) library verification. (a) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X SPRI purification of the ADT fraction after cDNA amplification reduces carryover of primers from cDNA amplification, and minimizes the amplification of this product during ADT-library amplification. To further enrich for ADT (or HTO) specific product the purified ADT library can be reamplified for ~3 additional cycles with ADT specific primer sets or P5/P7 generic primers. (**b**) A clean ADT (or HTO) library will contain a predominant single peak at around 180 bp.

Figure 2.

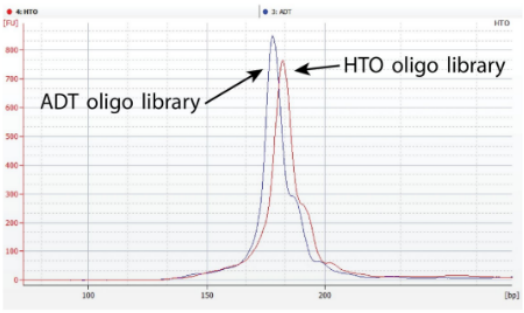


Figure 2. Verification of ADT and hashtag libraries. ADT and Hashtag libraries are very similar in size ~180bp but should appear as distinguishable products on a High Sensitivity Bioanalyzer, where the Hashtag library appears a few nucleotides larger compared to the ADT library.

Warnings

Please refer to the SDS (Safety Data Sheet) for hazard information.