CITE-seq

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Abstract

This protocol is for performing CITE-seq only.

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).

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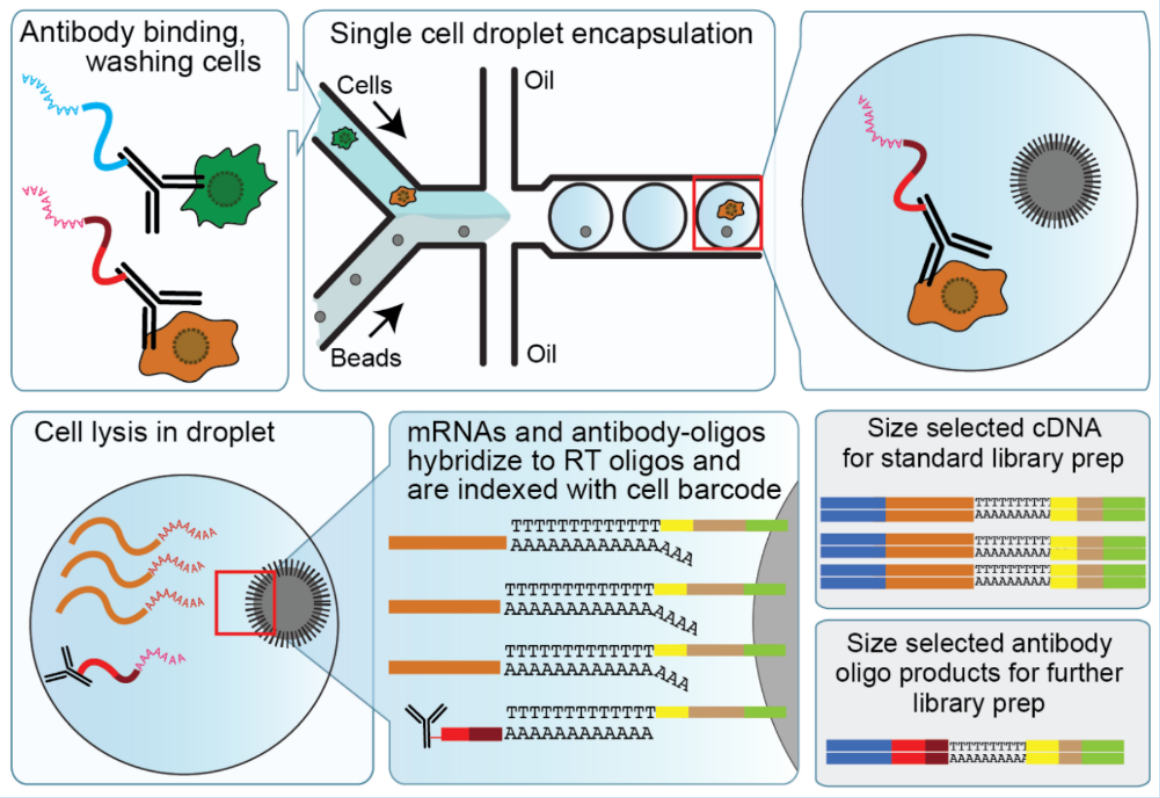

This allows for immunophenotyping of cells with a potentially limitless number of markers and unbiased transcriptome analysis using existing single-cell sequencing approaches.

Citation: Marlon Stoeckius CITE-seq. **protocols.io**
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Guidelines

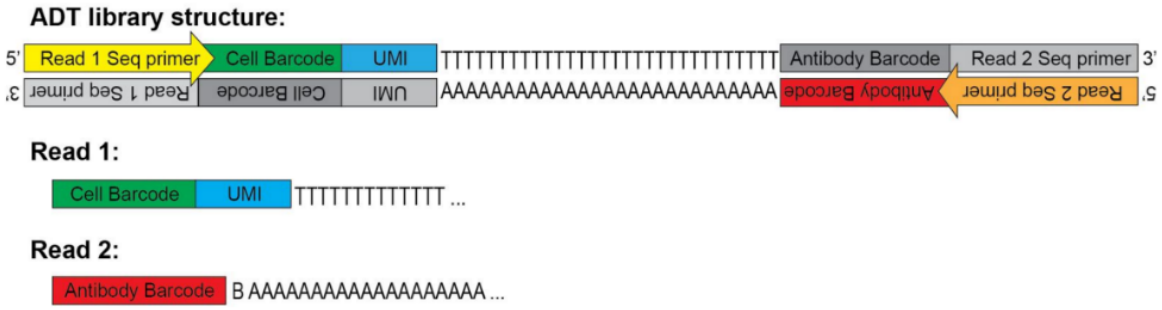
The protocol workflow is as follows:

1. Cell staining for Drop-seq or 10x Genomics
2. Drop-seq (Macosko et al. , 2015) or 10x Genomics single cell 3' v2 assay
3. cDNA amplification
4. Separating ADT-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
5. Amplifying ADT sequencing library

Sequencing CITE-seq libraries:

We estimate that an average of 100 molecules per ADT per cell is sufficient to achieve useful

information, we typically sequence our ADT / HTO libraries to obtain significantly more reads than this per cell. ADT and cDNA sequencing libraries can be pooled at desired proportions. We typically sequence ADT libraries in 5-10% of a lane and cDNA library fraction at 90% of a lane (HiSeq2500 Rapid Run).



Oligonucleotide sequences:

CITE-seq antibody-oligos (ADTs):

These 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'CCTTGGCACCCGAGAATTCCACATGATTGGCTCBAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Oligos required for ADT 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
- Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences © 2015 Illumina, Inc)
  5'CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A

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

Before start

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

Materials

Antibody-oligo conjugates for Cell Hashing and/or CITE-seq by BioLegend
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 and small RNA 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
✓ PBS by Contributed by users
✓ Tween20 by Contributed by users
✓ BSA by Contributed by users
✓ 80% Ethanol by Contributed by users

Protocol

Cell staining for Drop-seq or 10x Genomics
Step 1.

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. by FACS) is recommended.

Cell staining for Drop-seq or 10x Genomics
Step 2.

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 3.

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 4.

Incubate for 10 minutes at 4˚C.

TEMPERATURE

4 °C Additional info: Incubation

Cell staining for Drop-seq or 10x Genomics

Step 5.

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

Cell staining for Drop-seq or 10x Genomics

Step 6.

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

Cell staining for Drop-seq or 10x Genomics

Step 7.

Incubate for 30 minutes at 4˚C.

TEMPERATURE

4 °C Additional info: Incubation

Cell staining for Drop-seq or 10x Genomics

Step 8.

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 9.

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 10.

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 11.

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 12.

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 13.

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 14.

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].

Cell staining for Drop-seq or 10x Genomics

Step 15.

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

Cell staining for Drop-seq or 10x Genomics

Step 16.

Verify cell concentration by counting on hemocytometer after filtration.

Step 17.

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

cDNA amplification

Step 18.

Add “additive” primers to cDNA PCR to increase yield of ADT products:

ADT PCR additive primer (2 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-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)

Step 19.

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

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

Step 20.

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

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

Step 21.

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

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

Step 22.

Incubate 5 minutes and place on magnet.

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

Step 23.

● **Supernatant** contains ADTs.

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

mRNA-derived cDNA >300bp (beads fraction)

Step 24.

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

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

Purifying ADTs using two 2X SPRI purifications

Step 25.

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 26.

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

Purifying ADTs using two 2X SPRI purifications
Step 27.

Incubate 10 minutes at room temperature.

Purifying ADTs using two 2X SPRI purifications
Step 28.

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

Purifying ADTs using two 2X SPRI purifications
Step 29.

Carefully remove and discard the supernatant.

Purifying ADTs using two 2X SPRI purifications
Step 30.

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 31.

Carefully remove and discard the ethanol wash.

Purifying ADTs using two 2X SPRI purifications
Step 32.

Centrifuge tube briefly and return it to magnet.

Purifying ADTs using two 2X SPRI purifications
Step 33.

Remove and discard any remaining ethanol.

Purifying ADTs using two 2X SPRI purifications
Step 34.

Resuspend in beads in 50 µl water

AMOUNT
50 µl Additional info: Water

Purifying ADTs using two 2X SPRI purifications

Step 35.

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 36.

Mix by pipetting.

Purifying ADTs using two 2X SPRI purifications

Step 37.

Incubate 10 minutes at room temperature.

Purifying ADTs using two 2X SPRI purifications

Step 38.

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

Purifying ADTs using two 2X SPRI purifications

Step 39.

Carefully remove and discard the supernatant.

Purifying ADTs using two 2X SPRI purifications

Step 40.

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 41.

Carefully remove and discard the ethanol wash.

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 (2nd 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.

Centrifuge tube briefly and return it to magnet.

Purifying ADTs using two 2X SPRI purifications

Step 45.

Remove and discard any remaining ethanol.

Purifying ADTs using two 2X SPRI purifications

Step 46.

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

Purifying ADTs using two 2X SPRI purifications

Step 47.

Resuspend beads in 45 µl water.

AMOUNT

45 µl Additional info: Water

Purifying ADTs using two 2X SPRI purifications

Step 48.

Pipette mix vigorously.

Purifying ADTs using two 2X SPRI purifications

Step 49.

Incubate mix at room temperature for 5 minutes.

Purifying ADTs using two 2X SPRI purifications

Step 50.

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

Amplifying ADT sequencing library

Step 51.

To prepare 100uL PCR reaction with purified ADTs, first, add 45 µl purified ADT fraction to 50 µl 2x KAPA Hifi PCR Master Mix.

Reagent	Amount
purified ADT 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 fraction
AMOUNT
50 µl Additional info: 2x KAPA Hifi PCR Master Mix

Amplifying ADT sequencing library

Step 52.

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)

Amplifying ADT sequencing library

Step 53.

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 ADT sequencing library

Step 54.

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	

Purifying PCR product using 1.6X SPRI purification

Step 55.

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

AMOUNT
160 µl Additional info: SPRI reagent

Purifying PCR product using 1.6X SPRI purification

Step 56.

Incubate 5 minutes at room temperature.

Purifying PCR product using 1.6X SPRI purification

Step 57.

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

Purifying PCR product using 1.6X SPRI purification

Step 58.

Carefully remove and discard the supernatant.

Purifying PCR product using 1.6X SPRI purification

Step 59.

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 using 1.6X SPRI purification

Step 60.

Carefully remove and discard the ethanol wash.

Purifying PCR product using 1.6X SPRI purification

Step 61.

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 using 1.6X SPRI purification

Step 62.

Carefully remove and discard the ethanol wash.

Purifying PCR product using 1.6X SPRI purification

Step 63.

Centrifuge tube briefly and return it to magnet.

Purifying PCR product using 1.6X SPRI purification

Step 64.

Remove any remaining ethanol.

Purifying PCR product using 1.6X SPRI purification

Step 65.

Allow the beads to air dry for 2 minutes.

Purifying PCR product using 1.6X SPRI purification

Step 66.

Resuspend beads in 20 µl water.

AMOUNT

20 µl Additional info: Water

Purifying PCR product using 1.6X SPRI purification

Step 67.

Pipette mix vigorously.

Purifying PCR product using 1.6X SPRI purification

Step 68.

Incubate mix at room temperature for 5 minutes.

Purifying PCR product using 1.6X SPRI purification

Step 69.

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

Step 70.

ADT libraries are now ready to be sequenced.

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

EXPECTED RESULTS

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

Figure 1.

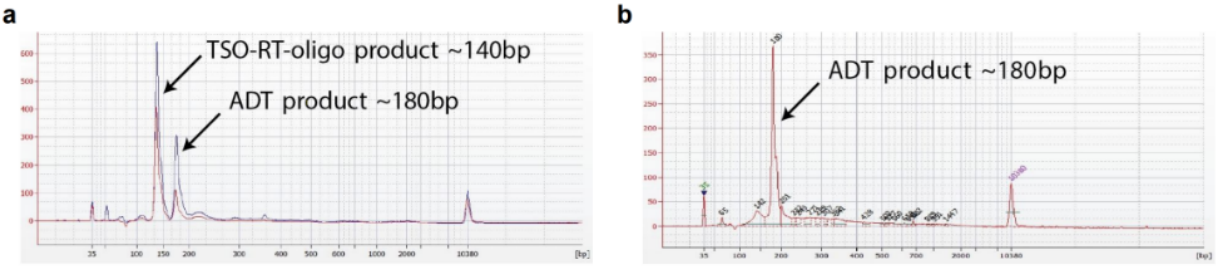


Figure 1. ADT library verification. (a) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X SPRI purification of the ADT fraction after cDNA amplification reduces carryover of primers from cDNA amplification, and minimizes the amplification of this product during ADT-library amplification. To further enrich for ADT 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 library will contain a predominant single peak at around 180 bp.

Warnings

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