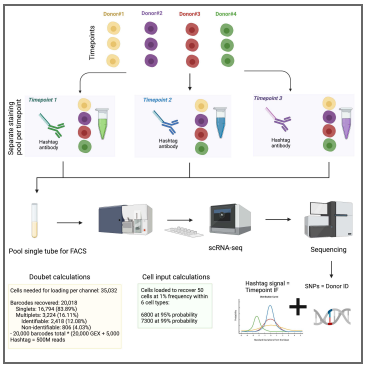

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Protocol status: Working
We use this protocol and it's working

Created: Aug 15, 2021

CITE-Seq PBMCs with demultiplexing

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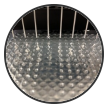
[1]Walter and Eliza Hall Institute



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DISCLAIMER

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This protocol has only been tested on PBMCs and constituent immune subsets. This protocol contains deviations from standard 10x Genomics SOPs and will not be supported by them.

Last Modified: Apr 02, 2024

PROTOCOL integer ID: 52348

Keywords: PBMCs, CITE-Seq, Total-Seq, scRNA-Seq, 10x Genomics

ABSTRACT

Total-Seq labelling with demultiplexing

This protocol reflects a common experimental design where PBMC samples from multiple human donors are pooled together for Total-Seq (CITE-Seq) labelling. Often multiple samples from the same donor are to be profiled. An example is timepoint or treatment.

Therefore we utilise 2 levels of multiplexing

- The distinct donors may be pooled and later demultiplexed using SNP genotypes. This cannot discriminate genetically identical samples.
- Hashtag antibodies are used to multiplex to the timepoint or treatment

The hashtag labelling must be done separately. We setup a distinct antibody staining pool for each timepoint or treatment.
When other extracellular markers are to be profiled with Total-Seq we make prepare these antibodies in a pool and then split the pool into aliquots, adding a different hashtag to the antibody subpools.

IMAGE ATTRIBUTION

Created with Biorender

GUIDELINES

Although PBMCs are a very robust cell type it is advised to work as quickly as possible after beginning thawing.

MATERIALS

- RPMI 1640 medium **Gibco - Thermo Fischer Catalog #**21875109 10% FCS
- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625
- 30% BSA
- BSA-Molecular Biology Grade - 12 mg **New England Biolabs Catalog #**B9000S
- Cell Staining Buffer **BioLegend Catalog #**420201
- TruStain FcX™ **BioLegend Catalog #**101319
- 0.1mg/mL
- 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #**D1306
- 50ml Falcon tubes **Corning Catalog #**352070
- 2 ml LoBind Tubes **Eppendorf Catalog #**0030108078
- 1.5mL Eppendorf tubes **USA Scientific Catalog #**Denville C-2171
- FACS Tubes 5mL polysterene, coat with 2% BSA
- Falcon™ Cell Strainers - Mesh size: 40um; blue **Thermo Fisher Scientific Catalog #**08-771-1
- Trypan Blue Solution, 0.4% **Thermo Fisher Catalog #**15250061
- 1x TotalSeq hashtag per timepoint or treatment. For example TotalSeq™-A0251 anti-human Hashtag 1 Antibody #399907
- Oligo conjugated antibodies against marker of interest. For example TotalSeq™-A Human Universal Cocktail, V1.0 #399907

Equipment

Eppendorf™ 5810R Centrifuge	NAME
Centrifuge	TYPE
Eppendorf	BRAND
02-262-8187	SKU
https://www.fishersci.com/shop/products/eppendorf-5810r-centrifuge-rotor-packages-16/022628187	LINK

Equipment

Countess II	NAME
Life Technologies	BRAND
AMQAX1000	SKU

SAFETY WARNINGS

- Provide prior notice to tissue culture room users before commencing processing of sample.
- Room will only be available to staff processing material from acutely infected individuals until work is completed, area disinfected and waste removed for decontamination
- Turn on BSCII and allow time to start up (5 minutes to allow air to purge)
- Disinfect the BSC with either 80% ethanol or Cavicide and have disinfectant close to BSC.
- Ensure that the BSCII cabinet is equipped with the following:

1. Biohazard waste bucket, with new autoclave bag
2. Biocan for tips and pipettes
3. Place only required items in cabinet

- Do not overfill biohazard bins (no greater than 80% filled).
- Work should be conducted in the centre of the BSCII.
- The grille of the BSCII must not be covered with any items that will restrict airflow.
- Minimise the amount of equipment in the BSCII.
- When using pipette tips, dispose directly into biocan within the BSCII.
- If gloves become contaminated, remove them within the BSCII and dispose of them in the biohazard bag.
- Liquid waste will be treated with 10,000ppm sodium hypochlorite for a minimum of 60 min prior to disposal down the sink with a strong flush of water.
- Use aerosol caps on centrifuge buckets

BEFORE START INSTRUCTIONS

Make all preparations prior to thawing cells.
Collect ice and chill all buffers on ice.
Warm RPMI 10% FCS media in water bath
Coat 5mL FACS polysterene tubes with 2% BSA in PBS before use to minimize cell loss.

Reconstituting the total-seq panel

15m

1 The large panels come lyophilized e.g TotalSeq-C Human Universal Cocktail, V1.0
The typical staining protocol is to block 500,000 cells in 25 uL of volume (cell staining buffer + Fc blocking reagent) and then add 25 uL of the resuspended antibodies.

Total staining volume is 50uL

Note

For 3 staining pools of a million cells total prepare **2 Total-Seq C vials**

1.1 Equilibrate the lyophilized panel vial(s) to room temperature for 5 minutes.

1.2 Place lyophilized panel vial(s) in an empty Eppendorf tube, pulse spin to collect powder to the bottom of the tube.

1.3 Add 27 µL cell staining buffer per tube and vortex 00:00:10 10s

1.4 Incubate 00:05:00 room temperature 5m 30s
vortex 00:00:30 then pulse centrifuge

1.5 Transfer entire volume of all antibody cocktails to a single DNA LoBind tube = 50 µL Total 10m
Centrifuge at 14000 x g, 4°C, 00:10:00

1.6 Split the antibody cocktail tube into multiple aliquots. For example for 3 timepoints / treatments aliquot 15 µL into separate tubes.

Note

Do not touch the bottom of the tube which could contain protein aggregates. Leave at least 2uL volume in centrifuged tube.

Preparing the Hashtag antibody

15m

2 We have tested staining with 0.25 µg of each hashtag antibody, 4x less than recommended by manufacturer which robustly labels PBMCs.
You will need a distinct hashtag antibody set for each sample.

> **Note**
>
> We currently label each sample with a unique combination of 2 hashtags at 1:4 dilution each. We have not compared with single hashtag labelled samples

2.1 Centrifuge the stock tube at 10000 x g, 4°C, 00:10:00

10m

> **Note**
>
> This is important to sediment protein aggregates

2.2 Add 0.5 µL of **distinct** hashtag antibodies to each antibody cocktail tube = 16 µL Total volume

0.5uL * 0.5ug/uL = 0.25 µg each hashtag

> **Note**
>
> Carefully record which hashtag antibodies are added to each antibody cocktail tube.
> Avoid touching the bottom of the antibody stock tubes

Prepare single-cell suspension

2h

3 This protocol has only been used for Human Peripheral Blood Mononuclear Cells (PBMCs).

3.1 We largely follow 10x Genomics guidelines: https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-fresh-frozen-human-peripheral-blood-mononuclear-cells-for-single-cell-rna-sequencing

We use Benzoase in the resuspension media at **50U/mL per sample** (1:5000 dilution). When washing cells normal media (without Benzoase) is used.

Note

Key is the slow addition of media to prevent a shock to cells during revival

3.2 Prior to counting resuspend in 9mL 9 mL Cell Staining buffer

Be sure to thoroughly mix cell suspensions before counting

Pooling cells for Total-Seq and hashtag staining

1h

4 Key to experimental design is ensuring the number of cells from each sample is as similar as possible. Refer to the cell pooling calculator.

4.1 Remove 500uL of cell suspension into 1.5mL tube and count cells. Make note of cell viability (>95%) making a count of live cells and total cells (live + dead) in separate columns.

Note

PBMCs are notoriously difficult to count with automated systems. Count manually or use a machine with fluorescence capability.

4.2 First prepare the pool of samples from **healthy donors** in a 10mL tube. This will subsequently be aliquotted into each of the staining pools.

Note

This design ensures more consistent representation of healthy donors across staining pools

4.3 Next pool the **experimental samples** that go into the antibody staining pools. You will have one staining pool per timepoint or treatment. Use a 10mL tube.

4.4 Add the healthy donor pool into each antibody staining pool.

Note

We want the final number of healthy controls to be the same as each timepoint sample. If there are 3 staining pools add the healthy donors to 1/3rd cell number in each pool.
In total we aim to recover equal cell numbers of controls and timepoints.

Total-seq and Hashtag staining

50m

5 Recommended cell concentration is 22000 cells/uL ($2x10^7$cells/mL) in staining buffer.
Concentrate pooled cell suspensions by centrifugation, leaving 15 µL supernatant approximately

Note

Be conservative with leaving volume behind, especially with a swinging bucket centrifuge. Better leave more rather than less supernatant

5.1 Aliquot 15 µL of cell suspension into **2mL Eppendorf tubes**.
You may have to perform 2 centrifugations to remove volume in the 10mL tube, transfer to 2mL tube then centrifuge again to reduce volume.

5.2 Add 1.7 µL Human TruStain FcX Blocking reagent and mix.

5.3 Incubate for 00:10:00 4C 10m

5.4 Add 16.7 µL TotalSeq antibody cocktail to the cell suspension.

5.5 Incubate for 00:30:00 on ice 30m

Snap freeze cell aliquot

30m

5.6 While incubating take minimum 500,000 cells into a new eppendorf tube. You can use the 1.5m 30m
used for cell counting to make this aliquot.
Pellet cells then snap freeze for the generation of bulk RNA-Seq libraries.

Note

This step is required if you want to recover the original donor ID from each sample. If "donor 1", "donor 2" is an acceptable sample ID you may omit this step.
This step may be substituted for a SNP array. For Infinium Global Screening Array, 200ng of gDNA is required

Total-seq and Hashtag washing

30m

5.7 Add 1.8 mL Cell Staining buffer carefully resuspending cells with pipette tip and 5m
400 x g, 4°C, 00:05:00 .
Use of swinging-bucket rotor is recommended for higher cell recovery.
Leave behind 100 µL supernatant approx

5.8 Resuspend cells in remaining 100uL and transfer to **new** 2mL eppendorf tube.

> **Note**
>
> 10x Genomics have recomended a tube change to reduce antibody background. This should remove unbound antibodies that have stuck to the plastic.
> https://kb.10xgenomics.com/hc/en-us/articles/360041942012-How-can-I-optimize-my-TotalSeq-antibody-labeling-protocol-

5.9 Repeat wash 2 more times go to step #5.7 = 3 washes total

5.10 Resuspend cells in the remaining 100 µL supernatant approx and combine the pools into a single 5mL FACS tube.

5.11

If cell suspension is clumpy filter cells through 40uM Flowmi Cell Strainer or FACS (blue top) Cell Strainer.

> **Note**
>
> I have compared the 2 filters, the main benefit of Flowmi is the reduced volume loss.

5.12 Add DAPI to a concentration of [M] 0.1 ug/mL DAPI , stock is typically 100x

Cell sorting by FACS

1h

6 Cells should be collected in 200uL volume in a 1.5-ml tube.
We typically recover 2/3 cells based on number of events captured

6.1 Top up volume of FACS tube to minimum 250uL with PBS + 2% BSA

6.2 Sort viable (DAPI negative) cells into 200 µL PBS + 2% BSA in a 1.5mL tube.
If necessary, the collected cells may be concentrated by centrifugation at 350 rcf and removing the supernatant.

6.3 Count cells with manual haemocytometer

Note

This is the last cell count and is critical for obtaining the desired cell output from scRNA-Seq. **I always count manually.**

6.4 Dilute cells as necessary for appropriate input into the 10X Chromium chip.
Use PBS + 0.04% BSA to dilute.

Capturing cells with 10x Genomics chromium controller

2h

7 Follow the guidelines of the relevant 10x Genomics protocol

7.1 35,000 cells will be needed for each of the 10x Genomics captures

Note

Approximately 20,000 barcode containing droplets, (17,000 singlets and 3,000 multiplets) will be obtained

7.2 34uL is loaded on the 10x Genomics machine. Therefore a concentration of **1,029 cells/uL** will need to be prepared per capture.

Note

Always prepare double the volume of cell suspension you will need in case of wetting failures or blockages of the 10x Genomics microfluidics chip.

7.3 Follow the guidelines of the relevant 10x Genomics protocol. The samples may be left overnight in the thermocycler after the reverse transcription step.