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 10X 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.
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)
| 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**

| REAGENT | SOURCE | IDENTIFIER |
|---------|--------|------------|
| Annexin V-APC** | BD Pharmingen | Cat#: 550474 |
| KAPA HiFi HotStart ReadyMix (2x) | Roche Diagnostics | Cat#: 7958935001 |
| 1X RBC Lysis Buffer** | Thermo Fisher Scientific | Cat#: 1277000 |
| RPMI 1640** | Life Technologies | Cat#: 21875-034 |
| Bovine Serum Albumin 5%** | Life Technologies | Cat#: 14200-067 |
| Dulbecco’s Phosphate Buffered Saline 10X (PBS)** | Life Technologies | Cat#: 10270-106 |
| Fetal Bovine Serum (FBS)** | Life Technologies | Cat#: 31350-010 |
| penicillin streptomycin | Life Technologies | Cat#: 15140122 |
| sodium pyruvate | Life Technologies | Cat#: 25030-024 |
| sodium pyruvate | Life Technologies | Cat#: 11360-039 |

**Critical commercial assay**

| Kit 10X 3’ version 2 | 10X Genomics | Cat#: 120267 |
| Agilent High Sensitivity DNA Kit | Agilent | Cat#: 50674626 |
| 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**

| STUDY | ORGANISM | STRAIN | SOURCE |
|-------|----------|--------|--------|
| Pten** mice | European Mouse Mutant Archive | EM:00406 |
| Myc** 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**

| STUDY | ORGANISM | STRAIN | SOURCE |
|-------|----------|--------|--------|
| 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 | 10X 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**

| STUDY | ORGANISM | STRAIN | SOURCE |
|-------|----------|--------|--------|
| HTO oligo biotinylated : /5Biosg/CTGTCTCTTATACACATCTC | Integrated DNA Technologies | n/a |
| HTO Additive: GTGACTGGAGTTCAGACGTGTGCTC | Integrated DNA Technologies | n/a |
| Trueq_D701_5*: CAAGCAAGACCGCATAACGGA | Integrated DNA Technologies | n/a |
| TCGAGTAATGTGACTGGAGTTCAGACGTGT*C | Integrated DNA Technologies | n/a |

**Software and algorithms**

| STUDY | ORGANISM | STRAIN | SOURCE |
|-------|----------|--------|--------|
| 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 essay |
| R version 3.5.3 | R PROJECT | R: The R Project for Statistical Computing (r-project.org) |
| CellRanger 3.0.1 | 10X Genomics | https://www.10xgenomics.com/ |

**Other**

| STUDY | ORGANISM | STRAIN | SOURCE |
|-------|----------|--------|--------|
| 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)
### MATERIALS AND EQUIPMENT

**PBS1 x**

| Reagent                                      | Final concentration | Amount     |
|----------------------------------------------|---------------------|------------|
| Dulbecco’s Phosphate Buffered Saline (PBS) 10x | 1x                  | 100 mL     |
| dH₂O                                         | n/a                 | Up to 1L   |
| Total                                        | n/a                 | 1 L        |

Store at 4°C for up to 1 month.

**PBS1 x/2%FBS**

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

Store at 4°C for up to 1 month.

**PBS1 x/0.04%BSA**

| Reagent                                | Final concentration | Amount    |
|----------------------------------------|---------------------|-----------|
| Bovine Serum Albumin (BSA) 5%          | 0.04%               | 60 µL     |
| PBS1 x                                 | 1x                  | 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               | 10x                 | 50 mL     |
| GlutaMax                         | 1x                  | 5 mL      |
| Sodium Pyruvate                  | 1x                  | 5 mL      |
| Penicillin Streptomycin          | 1x                  | 5 mL      |
| β-mercaptoethanol                | 50 µM               | 500 µL    |
| Total                            | n/a                 | 500 mL    |

Store at 4°C for up to 1 month.

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*The 17 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.*
**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).
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

Ø 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 x 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

Ø Timing: 2.5 days

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

△ CRITICAL: As described in 10X 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 10X 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 pM                       | See key resources table | 2.5 µL               |
| Si-PCR primer                              | PN-220111 or PN-200009S | 2.5 µL               |
| Transferred Supernatant Cleanup (step 2.3B-xiv) | n/a             | 5 µL               |
| Nuclease-free water                       | n/a             | 40 µL               |

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
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:

\[
\frac{\text{Qubit concentration (ng/μL)}}{660 \times \text{Average library size (bp)}} \times 10^6
\]

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 MULTSeqDemux 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 10x 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.
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 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-occurrence 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.
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.
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 demultiplexing (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%
If the cell type possesses a high quantity of RNA content this may result in 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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**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.
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.

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