Cell Hashing

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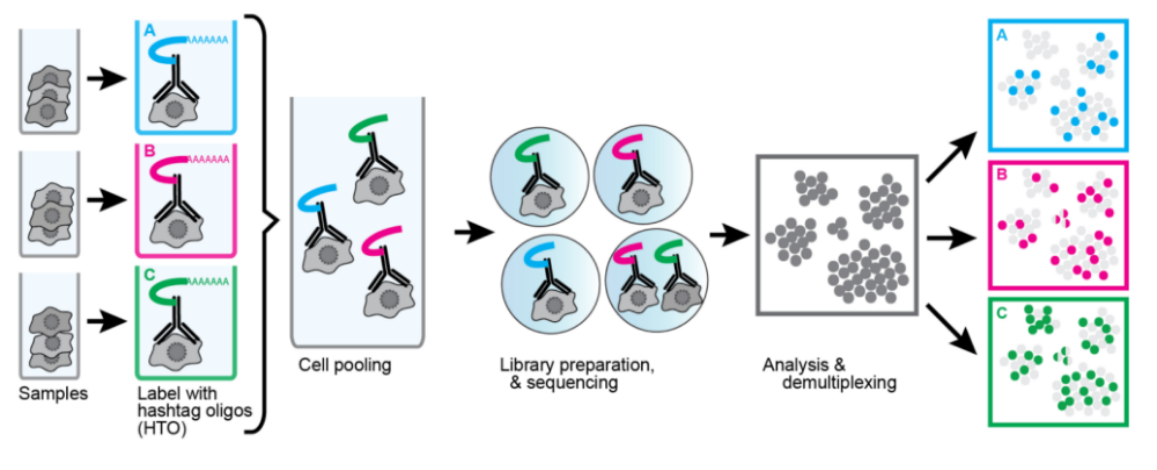
Abstract

This protocol is for performing Cell Hashing only.

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

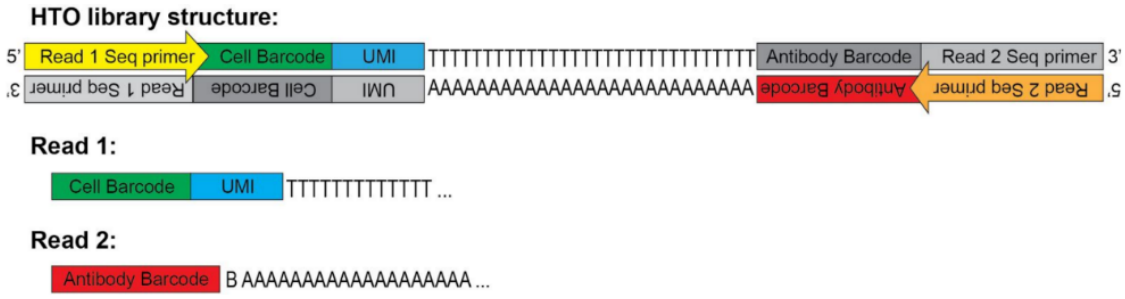
Satija lab to plan experiments, determine number of hashes, number of cells to load, expected doublet rates (detected and undetected) and cost considerations.

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 HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
5. Amplifying HTO sequencing library
6. Purification of PCR product

Sequencing Cell Hashing libraries:

We estimate that an average of 100 molecules of HTO per cell is sufficient to achieve useful information. The number of reads required to obtain 100 molecules depends on the complexity of the sequencing library (e.g. duplication rate). HTO and cDNA sequencing libraries can be pooled at desired proportions. To obtain sufficient read coverage for both libraries we typically sequence HTO libraries in 5-10% of a lane and cDNA library fraction at 90% of a lane (HiSeq2500 Rapid Run Mode Flowcell).



Oligonucleotide sequences:

Hashtag oligos (HTOs):

These 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′GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A

Oligos required for 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
- HTO cDNA PCR additive primer
  5′GTGACTGGAGTTCAGACGTGTGC*T*C
- 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

Desalting columns 732-6221 by BioRad Sciences

8-strip PCR tubes, emulsion safe (!) 1402-4700 by USA Scientific

Bioanalyzer chips and reagents (DNA High Sensitivity and small RNA kit) 5067-1548 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 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
- Biotin by Contributed by users
- TE pH 8.0 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.

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. by FACS) 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.

Add 1 µg of single cell hashing antibody to cells.

AMOUNT

1 µg Additional info: Single cell hashing antibody

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 (2%BSA/0.02%Tween, PBS). (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. (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. (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. (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. (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. (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 26 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 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.

Cell staining for Drop-seq or 10x Genomics

Step 17.

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

Step 18.

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

cDNA amplification step

Step 19.

Add “additive” primer to cDNA PCR to increase yield of HTO products:

HTO 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.

Separation HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
Step 20.

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

Separation HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
Step 21.

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

Separation HTO-derived cDNAs (<180bp) and mRNA-derived cDNAs (>300bp)
Step 22.

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

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

Incubate 5 minutes and place on magnet.

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

Supernatant contains hashtags.

Beads contain full length mRNA-derived cDNAs.

mRNA-derived cDNA >300bp (beads fraction)
Step 25.

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

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

Purify Hashtags using two 2X SPRI purifications
Step 26.

Purify Hashtags using two 2X SPRI purifications per manufacturer protocol. First, add 1.4X SPRI to supernatant to obtain a final SPRI volume of 2X SPRI.

Purify Hashtags using two 2X SPRI purifications
Step 27.

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

Purify Hashtags using two 2X SPRI purifications
Step 28.

Incubate 10 minutes at room temperature.

Purify Hashtags using two 2X SPRI purifications
Step 29.

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

Purify Hashtags using two 2X SPRI purifications
Step 30.

Carefully remove and discard the supernatant.

Purify Hashtags using two 2X SPRI purifications
Step 31.

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

Purify Hashtags using two 2X SPRI purifications
Step 32.

Carefully remove and discard the ethanol wash.

Purify Hashtags using two 2X SPRI purifications
Step 33.

Centrifuge tube briefly and return it to magnet.

Purify Hashtags using two 2X SPRI purifications
Step 34.

Remove and discard any remaining ethanol.

Purify Hashtags using two 2X SPRI purifications
Step 35.

Resuspend in beads in 50 µl water.

AMOUNT
50 µl Additional info: Water

Purify Hashtags using two 2X SPRI purifications
Step 36.

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

AMOUNT

100 µl Additional info: SPRI reagent

Purify Hashtags using two 2X SPRI purifications

Step 37.

Mix by pipetting.

Purify Hashtags using two 2X SPRI purifications

Step 38.

Incubate 10 minutes at room temperature.

Purify Hashtags using two 2X SPRI purifications

Step 39.

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

Purify Hashtags using two 2X SPRI purifications

Step 40.

Carefully remove and discard the supernatant.

Purify Hashtags using two 2X SPRI purifications

Step 41.

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

Purify Hashtags using two 2X SPRI purifications

Step 42.

Carefully remove and discard the ethanol wash.

Purify Hashtags using two 2X SPRI purifications

Step 43.

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

Purify Hashtags using two 2X SPRI purifications

Step 44.

Carefully remove and discard the ethanol wash.

Purify Hashtags using two 2X SPRI purifications

Step 45.

Centrifuge tube briefly and return it to magnet.

Purify Hashtags using two 2X SPRI purifications
Step 46.

Remove and discard any remaining ethanol.

Purify Hashtags using two 2X SPRI purifications
Step 47.

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

Purify Hashtags using two 2X SPRI purifications
Step 48.

Resuspend beads in 45 µl water.

AMOUNT
45 µl Additional info: Water

Purify Hashtags using two 2X SPRI purifications
Step 49.

Pipette mix vigorously and incubate at room temperature for 5 minutes.

Purify Hashtags using two 2X SPRI purifications
Step 50.

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

Amplify HTO sequencing library
Step 51.

Prepare 100 µL PCR reaction with purified small fraction as follows:

First, add 45 µl purified Hashtag fraction.

Reagent	Amount
purified Hashtag fraction	45 µl
2x KAPA Hifi PCR Master Mix	55 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 Hashtag fraction

Amplify HTO sequencing library

Step 52.

Add 50 µl 2x KAPA Hifi PCR Master Mix.

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

Amplify HTO sequencing library

Step 53.

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

Amplify HTO sequencing library

Step 54.

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.

Amplify HTO sequencing library

Step 55.

Cycling conditions:

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

Purification

Step 56.

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

AMOUNT
160 µl Additional info: SPRI reagent

Purification

Step 57.

Incubate 5 minutes at room temperature.

Purification

Step 58.

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

Purification

Step 59.

Carefully remove and discard the supernatant.

Purification

Step 60.

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

Purification

Step 61.

Carefully remove and discard the ethanol wash.

Purification

Step 62.

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

Purification

Step 63.

Carefully remove and discard the ethanol wash.

Purification

Step 64.

Centrifuge tube briefly and return it to magnet.

Purification

Step 65.

Remove and discard any remaining ethanol.

Purification

Step 66.

Allow the beads to air dry for 2 minutes.

Purification

Step 67.

Resuspend beads in 20 µl water.

AMOUNT

20 µl Additional info: Water

Purification

Step 68.

Pipette mix vigorously and incubate at room temperature for 5 minutes.

Purification

Step 69.

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

Purification

Step 70.

Hashtag libraries are now ready to be sequenced.

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

EXPECTED RESULTS

Hashtag library will be around 180 bp (Figure 1).

Figure 1.

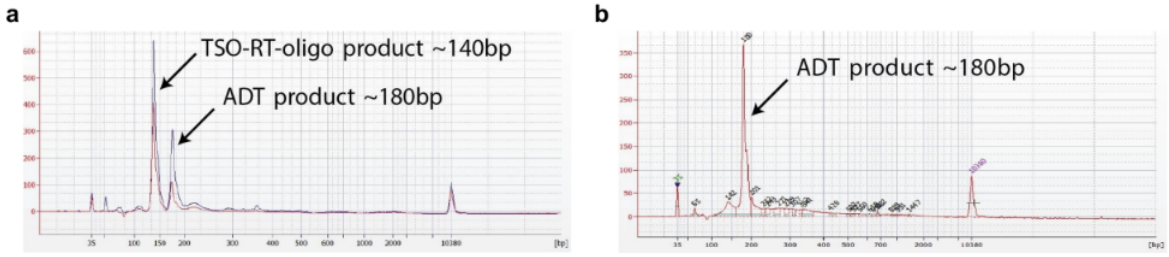


Figure 1. Hashtag library verification. (a) A TSO-RT-oligo product (~140 bp) can be amplified during the HTO PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. This example figure shows ADT libraries. Sequential 2X SPRI purification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

Warnings

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