Feb 28, 2019 Version 2

Cell Hashing V.2

Version 1 is forked from Cell Hashing

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Protocol status: In development
We are still developing and optimizing this protocol

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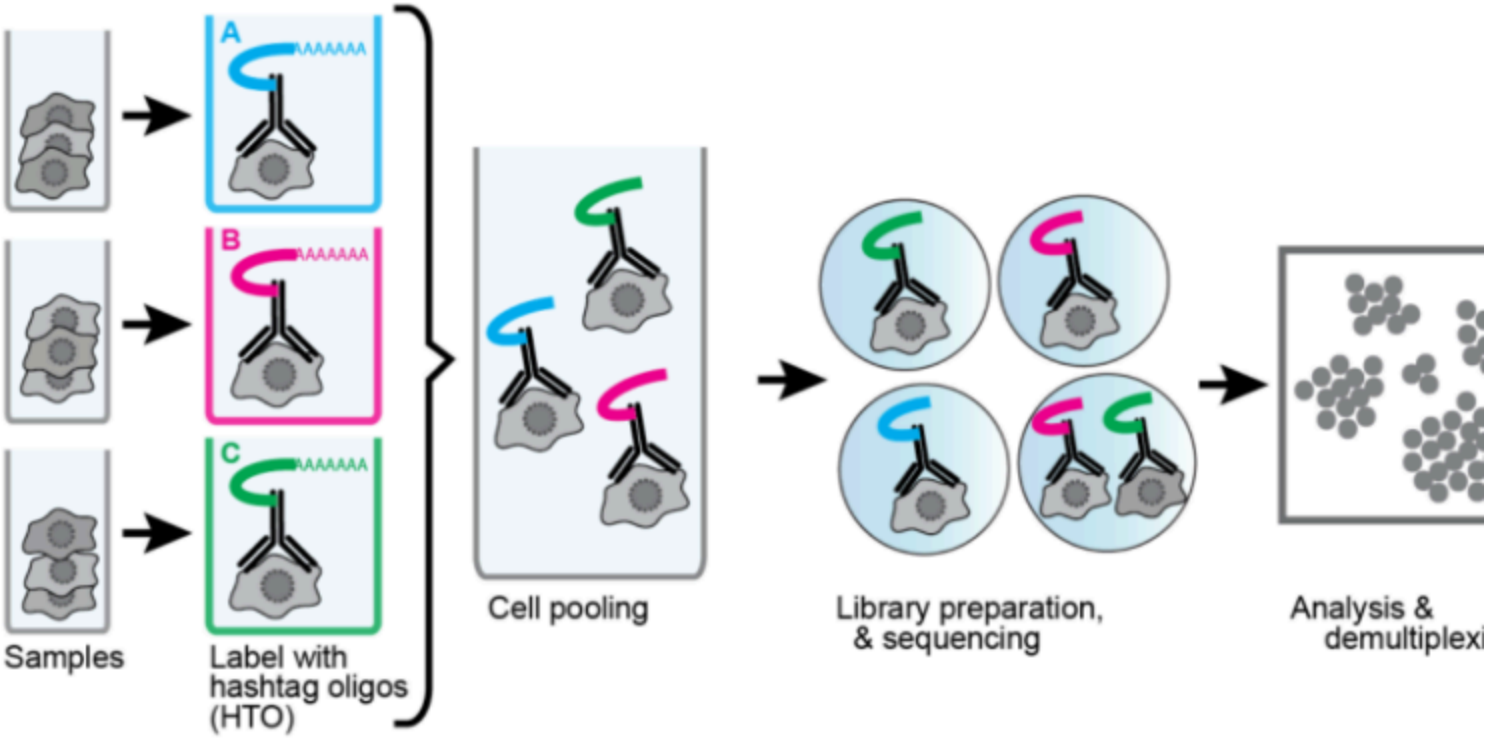
Abstract

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.



Attachments

PDF

hashing_protocol_180...

498KB

Guidelines

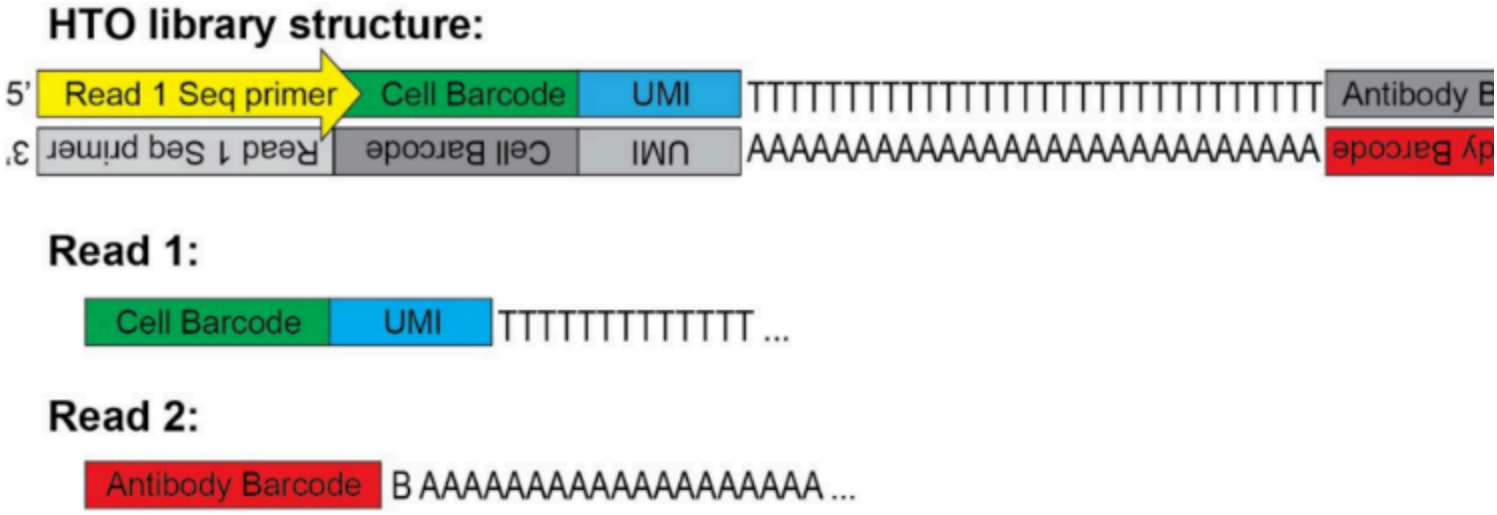
For experiments involving cell hashing, we recommend using the **cost per cell calculator** from the Satija lab to plan experiments, determine number of hashes, number of cells to load, expected doublet rates (detected and undetected) and cost considerations.

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′GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGGACCATCCAABAAAAAAAAAAAA

Oligos required for HTO library amplification:

- Drop-seq P5-SMART-PCR hybrid primer (for Drop-seq only)

5′AATGATACGGCGACCACCGAGATCTACACGCCTGTCCGCGGAAGCAGTGGT

- 10x Genomics SI-PCR primer (for 10x Single Cell Version 2 only)

5′AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTA

- 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′CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCA

* Phosphorothioate bond

B C or G or T; not A nucleotide

Materials

MATERIALS

- FC blocking reagent (FcX) **BioLegend**
- Desalting columns **Bio-Rad Laboratories Catalog #**732-6221
- 8-strip PCR tubes, emulsion safe (!) **USA Scientific Catalog #**1402-4700
- Bioanalyzer chips and reagents (DNA High Sensitivity and small RNA kit) **Agilent Technologies Catalog #**5067-1548
- SPRIselect reagent **GE Healthcare Catalog #**B23317
- E-gel 4% **Invitrogen - Thermo Fisher**
- Low-bind 1.5 mL tubes
- PCR Thermocycler **Bio-Rad Laboratories Catalog #**T100
- Magnetic tube rack **Invitrogen - Thermo Fisher**
- Qubit **Invitrogen - Thermo Fisher**
- Hemocytometer (e.g. Fuchs Rosenthal)
- DMSO
- PBS
- Tween20
- Biotin
- TE pH 8.0
- BSA
- 80% Ethanol

Safety warnings

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

Before start

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

Cell staining for Drop-seq or 10x Genomics

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.

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!
Record cell count **in cell count table**

3 Resuspend all cells in 100 µl Staining buffer (2%BSA/0.02%Tween, PBS).

- We assume ~200K to 1 million cells per well, which is less than max of what can be stained
- Note this much more hashing Ab than needed. Biolegends says it can stain 1-2 million cells

100 µL Staining buffer

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

10 µL Fc Blocking reagent

5 Incubate for 10 minutes at 4˚C.

4 °C Incubation

00:10:00 Incubation

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

To maximize performance, centrifuge the antibody pool at 14,000xg at 2 – 8°C for 10 minutes before adding to the cells.

Carefully pipette out the liquid, avoiding the bottom of the tube, and add the TotalSeq™ antibody cocktail to the cell suspension.

7 Add 2 uL (1 µg) of single cell hashing antibody to each tube of cells.

1 µg Single cell hashing antibody

8 Incubate for 30 minutes at 4˚C.

4 °C Incubation

00:30:00 Incubation

9 Wash cells with 1 mL Staining buffer (2%BSA/0.02%Tween, PBS). (1/3)

1 mL Staining buffer

10 Spin 5 minutes 400g at 4˚C. (1/3)

4 °C Spinning

00:05:00 Spinning

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

1 mL Staining buffer

12 Spin 5 minutes 400g at 4˚C. (2/3)

4 °C Spinning

00:05:00 Spinning

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

1 mL Staining buffer

14 Spin 5 minutes 400g at 4˚C. (3/3)

4 °C Spinning

00:05:00 Spinning

15 Resuspend cells in Cell capture buffer at appropriate concentration for downstream application.
We estimate resuspending in 200 uL if we have ~300,000 cells reatined after staining and washing

Note

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

Prior to experiment estimate min and max cells expected for resuspension

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

17 Verify cell concentration by counting on Countess after filtration.

Record counts in **cell count table**

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

19 Count pooled cell suspension. For 10x superloading ~1500 cells/uL is ideal (or 1.5e6 cells/mL).
Record counts **in cell count table**

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

cDNA amplification step

21 **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)

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

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

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

25 Incubate 5 minutes and place on magnet.
00:05:00 Incubation on magnet

26 **Supernatant** contains hashtags.
Beads contain full length mRNA-derived cDNAs.

mRNA-derived cDNA >300bp (beads fraction)

27 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

28 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.

From Dani/KCO we expect 100 uL initial volume, but got 150 uL so added 300 uL beads

300 µL SPRI beads

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

30 Incubate 10 minutes at room temperature.

00:10:00 Incubation

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

00:02:00 Magnet

32 Carefully remove and discard the supernatant.

33 Add 450 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (only one Ethanol wash).
This is the same total volume as beads + supernatant

450 µL 80% Ethanol

00:00:30 Ethanol wash

34 Carefully remove and discard the ethanol wash.

35 Centrifuge tube briefly and return it to magnet.

36 Resuspend in beads in 50 µl water.
deviation form KAtie becuase the 1.5 mL tub is too large to elute in 15 uL

50 µL Water

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

deviation form Katie - she uses 30 uL beads w/15 uL eluted H2O

100 µL SPRI reagent

38 Mix by pipetting.

39 Transfer to PCR strip tube

40 Incubate 10 minutes at room temperature.

00:10:00 Incubation

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

00:02:00 Magnet

42 Carefully remove and discard the supernatant.

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

200 µL 80% Ethanol

00:00:30 1. Ethanol wash

44 Carefully remove and discard the ethanol wash.

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

200 µL 80% Ethanol

00:00:30 2. Ethanol wash

46 Carefully remove and discard the ethanol wash.

47 Centrifuge tube briefly and return it to magnet.

48 Remove and discard any remaining ethanol.

49 Allow the beads to air dry for 2 minutes (do not over dry beads).
00:02:00 Air drying

50 Resuspend beads in 15 µl water.
15 µL Water

51 Pipette mix vigorously and incubate at room temperature for 5 minutes.
00:05:00 Incubation

52 Place tube on magnet and transfer clear supernatant into PCR tubes. ~2-5 min

Note

For PCR
typically add ~5 ng DNA into each PCR reaction

set up 3 different reactions with different cycles 12, 15, 18 cycles to not over amplify
1 uL of elution, or 10 ng total input if concentrated
typical input range 2-30 ng/uL - but doesn't necessarily correlate to number of cycles

53 Quantify using qubit or nano drop (less sensitive, but usally ok)

Amplify HTO sequencing library

54 Prepare 50 µL PCR reaction with purified small fraction as follows:
add ~1 µl purified Hashtag fraction.
1 µL Hashtag fraction

	Reagent	Amount
	purified Hashtag fraction	~1 µl
	2x KAPA Hifi PCR Master Mix	25 µl
	TruSeq DNA D7xx_s primer (containing i7 index) 10 µM	1.25 µl
	SI PCR oligo* 10 µM	1.25 µl
	H2O	to 50 uL ~21.5 uL if 1uL hashing fraction

	used

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

55 Cycling conditions:

95˚C 3 min 95˚C 20 sec 64˚C 30 sec 72˚C 20 sec 72˚C 2-5 min	10, 15, 18 cycles

can decrease final extenstion if over amplified.
saved cycling conditons on the DepMap person, made Apollo folder
18 cycle run is ~35 min

Purification

56 Purify PCR product using 2X SPRI purification by adding 100 µl SPRI reagent.

100 µL SPRI reagent

can do a 0.8x SPRI to clean up if primer dimer persists after QC in a pinch, but better to reamplify with reserved Hashing template

57 Incubate 5 minutes at room temperature.

00:05:00 Incubation

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

00:05:00 Magnet

59 Carefully remove and discard the supernatant.

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

200 µL 80% Ethanol

00:00:30 1. Ethanol wash

61 Carefully remove and discard the ethanol wash.

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

200 µL 80% Ethanol

00:00:30 2. Ethanol Wash

63 Carefully remove and discard the ethanol wash.

64 Centrifuge tube briefly and return it to magnet.

65 Remove and discard any remaining ethanol.

66 Allow the beads to air dry for 2 minutes.
00:02:00 Air drying

67 Resuspend beads in 15 µl water.
15 µL Water

68 Pipette mix vigorously and incubate at room temperature for 5 minutes.
00:05:00 Incubation

69 Place tube on magnet and transfer clear supernatant to PCR tube.
NOTE did 1 PCR tube at a time holding bottom of tube at top edge of magnet

70

Note

90 bp peak is primer if under amp'd, larger products than 186 are generated when over amplified

Hashtag libraries are now ready to be sequenced.
Quantify library by standard methods (QuBit, BioAnalyzer, qPCR).

Expected result

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

Figure 1.

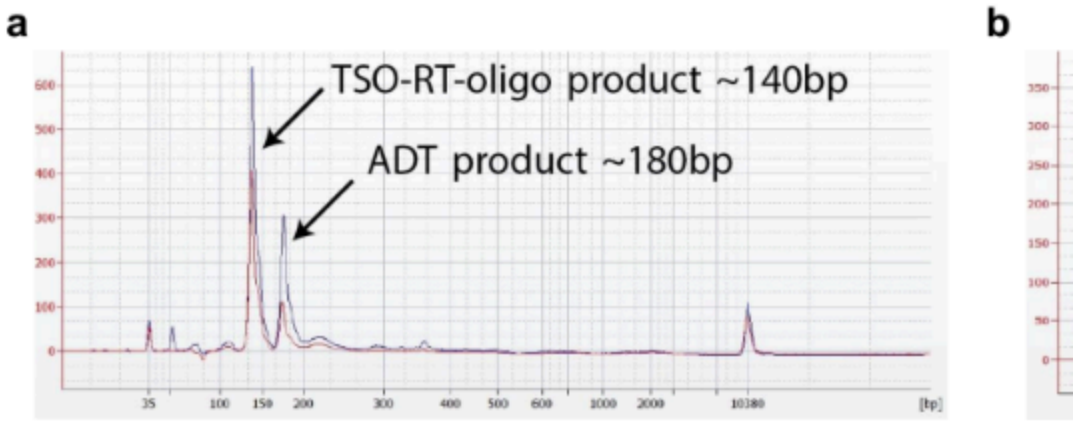


Figure 1. Hashtag library verification. (a) A TSO-RT-oligo pr
PCR by carryover primers from cDNA amplification. The product
This example figure shows ADT libraries. Sequential 2X SI
amplification reduces carryover of primers from cDNA amplificat
during HTO-library amplification. To further enrich for HTO s
reamplified for ~3 additional cycles with HTO specific primer sets
will contain a predominant single peak at around 180 bp.