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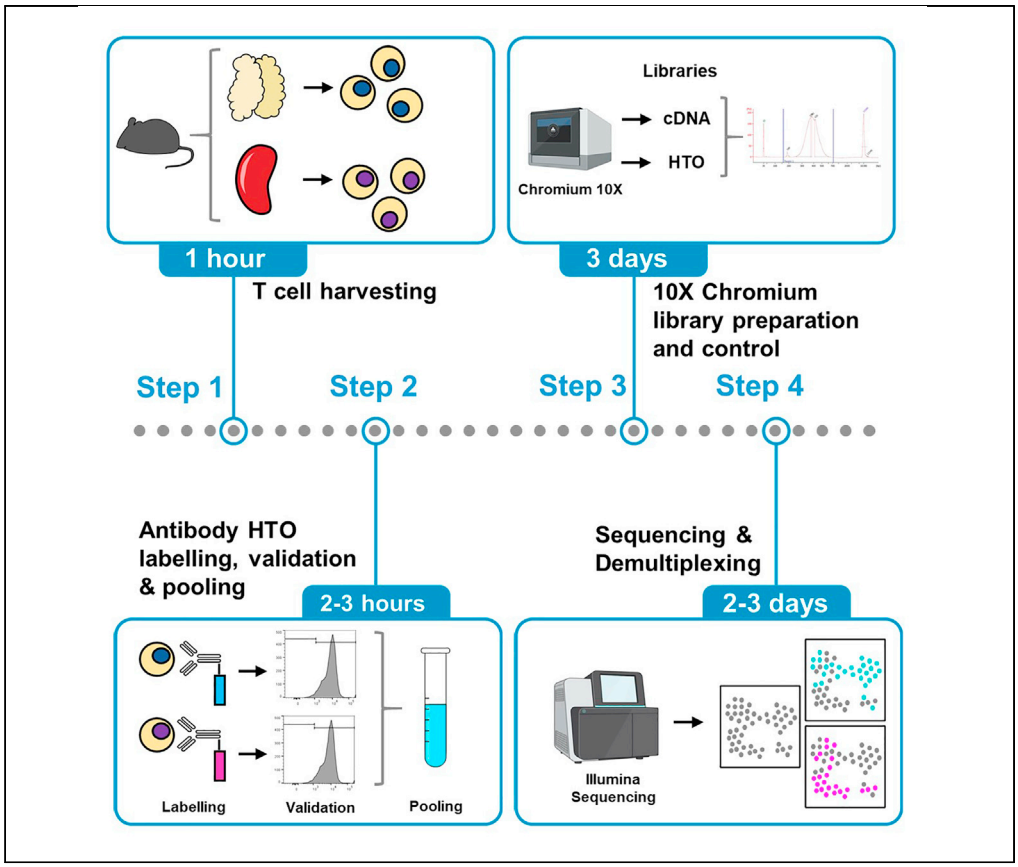
Protocol

Multiplexed single-cell RNA-sequencing of mouse thymic and splenic samples



Multiplexed single-cell RNA-sequencing (scRNA-seq) enables investigating several biological samples in one scRNA-seq experiment. Here, we use antibodies tagged with a hashtag oligonucleotide (HTO) to label each sample, and 10× Genomics technology to analyze single-cell gene expression. Advantages of sample multiplexing are to reduce the cost of scRNA-seq assay and to avoid batch effect. It may also facilitate cell-doublet removal and the merging of several scRNA-seq assays. Herein, we apply multiplexed scRNA-seq to investigate mouse thymocytes and splenic T lymphocytes development.

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Highlights

Cell labelling with antibodies coupled with oligonucleotides (Ab-HTO)

Validation of Ab-HTO labelling by flow cytometry

Multiplexing and analysis of Ab-HTO-labelled samples by scRNAseq

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Multiplexed single-cell RNA-sequencing of mouse thymic and splenic samples

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SUMMARY

Multiplexed single-cell RNA-sequencing (scRNA-seq) enables investigating several biological samples in one scRNA-seq experiment. Here, we use antibodies tagged with a hashtag oligonucleotide (Ab-HTO) to label each sample, and 10× Genomics technology to analyze single-cell gene expression. Advantages of sample multiplexing are to reduce the cost of scRNA-seq assay and to avoid batch effect. It may also facilitate cell-doublet removal and the merging of several scRNA-seq assays. Herein, we apply multiplexed scRNA-seq to investigate mouse thymocytes and splenic T lymphocytes development.
For complete details on the use and execution of this protocol, please refer to Nozais et al. (2021).

BEFORE YOU BEGIN

The protocol below outlines the procedures starting from sample preparation to the demultiplexing of sequencing data. Here, the procedure was performed with Chromium™ Single Cell 3′ Library & Gel Bead Kit v2 and has been optimized for T lymphocytes from mouse models according to Nozais et al. (2021). However, this protocol has also been implemented with Chromium™ Single Cell 5′ Kit. Moreover sample multiplexing can be applied to a wide range of cells, notably human cells (Rabilloud et al., 2021). In this protocol, we used both female and male mice aged between 4 to 6 weeks. Mice were bred and housed in specific pathogen-free conditions in CIML animal facilities and were handled in accordance with French and European guidelines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Streptavidin BV421 1:1000	BD Pharmingen	Cat# : 3563259; RRID: AB_2869475
TotalSeqA0301 anti-mouse Hashtag 1 antibody 1:50	BioLegend	Cat# : 155801; RRID: AB_2750032
TotalSeqA0302 anti-mouse Hashtag 2 antibody 1:50	BioLegend	Cat# : 155803; RRID: AB_2750033
TotalSeqA0303 anti-mouse Hashtag 3 antibody 1:50	BioLegend	Cat#: 155805; RRID: AB_2750034
TotalSeqA0304 anti-mouse Hashtag 4 antibody 1:50	BioLegend	Cat#: 155807; RRID: AB_2750035
TotalSeqA0305 anti-mouse Hashtag 5 antibody 1:50	BioLegend	Cat#: 155869; RRID: AB_2750036
TotalSeqA0306 anti-mouse Hashtag 6 antibody 1:50	BioLegend	Cat#: 155811; RRID: AB_2750037
TotalSeqA0307 anti-mouse Hashtag 7 antibody 1:50	BioLegend	Cat#: 155813; RRID: AB_2750039

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TotalSeqA0308 anti-mouse Hashtag 8 antibody 1:50	BioLegend	Cat#: 155815; RRID: AB_2750040
CD16/CD32 Monoclonal Antibody 1:1000	Thermo Fisher Scientific	Cat#: 14-0161-85; RRID: AB_467134
Chemicals, peptides, and recombinant proteins		
Annexin V-APC**	BD Pharmingen	Cat#: 550474
KAPA HiFi HotStart ReadyMix (2×)	Roche Diagnostics	Cat#: 7958935001
1× RBC Lysis Buffer**	Thermo Fisher Scientific	Cat#: 12770000
RPMI 1640**	Life Technologies	Cat#: 21875-034
Bovine Serum Albumin 5%**	Thermo Fisher Scientific	Cat#: AM2616
Dulbecco's Phosphate Buffered Saline 10× (PBS)**	Life Technologies	Cat#: 14200-067
Fetal Bovine Serum (FBS)**	Life Technologies	Cat#: 10270-106
β-mercaptoethanol	Life Technologies	Cat#: 31350-010
Penicillin Streptomycin	Life Technologies	Cat#: 15140122
GlutaMax	Life Technologies	Cat#: 25030-024
Sodium Pyruvate	Life Technologies	Cat#: 11360-039
Critical commercial assay		
Kit 10× 3′ version 2	10× Genomics	Cat#: 120267
Agilent High Sensitivity DNA Kit	Agilent	Cat#: 5067-4626
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat#: Q32851
EasySep Mouse T cell isolation kit	Life Technologies	Cat#: 19851
EasySepfdg Dead Cell Removal (Annexin V) Kit	Life Technologies	Cat#: 17899
Experimental models: Organisms/strains		
$Pten^{flox/flox}$ mice	European Mouse Mutant Archive	EM:00406
$Myc^{flox/flox}$ mice	Andreas Trumpp (DKFZ)	Bishop JM. Nature. 414, 768-73 (2001).
CD4-Cre mice	European Mouse Mutant Archive	EM: 01139
ROSA26-eYFP reporter mice	The Jackson Laboratory	MGI: 2449038
Deposited data		
Raw and analyzed data	Nozais et al., 2021	GEO: GSE169374, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169374
Zenodo	Nozais et al., 2021	Zenodo: https://zenodo.org/record/4636520
Mouse reference MM10 (Ensembl 93) 3.0.0	10× Genomics	ftp://ftp.ensembl.org/pub/release-93/fasta/mus_musculus/dna/Mus_musculus.GRCm38.dna.primary_assembly.fa.gz
Code and dataset	Nozais et al., 2021	https://github.com/mathisnozais/MycPten
Oligonucleotides		
HTO oligo biotinylated : /5Biosg/CTGTCTCTTATACACATCTC	Integrated DNA Technologies	n/a
HTO Additive : GTGACTGGAGTTCAGACGTGTGCTC	Integrated DNA Technologies	n/a
Truseq_D701_s*: CAAGCAGAAGACGGCATACGAGA**TCGAGTAAT**GTGACTGGAGTTCAGACGTGT*G*C	Integrated DNA Technologies	n/a
Software and algorithms		
FlowJo version 10	FlowJo	https://www.flowjo.com/
Diva version 8.0.1	BD Biosciences	https://www.bdbiosciences.com/
Citeseq count version 1.4.3	NYGCtech	GitHub - Hoohm/CITE-seq-Count: A tool that allows to get UMI counts from a single cell protein assay
R version 3.5.3	R PROJECT	R: The R Project for Statistical Computing (r-project.org)
Cell Ranger 3.0.1	10× Genomics	https://www.10xgenomics.com/
Other		
6-well plate**	Falcon	Cat#: 353046
5 mL polystyrene tubes**	Falcon	Cat#: 352054
50 mL conical tubes**	Sarstedt	Cat#: 62.547.254
70 μm cell strainer**	Sarstedt	Cat#: 83.3945.070
EasySep Magnet	STEMCELL Technology	Cat#: 18000

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hemocytometer**	KOVA International	Cat#: 87144F
Microscope TS100**	Nikon Eclipse	This model is discontinued and any similar inverted microscope (such as Nikon Eclipse Ts2) can be used.
Centrifuge**	Eppendorf	Cat#: G5810R
1.5 mL microcentrifuge**	Eppendorf	Cat#: G5415R
BD FACSCanto II Cell Analyzer**	Becton Dickinson	n/a
10× Chromium Controller	10× Genomics	Cat#: 1000202
2100 Bioanalyzer	Agilent	Cat#: G2938C

*The i7 index 1 sequence is written in bold, additional index sequences can be found on Illumina website.
**Similar reagents or equipment from other companies can be used for this protocol.

MATERIALS AND EQUIPMENT

PBS1×

Reagent	Final concentration	Amount
Dulbecco's Phosphate Buffered Saline (PBS) 10×	1×	100 mL
dH_2O	n/a	Up to 1L
Total	**n/a**	**1 L**

Store at 4°C for up to 1 month.

PBS1×/2%FBS

Reagent	Final concentration	Amount
Fetal Bovine Serum (FBS)	2%	8 mL
PBS1×	1×	Up to 400 mL
Total	**n/a**	**400 mL**

Store at 4°C for up to 1 month.

PBS1×/0.04%BSA

Reagent	Final concentration	Amount
Bovine Serum Albumin (BSA) 5%	0.04%	60 µL
PBS1×	1×	Up to 7.5 mL
Total	**n/a**	**7.5 mL**

Store at 4°C until use.

Complete RPMI medium

Reagent	Final concentration	Amount
RPMI 1640	n/a	435 mL
Fetal Bovine Serum	10×	50 mL
GlutaMax	1×	5 mL
Sodium Pyruvate	1×	5 mL
Penicillin Streptomycin	1×	5 mL
β-mercaptoethanol	50 µM	500 µL
Total	**n/a**	**500 mL**

Store at 4°C for up to 1 month.

STEP-BY-STEP METHOD DETAILS

Harvesting T cells from thymus and spleen

Timing: 2 h

This section describes how to obtain mouse T cells from thymus and spleen.

1. Decontaminate working spaces with 70% ethanol before beginning.
2. Euthanize mice with carbon dioxide.

Note: Use this method instead of cervical dislocation to preserve the thymus.

3. Sterilize the skin with 70% ethanol.
4. Dissect the mice, collect thymus and spleens, and place each organ in 5 mL of PBS1×/2%FBS.
5. Place a 70 μm cell strainer on the top of a 50 mL conical tube, then moisten the strainer by adding 2 mL of PBS1×/2%FBS. Grab the organ with tweezers and transfer it onto the cell strainer.
6. With the flat end of a syringe piston, dilacerate the organ and add around 10 mL of PBS1×/2% FBS with a pipette to help the dilaceration and to wash the strainer.
7. Remove the cell strainer and centrifuge (7 min, 450 rcf, 15°C) the 50 mL conical tube containing the filtered cell suspension.
8. Remove the supernatant.
9. Resuspend splenic cell pellet in 2 mL of Red Blood Cell (RBC) lysis buffer, and incubate at room temperature (RT; 20°C–25°C) for 10 min. Then, add 10 mL of PBS1×/2%FBS and centrifuge (7 min, 450 rcf, 4°C). Remove the supernatant and resuspend cell pellet in 10 mL of PBS1×/2%FBS.
10. In parallel to the RBC treatment of splenic samples, resuspend the thymic cell pellet in 10 mL of PBS1×/2%FBS.

Note: RBC lysis is optional for thymic cells, as thymus usually contains low numbers of erythrocytes.

11. Take 5 μL of cells and mix them with 45 μL of trypan blue (previously diluted at 1:1 ratio with PBS1×).
12. Load cell mixture onto a hemocytometer. Using a microscope, count viable cells which correspond to bright cells that are not stained in blue.
13. Centrifuge 50 mL conical tubes containing 10 mL of cell suspension (7 min, 450 rcf, 4°C).
14. Resuspend the pellet in PBS1×/2%FBS at 10 × 10^6 cells/mL or 100 × 10^6 cells/mL when a purification step is required (see note below).

Note: To avoid the sequencing of unwanted cells, a cell purification step might be required to enrich the sample for a cell population of interest, or to remove dead cells. We use EasySep™ Mouse T Cell Isolation Kit to purify T lymphocytes from the spleen. Moreover, if more than 20% dead cells are observed, EasySep™ Dead Cell Removal-Annexin V Kit can be applied to remove dead cells. As an alternative, the autoMACS® Pro Separator (Miltenyi Biotec technology; cat#: 130-092-545) with Dead cell removal kit (Miltenyi Biotec technology; Cat#: 130-090-101) can be used.

Sample labelling with Ab-HTO

Timing: 2 h

In this procedure, we utilized antibodies tagged with hashtag oligonucleotides (Ab-HTO) from BioLegend. These antibodies are a mix of anti-CD45 and anti-MHC-I antibodies. TotalSeq™-A

anti-mouse Hashtag reagents are used with Chromium™ Single Cell 3′ kit, while TotalSeq™-C with Single Cell 5′ kit.

15. Transfer approximately 500,000 cells (50 µL of 10 × 10^6 cells/mL suspension) of each sample into a 1.5 mL microcentrifuge tube.

Note: We advise to perform the assay with more than 50,000 cells. With a small number of cells (less than 50,000 cells) we noticed a tendency to lose cells during washing steps.

Compared to 5 mL polystyrene tubes (FACS tubes), we observed better outcomes with microcentrifuge tubes. Indeed in the latter, cell pellet is clearly visible and the supernatant can be thoroughly and safely removed.

16. Dilute Fc block (CD16/CD32 antibody) to 1/100 in PBS1×/2%FBS, take 5 µL and add it to the cell suspension.
17. Incubate 10 min at 4°C.
18. Add 1 µL of a distinct Ab-HTO (0.5 µg) to each sample and record their assignment.
19. Incubate 30 min at 4°C.
20. Transfer 1/10 of your cell volume to a FACS tube and proceed with quality control of Ab-HTO labelling (steps 26 to 32). For the remainder of the sample proceed to step 21.
21. Add 1 mL of PBS1×/2%FBS and centrifuge (5 min, 700 rcf, 4°C).
22. Remove the supernatant and resuspend in 1 mL of PBS1×/2%FBS. Centrifuge (5 min, 700 rcf, 4°C).
23. Repeat the washes (step 22) three more times. The last wash is performed in 1 mL PBS1×/0.04% BSA.

Note: Thorough washing steps are necessary to remove free Ab-HTO (see troubleshooting 1).

24. After the last wash, resuspend cell pellet in 100 µL of PBS1×/0.04%BSA.
25. Count cells as described in steps 11 & 12. (We advise to perform cell counting in duplicate).

⚠ CRITICAL: Follow the washing procedure in order to eliminate free Ab-HTO.

Validation of Ab-HTO labelling

Timing: 1H30

This section can be skipped; however it is highly recommended (see troubleshooting 2). Indeed, if cells are not properly labeled with Ab-HTO, samples will not be accurately demultiplexed and the full scRNA-seq experiment is at stake. This section is carried out in parallel to 'sample labelling with ab-HTO'.

26. Add 5 µL of biotinylated oligo (previously diluted to 1 µM in PBS 1×) to the cell suspension from step 20.
27. Incubate 30 min at RT.
28. Add 1 mL of PBS1×/2%FBS and centrifuge (5 min, 700 rcf, 4°C). Remove the supernatant.
29. Resuspend cell pellet in 50 µL PBS/2%FBS and add 0.1 µg of fluorescent Streptavidin (herein we used 5 µL of Streptavidin-BV421 previously diluted at 1/100).
30. Incubate 15 min at 4°C in the dark.
31. Add 1 mL of PBS1× and centrifuge (5 min, 700 rcf, 4°C).
32. Use flow cytometry to check the percentage of Streptavidin-BV421 positive cells (Figure 1).

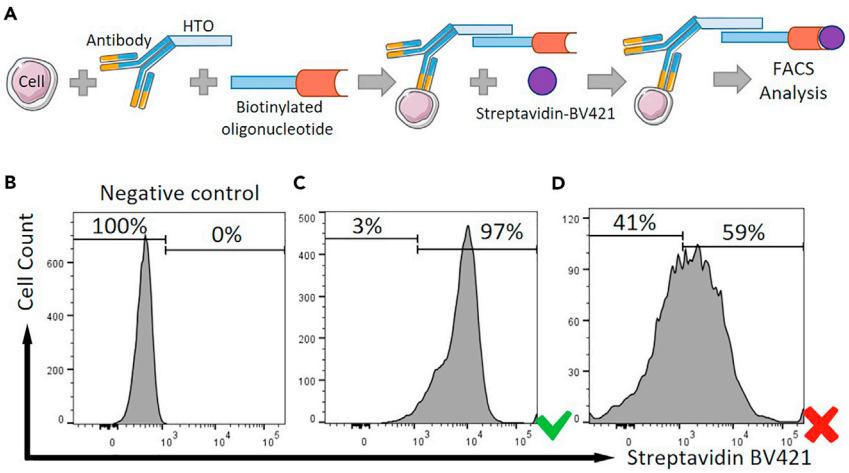

Figure 1. Quality control of cell labelling with antibodies coupled to HTO (Ab-HTO)

(A) Scheme of the Ab-HTO labelling validation workflow. Cells labelled with Ab-HTO are incubated with complementary biotinylated oligonucleotides. After a wash in PBS1×/2%FBS, streptavidin coupled to BV421 was added.

(B–D) FACS analysis. (B) Negative control corresponds to cell incubated only with biotinylated oligonucleotides and streptavidin-BV421 (without Ab-HTO). (C and D) Cells incubated with an Ab-HTO, biotinylated oligonucleotides and streptavidin-BV421. (C) Cells are properly labelled with Ab-HTO. (D) 40% of cells are unlabelled. We consider that this sample does not pass the quality control of the 'Ab-HTO labelling' step and thus should not be used for multiplexed scRNA-seq experiment.

Note: We expect all cells to be labelled with Ab-HTO. Do not proceed to scRNA-seq assay if less than 90% of cells of interest are labelled.

Pooling

Timing: 15 min

33. Pool the different samples at your chosen ratio. In Nozais et al. (2021) up to 8 samples were mixed in PBS1×/0.04%BSA for a total of 25 000 cells.

Proceed to single-cell emulsion (step 34) as soon as possible because keeping cells for extended time may increase cell death. To save time, we recommend that two investigators carry out sample preparation up to step 33 (samples pooling), especially if many samples are processed.

Libraries preparation

Timing: 2.5 days

34. Proceed with single-cell emulsion using the 10× Genomics Chromium Controller according to manufacturer instructions (CG000185 Rev B user guide).

⚠ CRITICAL: As described in 10× Genomics user guide, you should obtain a uniform emulsion. The presence of a large 'clear' phase indicates a problem in gel beads-in-emulsion (GEM) generation, putting at stake the success of the assay.

35. Prepare libraries for scRNA-Seq using Chromium™ Single Cell 3′ Library & Gel Bead Kit v3 (ref: PN 1000075). Follow 10× Genomics CG000185 Rev B user guide, except for cDNA amplification mix and for HTO library construction (see below).

36. cDNA amplification (step 2.2.a. from CG000185 Rev B user guide). Proceed with the following change (in order to amplify the HTO along with the cDNA):

Reagent	10× Reference	Volume for 1 sample
Amp Mix	2000047	50 µL
HTO additive primer (20 µM)	See key resources table	0.5 µL (0.1 µM final)
cDNA primer mix	2000089	15 µL

37. Cell surface protein (or HTO) Library construction (step 4.1 from CG000185 Rev B). Proceed with the following changes:
    a. Prepare sample Index PCR mix (we used KAPA HiFi PCR master mix as it gives rise to the same quality results as the 10× Genomics Amp Mix. KAPA HiFi has the advantage of being available independently of the 10× library kit).

Sample index PCR mix	10× Reference	Volume for 1 sample
2× KAPA HiFi PCR master mix	See key resources table	50 µL
Truseq_D701_s 10 µM	See key resources table	2.5 µL
SI-PCR primer	PN-220111 or PN-2000095	2.5 µL
Transferred Supernatant Cleanup (step 2.3B-xiv)	n/a	5 µL
Nuclease-free water	n/a	40 µL

PCR cycling conditions (Lid temperature 105°C)			
Steps	Temperature	Time	Cycles
1	95°C	3 min	1
2	95°C	20 s	10 cycles
3	64°C	30 s	
4	72°C	20 s	
5	72°C	5 min	1
6	4°C	Hold	

    b. Incubate in a thermal cycler with the following parameters (adapted to the use of KAPA HiFi).

Note: When using 10× Genomics 5′ kit we use the protocol CG000186 Rev A.

Quality control of library

Timing: 1 h

After preparation of the libraries, size verification is required (Figure 2).

38. Measure the concentration of the libraries with Qubit dsDNA HS™ Assay Kit as described by the manufacturer's instructions.
39. Load 1 µL of your libraries onto a High Sensitivity DNA chip (Agilent™) (concentration should not exceed 10 ng/µL – otherwise dilute in nuclease free water before loading).
40. Check the quality of HTO and cDNA libraries (see Figure 2 that displays good quality library profiles, and troubleshooting 3) and determine the average fragment size.

Library pooling

Timing: 30 min

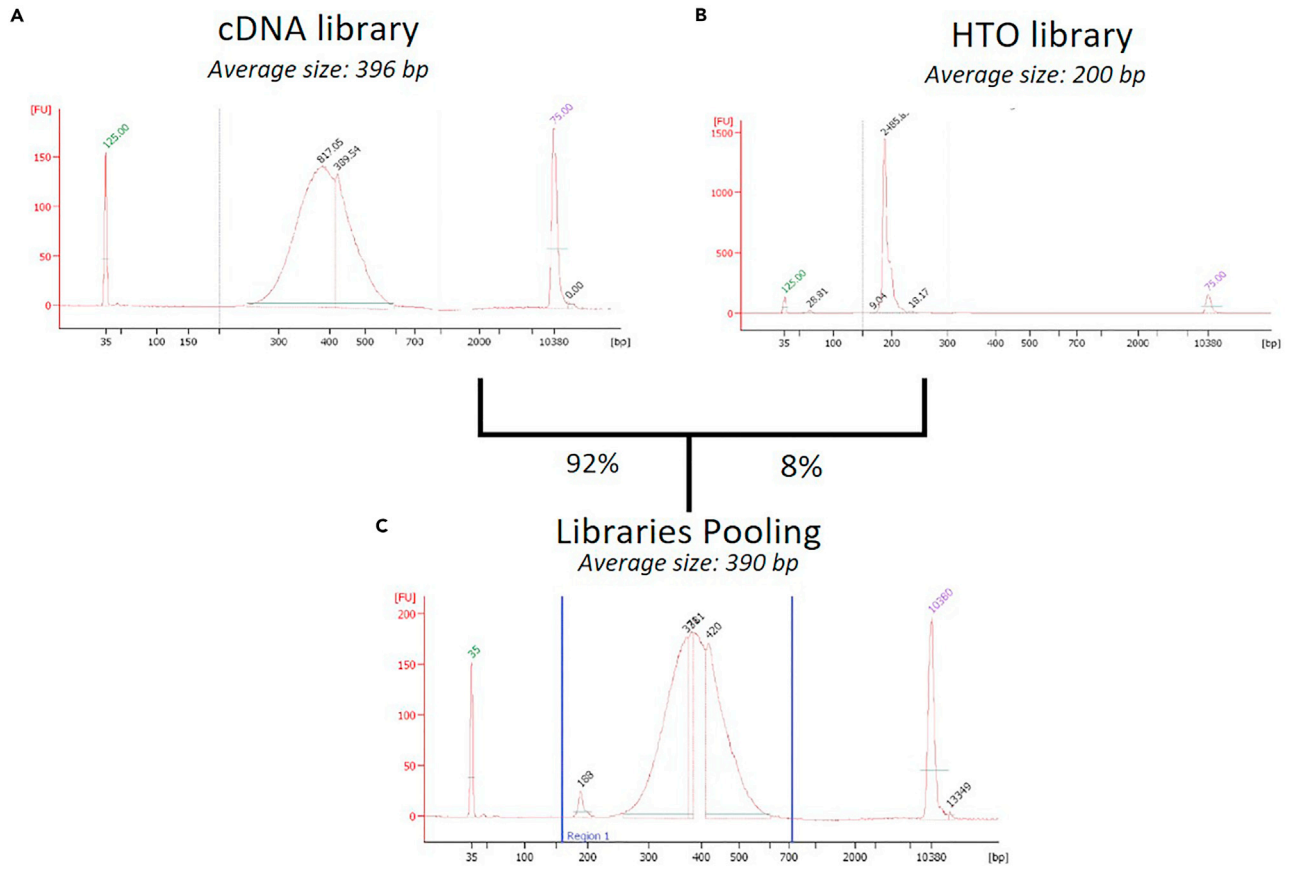

Figure 2. Quality control of cDNA and HTO libraries

After library construction, 1 μL of each library is loaded onto Agilent Bioanalyzer High Sensitivity DNA chip.

(A and B) Typical examples of Bioanalyzer electropherogram traces of cDNA (A) and HTO (B) libraries are shown. From these traces we define average library size: 200 bp for HTO and usually around 400 bp for cDNA.

(C) These sizes are used to calculate the molar concentration of libraries. The two libraries are pooled and reanalyzed on the Bioanalyzer (C) and then sequenced.

This section details the method used for the pooling of libraries.

41. Dilute both HTO and cDNA libraries to a final concentration of 4 nM.

Note: We use this formula to calculate molar concentration:

$$\frac{Qubit\ concentration\ (ng/\mu L)}{660 \times Average\ library\ size\ (bp)} \times 10^6$$

In the example in Figure 2, the average size of the cDNA library is 396 bp and the Qubit concentration is 30.6 ng/μL, thus molar concentration of the cDNA library is 117 nM. Dilute 3.42 μL of the library up to 100 μL with nuclease-free water to obtain a solution of 4 nM. Check the concentration of the diluted library with Qubit dsDNA HS™ Assay Kit.

42. Pool the 4 nM libraries. We usually use a ratio of 8% HTO and 92% of cDNA, thus in our example 92 μL of cDNA library are mixed with 8 μL of HTO library. Then quantify DNA concentration of the final pool with Qubit dsDNA HS™ Assay Kit as described at step 38.

Sequencing and demultiplexing

Timing: 2–3 days

This section addresses the main steps of sequencing data processing in order to demultiplex the different samples. For more details on all the pre-processing steps, please refer to the GitHub repository (see data and code availability). This process requires the use of UNIX-based tools and R.

Note: We obtain FASTQ files from the sequencing facility however one can start from BCL requiring an extra step for FASTQ generation.

43. Sequence the pool. We used Illumina Next-seq 500 system and the following parameters: Read 1: 26 cycles, i7: 8 cycles & Read 2: 57 cycles.
44. Reads from cDNA library are aligned to the mm10 version of the mouse genome and quantified using Cell Ranger count function (Zheng et al., 2017).

Note: The summary of reads assembly and quantification process from Cell Ranger can be found on the web_summary.html file after the run. Based on our experience, the expected number of cells is approximately one third of the number of loaded cells and the number of median genes per cell is 1000-1 500 (these numbers are cell type specific). A lower number of captured cells may be caused by the inclusion of too many dead/damaged cells.

45. Reads from HTO library are quantified using CITE-seq-count (Roelli et al., 2019). We utilized -cbf 1, -cbl 16, -umif 17, -umil 26 and -max-error 2 as parameters (see CITE-seq-count documentation for more information about the parameters).
46. Load HTO and cDNA matrices in Seurat (Stoeckius and Zheng, 2018) and keep only cell-barcodes that are found in both matrices. To create a Seurat object, we use these parameters:
    a. Remove low quality cells expressing less than 200 genes and/or more than 10% mitochondrial genes.
    b. Remove genes that are expressed in less than 3 cells.

Note: The detailed code to setup a Seurat object comprising cDNA and HTO data can be found on Seurat website "Demultiplexing with hashtag oligos (HTO)".

Note: These thresholds can be modified according to the cell type present in a given experiment. Mitochondrial fractions can also vary experiment-to-experiment, so thresholds should be based on the observed distributions.

47. Normalize HTO matrices using the Centered Log Ratio (CLR) transformation across cells.
48. Demultiplex your sample to identify the droplet samples of origins.

Note: For demultiplexing we use MULTIseqDemux function (McGinnis et al., 2019). An alternative is HTODemux which is used in Seurat.

49. Remove from the Seurat object cell-barcodes identified as negative or doublet.

Note: The use of Ab-HTO facilitates inter-sample doublet removal. Indeed, two different HTO harboring the same 10× barcode indicates that two cells were simultaneously encapsulated in one droplet (Figure 3). Yet there is still the possibility to have intra-sample doublets.

Quality control of sequencing data

In this section, we describe some critical metrics from Cell Ranger report. The parameters given below are for T cells and might differ for other cell types.

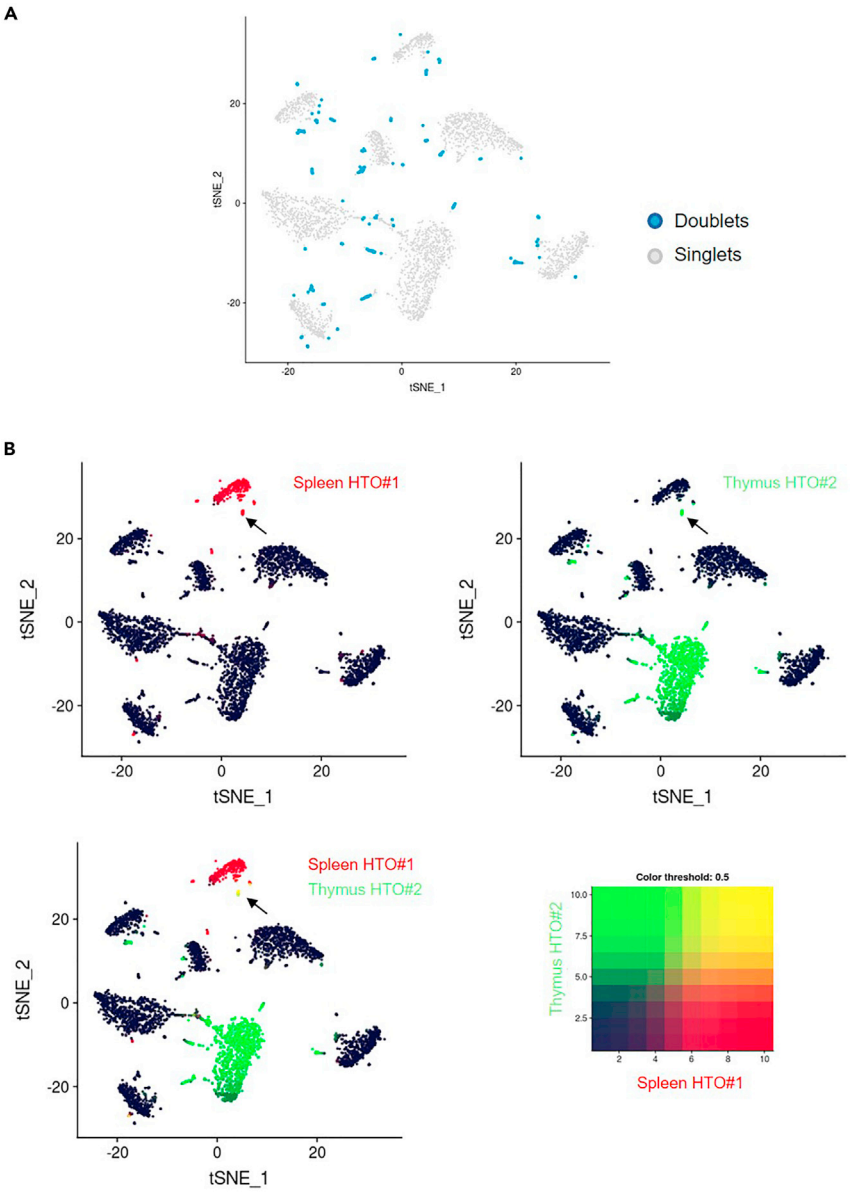

Figure 3. Labelling of samples with different Ab-HTO facilitates the removal of cell doublets

(A) tSNE plot highlighting the position of cells identified as doublet after demultiplexing.

(B) Example of the co-occurence of two HTO. Upper panels are tSNE plot showing the normalized counts of "Spleen HTO#1" in red (on the left) and " Thymus HTO#2' in green (on the right) in each cells. The bottom panel shows the tSNE colored according to the normalized counts for the two HTO of interest. The arrows are pointing a group of doublet cells from "Spleen HTO#1" and "Thymus HTO#2 " samples. The color code is indicated.

50. We expect a minimum of 400,000,000 reads to be sequenced yielding approximately 1000 median genes per cell and 100,000 read per cell. To identify a meaningful number of genes, we attempt to reach around 80% of saturation (see troubleshooting 4).
51. Among other important parameters, we check the sequencing read and mapping quality. The percentage of reads (barcode, UMI and RNA reads) having a Q-score > 30 should be > 80%, and the percentage of reads mapped confidently to the genome should be higher than 80%.

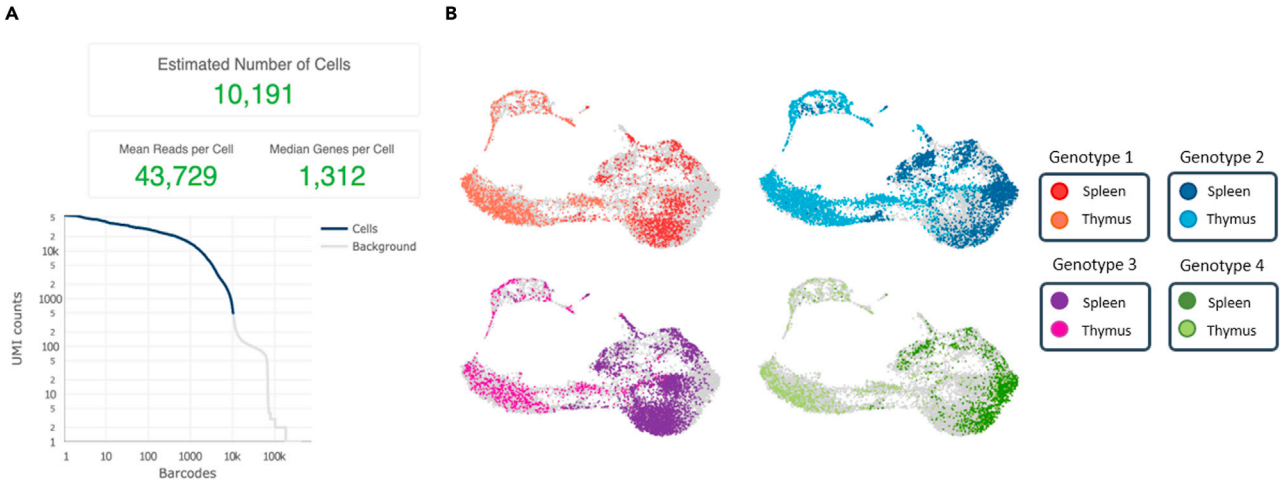

Figure 4. Expected results from Cell Ranger and data demultiplexing

(A) Output from Cell Ranger indicating the number of estimated cells, means reads per cell, median genes per cells, and the distribution UMI counts across barcodes.

(B) The Uniform Manifold Approximation and Projection (UMAP) plot, colored according to tissue (thymus or spleen) and mouse genotype. Ab-HTO labelling and the demultiplexing procedure described herein allowed us to identify the origin of each single cell.

52. Moreover, the fraction of reads that contains a valid cell barcode should be higher than 70%. Lower percentage suggests that a high fraction of reads (coming from ambient RNA) are not associated with a detected cell (empty droplets), leading to a noisier dataset.

Note: Depending on the cell type, those parameters may vary. Typically, cells expressing many genes such as tumoral cells may require to sequence more reads or to load less cells (see troubleshooting 4).

EXPECTED OUTCOMES

The aim of this protocol is to perform a scRNA-seq experiment with multiple samples that can be efficiently demultiplexed through bioinformatic analysis. We performed a scRNA-seq experiment with 8 samples from thymus and spleen of 4 mice. Following cell harvesting, thymic and splenic samples were individually labelled with an Ab-HTO. We recommend performing a quality control for the Ab-HTO labelling step, as this gives the opportunity to the investigator to exclude (prior cell loading onto 10× Chromium Controller) samples that are not properly labelled (Figure 1). Indeed, unlabelled cells cannot be assigned to a given sample during the sequencing data pre-processing step. After Ab-HTO labelling, samples are pooled and analyzed by scRNA-seq using Chromium 10× platform. Two libraries are produced: cDNA and HTO, those are quantified with the Qubit system and we expect to obtain a concentration ranging from 15–30 ng/μL for cDNA library and 5–20 ng/μL for HTO library. The quality and the average size of libraries are assessed using Agilent Bioanalyzer. We expect the average size for cDNA and HTO libraries to be around 400 bp and 200 bp respectively (Figure 2). The two libraries are pooled, sequenced and finally sequencing reads are processed with Cell Ranger (Figure 4) and CITE-seq-count. Then demultiplexing is done in R with MULTI-seq function. Figures 5A and 5B displays a successful demultiplexed experiment in which cells are properly separated between the 8 samples. In a former experiment performed with 6 samples, we could not assign cells to sample (Figures 5C and 5D) due to the over-representation of one HTO (HTO#5) in all samples and the under-representation of other HTO (HTO#1, #3 and #6) (see troubleshooting section).

LIMITATIONS

In this protocol, murine cells are labelled with TotalSeq-A antibody from BioLegend which is a mix of anti-CD45 and MHC-I antibodies. Consequently, cells must express CD45 and/or MHC-I at the cell

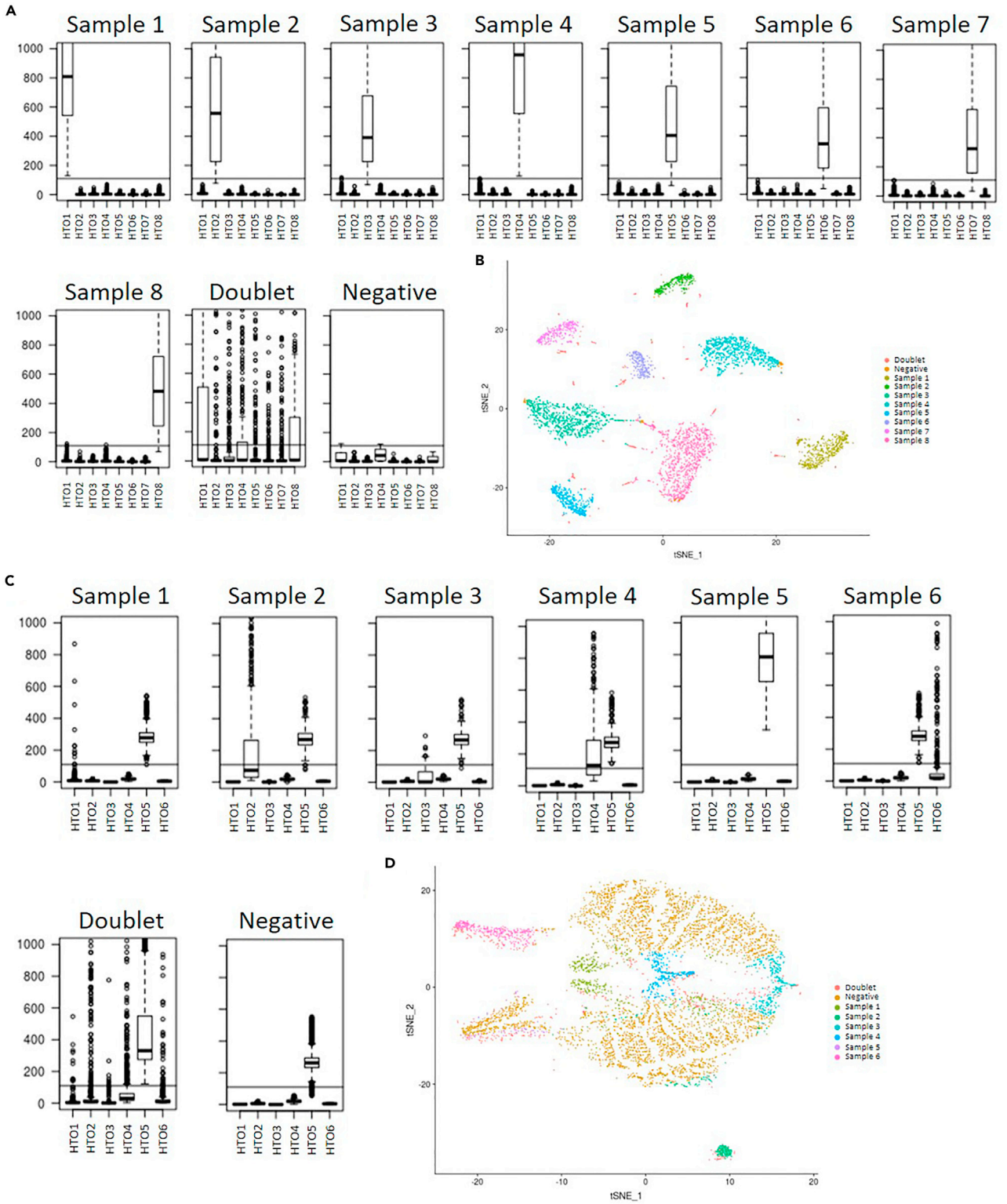

Figure 5. Typical examples of successful and failed demultiplexing

(A and B) Successful demultiplexing. (A) Box plots showing HTO raw counts in each cells for each samples that were assigned by the demultiplexing tool. As expected, counts for HTO#1 are high in sample 1 while they are low for the other samples. Similar results are obtained for the other HTO: their counts are high in one specific sample. In doublets, several HTO are detected, while for negative cells no HTO is clearly detected.

Figure 5. *Continued*
(B) t-SNE plot based on HTO CLR normalized counts matrix. Cells are colored according to MULTI-seq demultiplexing identification.
(C and D) Failed demultiplexing. (C) Box plots showing HTO raw counts in each cells for each samples that were assigned by the demultiplexing tool. In this example, 6 samples were mixed in a scRNAseq assay. High counts for HTO#5 were found in all samples while counts for HTO#1, #3 and #6 were systematically low. (D) t-SNE plot based on HTO CLR normalized counts matrix. Cells are colored according to MULTI-seq demultiplexing identification.

surface. An alternative is to generate home-made Ab-HTO (Rabilloud et al., 2021; van Buggenum et al., 2016).

TROUBLESHOOTING

Problem 1

HTO contamination

One major problem is the contamination of all samples by a given HTO. This impedes proper sample demultiplexing. Indeed most cells will appear as 'negative' or as 'doublets' (Figures 5C and 5D; step 18).

Potential solution

This problem is due to a carryover of free Ab-HTO during samples pooling. To overcome this problem, we thoroughly wash the cells after Ab-HTO labelling (steps 21 to 23). Alternatively, cells can be sorted using a FACS cytometer, this will remove free Ab-HTO.

Problem 2

Low level of cell labelling with Ab-HTO

Low level of cell labelling with Ab-HTO compromises efficient sample demultipexing (step 32).

Potential solution

Perform the 'validation of Ab-HTO labelling' assay (described in steps 26 to 32). If more than 10% of cells of interest are unlabeled, you may increase the quantity of Ab-HTO or titrate Ab-HTO using the validation assay. We encountered this problem when we put too many cells in respect to the amount of Ab-HTO.

Problem 3

Poor quality of the Bioanalyzer profile

Quality control of libraries using Agilent Bioanalyzer does not result in typical electropherogram traces shown in Figure 2 (step 39).

Potential solution

Poor quality profiles are shown in Figure 6.

In Figure 6A, the HTO library does not consist of a single 200 bp peak. First, repeat the construction of the library starting from step 37. If the problem persists, this indicates a problem of Ab-HTO labelling. In the example displayed in Figure 6A the problem comes from insufficient cell labelling (see problem 2 above).

Few times, we observed a poor quality profile of the cDNA library (Figure 6B). Reload the sample on a new Agilent chip and freshly prepared gel. You can also reload the library at a lower concentration. If the problem is not solved, the libraries must be prepared again.

Problem 4

The sequencing saturation in Cell Ranger report is below 80%

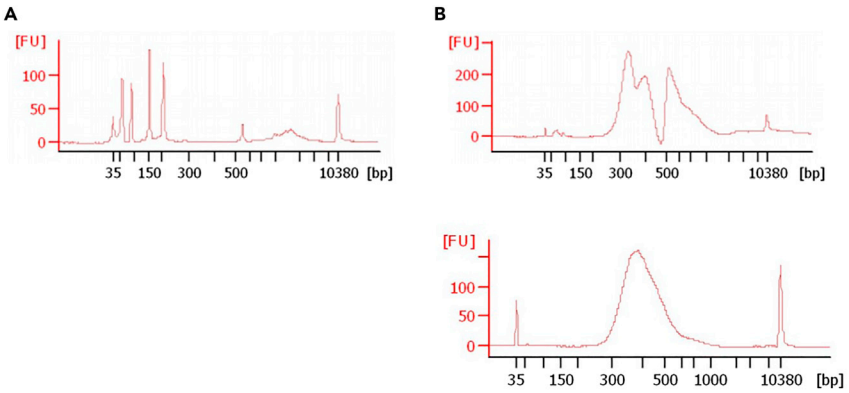

Figure 6. Problems with quality control of the libraries

(A) Faulty HTO library. Several peaks are observed on the Bioanalyzer electropherogram, consequently this experiment was not sequenced.

(B) Bioanalyzer electropherogram of a cDNA library. In the first analysis (top panel) the profile obtained was unsatisfactory. The same sample was reloaded at a lower concentration on a new Bioanalyzer Chip (bottom panel). It passed the quality control and thus was sequenced.

If the cell type possesses a high quantity of RNA content this may result to a sequencing saturation below 80% (step 43).

Potential solution

Perform an additional sequencing run using the same library. The concatenation of the two sequencing runs will give rise to higher saturation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dominique PAYET-BORNET (payet@ciml.univ-mrs.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The accession numbers for raw sequences and data reported in this paper are GEO: GSE169374 and Zenodo: 4636520, respectively. All the codes used in this study are available at https://github.com/mathisnozais/MycPten.

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AUTHOR CONTRIBUTIONS

M.N., C.G., J.Q., M.L., and D.P.B. performed the experiments. S.P., D.P., and M.N. performed bioinformatic analysis. S.P., M.N., and D.P.B. wrote the paper. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

van Buggenum, J.A.G.L., Gerlach, J.P., Eising, S., Schoonen, L., van Eijl, R.A.P.M., Tanis, S.E.J., Hogeweg, M., Hubner, N.C., van Hest, J.C., Bonger, K.M., and Mulder, K.W. (2016). A covalent and cleavable antibody-DNA conjugation strategy for sensitive protein detection via immuno-PCR. Sci. Rep. *6*, 22675. https://doi.org/10.1038/srep22675.

McGinnis, C.S., Patterson, D.M., Winkler, J., Conrad, D.N., Hein, M.Y., Srivastava, V., Hu, J.L., Murrow, L.M., Weissman, J.S., Werb, Z., et al. (2019). MULTI-seq: sample multiplexing for single-cell RNA sequencing using lipid-tagged indices. Nat. Methods *16*, 619–626. https://doi.org/10.1038/s41592-019-0433-8.

Nozais, M., Loosveld, M., Pankaew, S., Grosjean, C., Gentil, N., Quessada, J., Nadel, B., Mionnet, C., Potier, D., and Payet-Bornet, D. (2021). MYC deficiency impairs the development of effector/memory T lymphocytes. iScience *24*, 102761. https://doi.org/10.1016/j.isci.2021.102761.

Rabilloud, T., Potier, D., Pankaew, S., Nozais, M., Loosveld, M., and Payet-Bornet, D. (2021). Single-cell profiling identifies pre-existing CD19-negative subclones in a B-ALL patient with CD19-negative relapse after CAR-T therapy. Nat. Commun. *12*, 865. https://doi.org/10.1038/s41467-021-21168-6.

Roelli, P., Flynn, B., and Gui, G. (2019). Hoohm/CITE-seq-Count: 1.4.2. Zenodo. https://doi.org/10.5281/zenodo.2590196.

Stoeckius, M., and Zheng, S. (2018). Cell hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. Genome Biol. *19*, 1–12. https://doi.org/10.1186/s13059-018-1603-1.

Zheng, G.X.Y., Terry, J.M., Belgrader, P., Ryvkin, P., Bent, Z.W., Wilson, R., Ziraldo, S.B., Wheeler, T.D., McDermott, G.P., Zhu, J., et al. (2017). Massively parallel digital transcriptional profiling of single cells. Nat. Commun. *8*, 14049. https://doi.org/10.1038/ncomms14049.