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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

Protocol to isolate mature thymic T cell subsets using fluorescence-activated cell sorting for assessing gene expression by RNA-seq and transcription factor binding across the genome by CUT&RUN

Dimitris Theofilatos,1,4,* Tarmo Äijö,1 and Ageliki Tsagaratou1,2,3,5,*

1Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
2Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
3Department of Microbiology, University of North Carolina, Chapel Hill, NC 27599, USA
4Technical contact
5Lead contact
*Correspondence: dimitris_theofilatos@med.unc.edu (D.T.), ageliki_tsagaratou@med.unc.edu (A.T.)
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SUMMARY

Here, we describe steps to isolate mature thymic T cell subsets, namely CD4 single positive (SP), CD8 SP, and invariant natural killer T (iNKT) cells starting from murine total thymocytes using fluorescence-activated cell sorting. We detail protocols to study gene expression by RNA-seq and assess binding of transcription factors across the genome using CUT&RUN. This approach deciphers the molecular principles that govern T cell lineage specification and function. This protocol works well with limited starting material.

For complete details on the use and execution of this protocol, please refer to Äijö et al. (2022).¹

BEFORE YOU BEGIN

The protocol below describes the steps to isolate highly pure, mature subsets from murine thymocytes. Then, details are provided to prepare libraries for RNA-seq in order to assess gene expression and CUT&RUN, in order to assess transcription factor binding, from 500,000 FACS sorted CD4 SP cells.¹ However, we have also used this protocol to isolate CD8 SP cells and iNKT cells.²,³ We have isolated total RNA and performed RNA-seq.³

Institutional permissions

All the experiments described in this protocol are compliant with the ethical regulations approved by the University of North Carolina (UNC) Institutional Animal Care and Use Committee (IACUC) and the protocol 20-013. Researchers who aim to perform similar experiments using cells isolated from mice must first obtain permission from their institution and comply with the specific regulations of their institute. If the researchers work on animals or cells treated with hazardous materials [Biosafety level (BSL) 2 or above], they must obtain authorization and perform these experiments in designated approved areas, following standard operating procedures that are compliant with the safety rules of their institution.

Ensure that you have available the required reagents and the necessary equipment

© Timing: On the day of the experiment

1. Order commercial reagents and kits described in this protocol (summarized in the key resources table).
a. Make sure you have sufficient amount of the reagents before you start.
b. Prepare enough amount of 1× PBS.
   i. Prepare enough FACS buffer for the enrichment and purification steps.
2. Set up and save in your thermocycler the relevant programs to be utilized in the RNA-seq and CU-T&RUN protocols.
3. Make any necessary reservations of common equipment if needed.
   a. Reserve a sorter for the FACS sorting step.
4. Pre-chill centrifuges at 4°C.
5. Set up a water bath at 37°C to thaw normal rat serum from the EASYSEP RapidSpheres kit.
6. Thoroughly clean the RNA dedicated bench where you will perform RNA isolation and cDNA synthesis using RNase ZAP solution. Clean your pipettes. Use RNase free tubes.
7. Clean the bench that you will use to perform CUT&RUN experiments.

△ CRITICAL: Unless a safe stopping point is mentioned in this protocol, proceed to the next step.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Biotin anti-mouse CD24, clone:M1/69 (1:167) | BioLegend | Cat# 101804 RRID: AB_312837 |
| Biotin anti-mouse TER119, clone: TER119 (1:500) | BioLegend | Cat# 116204, RRID: AB_313705 |
| AF488 anti-mouse CD4, clone: RM4-5 (dilution 1:200) | BioLegend | Cat # 100529, RRID: AB_389303 |
| anti-mouse CD8, APC, clone:53-6.7 (1:200) | BioLegend | Cat # 100712, RRID: AB_312751 |
| Anti-mouse TCRβ, PERCP/Cy5.5, clone: H57-597 (1:200) | BioLegend | Cat # 109228, RRID: AB_1575173 |
| aGalactosyl-Ceramide loaded tetramer^® (1:400) | Tetramer Core National Institute of Health | Not applicable |
| GATA3 (clone: D13C9) XP Rabbit mAb (0.8 µL of antibody in 50 µL of antibody buffer) | Cell Signaling | Cat # 5852, RRID: AB_10835690 |
| CUTANA rabbit IgG CUT&RUN negative control antibody (prepare working stock 1 mg/mL and add 0.5 µL of antibody in 50 µL antibody buffer, dilution 1:100) | EpiCypher | Cat # 13-0042, RRID: AB_2923178 |
| Biological samples |        |            |
| Murine thymus       |        |            |
| Ethyl Alcohol, Pure | Sigma-Aldrich | Cat# E7023-500ML |
| PBS 10x             | Corning | Cat# 46-013-CM |
| DMEM without pyruvate 1x | Corning | Cat# 10-017-CV |
| FBS                 | Avantor Seradigm, VWR | Cat# 97068-085 |
| 2-Mercaptoethanol (stock concentration 55 mM) | Gibco | Cat# 21985-023 |
| HEPES (1 M)         | Gibco | Cat# 15630-023 |
| Glutamax            | Gibco | Cat# 35050-061 |
| Sodium pyruvate     | Gibco | Cat# 11360-070 |
| Penicillin/ Streptomycin | Sigma | Cat# P0781 |
| Trypan Blue solution 0.4% | Sigma | Cat# T8154 |
| UltraPure Distilled Water | Life Technologies | Cat# 10977 |
| 1 M Tris, pH 8.0    | Thermo Fisher | Cat# AM 9585 |

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## Materials and Equipment

The key resources table summarizes reagents, software and equipment that we have validated and used for this protocol. However, for some steps, alternative products or approaches may be used and some examples are indicated below. The precise outcomes of these alternatives have not been tested in our hands.

### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Fixable Viability Dye eFluor 780 | eBioscience | Cat# 65-0865-18 |
| RNaseZap RNase Decontamination solution | Invitrogen | Cat# AM9780 |
| cOmplete, EDTA-free Protease Inhibitor Cocktail | Roche | Cat#11873580001 |

### Critical Commercial Assays

- **EasySep Mouse Streptavidin RapidSphere Isolation Kit**
  - **Source:** STEMCELL Technologies
  - **Identifier:** Cat# 19860

- **RNA plus mini kit**
  - **Source:** Qiagen
  - **Identifier:** Cat# 74134

- **RNAasy plus micro kit**
  - **Source:** Qiagen
  - **Identifier:** Cat# 74034

- **Qubit RNA**
  - **Source:** Invitrogen
  - **Identifier:** Cat#5067-1513

- **RNA 6000 pico kit**
  - **Source:** Agilent
  - **Identifier:** Cat#5067-5584

- **Qubit 1 x double stranded (ds) DNA High Sensitivity Tape assay**
  - **Source:** Agilent
  - **Identifier:** Cat#5067-5588

- **D5000 Screen Tape assay**
  - **Source:** Agilent
  - **Identifier:** Cat#5067-5588

- **SMARTseq kit v4 Ultra Low Input RNA kit for sequencing**
  - **Source:** Clontech Takara
  - **Identifier:** Cat#634888

- **CUTANA ChIC/CUT&RUN Kit**
  - **Source:** EpiCypher
  - **Identifier:** Cat#14-1048

- **NEBNext Ultra II DNA Library Prep Kit**
  - **Source:** NEB
  - **Identifier:** Cat#: E7645

### Deposited Data

- **GSE206450**
  - **Accession:** Aijo 2022

- **GSE190230**
  - **Accession:** Aijo 2022

- **GSE190228**
  - **Accession:** Aijo 2022

### Experimental Models: Organisms/strains

- **C57/BL6J** (male and female mice are used, age 3–6 weeks old)
  - **Source:** The Jackson Laboratories (purchased during the past 4 years and bred in house at UNC)
  - **Identifier:** Cat#000664

### Oligonucleotides

- **NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 2)**
  - **Source:** NEB
  - **Identifier:** Cat#: E77805

### Software and Algorithms

- **FlowJo**
  - **Source:** Tree Star

- **FACS Diva**
  - **Source:** BD

- **NovoExpress**
  - **Source:** Agilent

- **Tapestation software**
  - **Source:** Agilent

### Other

- **Qubit 4 Fluorometer**
  - **Source:** Thermo Fisher Scientific
  - **Identifier:** N/A

- **Novocye 300S**
  - **Source:** ACEA Agilent
  - **Identifier:** N/A

- **BD LSR Fortessa**
  - **Source:** BD
  - **Identifier:** N/A

- **BD FACS Aria**
  - **Source:** BD
  - **Identifier:** N/A

- **Tapestation 4150**
  - **Source:** Agilent
  - **Identifier:** N/A

- **Bioanalyzer**
  - **Source:** Agilent
  - **Identifier:** N/A

- **T1000 Thermocycler**
  - **Source:** Bio-Rad
  - **Identifier:** N/A

- **EasyEights EasySep magnet**
  - **Source:** STEMCELL Technologies
  - **Identifier:** Cat#18103
**Alternatives:** We harvest organs in T cell medium (TCM) containing 1% FBS to ensure optimal viability of the cells. However, organs can also be harvested in ice-cold PBS. Make sure, if you use the latter option, to work fast and not incubate the cells for prolonged period in PBS since this can impact cell viability.

**Alternatives:** The protocol uses EASYSep mouse streptavidin RapidSpheres isolation kit for the enrichment of cells by depleting biotin labeled unwanted cells (negative selection). This protocol is very efficient and reduces significantly the incubation times. In addition, it allows the isolation of untouched cells. However, there are alternative enrichment kits available offered by other companies such as Dynabeads streptavidin beads (Invitrogen) or MojoSort Streptavidin Nanobeads (BioLegend).

**Alternatives:** For the preparation of unstained and single-fluorophore stained controls for the FACS sorting experiment we used cells. Alternatively, one can use beads for compensation (such as UltraComp ebeads, compensation beads cat no:01-2222-41, Thermo Fisher Scientific).

**Alternatives:** Cells can be counted using a hemocytometer. Alternatively, cells can be counted using automated cell counters such as Countess (Thermo Fisher Scientific, Invitrogen) or TC20 Cell counter (Bio-Rad) to mention some options. In addition, if available, a flow cytometer such as BD Accuri, Novocyte 3005 (ACEA Agilent) can also be used.

**Alternatives:** For the FACS sorting step we used a BD FACS Aria sorter (UNC Flow Cytometry Core). Other FACS sorters equipped with laser configuration to detect multiple fluorophores can also be used.

- As T cells are small in diameter cells, sorting is performed using a 70um nozzle. It is highly recommended to discuss with the personnel of your collaborating Flow cytometry core, explain them your specific experimental goals and follow their advice, regarding sample preparation and optimal strategies to sort your cell population of interest.
- Typically, for larger in size cells it is recommended to use a 100um nozzle.
- Another consideration is whether the cells will be directly used to isolate DNA/RNA/ protein or if they will be used for functional assays or adoptive transfers to recipient mice. In the latter case, one might consider to sort the cells using the larger nozzle to minimize any stress for the cells.

**Alternatives:** The cells can be collected in tubes containing T cell medium or FACS buffer or serum. In our hands, presence of serum ensures optimal viability.

**Alternatives:** For RNA isolation we used the QIAGEN RNeasy plus micro kit. However, this protocol will work also for RNA isolated for instance with Quick-RNA microprep kit (Zymo), Purelink RNA micro-scale kit (Thermo Scientific) or TRizol (Thermo Scientific). If you are sorting directly into Trizol you can use TRizol LS, as it has the advantage to be more concentrated.

**Note:** Keep in mind that TRizol and RLT lysis have an upper limit regarding the maximum volume of water phase they can contain. For example, for regular TRizol this is about 20% and for TRizol LS is about 30%. Thus, if you are sorting directly in TRizol LS, you can estimate 300 µL of total sorted sample in 700 µL of TRizol LS. If you are sorting in QIAGEN RLT lysis buffer, you can sort at a ratio of 100 µL of sample to 350 µL of RLT lysis buffer.

It is highly recommended to perform a pilot experiment to evaluate the yield of isolated RNA for your specific needs, before proceeding with large scale experiments.

**Alternatives:** For assessing size and quality of RNA we have used both Bionalyzer and Tapestation (both from Agilent). Similarly, for evaluating DNA samples and for the quality check (size
distribution, concentration) of libraries we have used Bionalyzer and Tapestation. Tapestation allows to process simultaneously multiple samples. Moreover, if the researcher does not have to run simultaneously multiple samples the tape can be carefully stored at 4°C and be reused within 16 days. However, this is not feasible when using Bioanalyzer since the chips, cannot be reused. Thus, Tapestation offers more flexibility. In addition, preparing and loading the chip for Bioanalyzer is a time sensitive process and steps must be executed fast, as instructed by the manufacturer. Thus, for researchers with limited experience Tapestation is more user-friendly.

**Alternatives:** For DNA cleanup and size selection we typically use Agencourt AMPure XP beads (Beckman Coulter). However, other beads are available. For instance, Kapa Pure Beads (Kapa Biosystems, Roche) have been reported to perform well.

**Alternatives:** For the CUT&RUN assay we used the CUTANA kit (EpiCypher) however other commercially available kits are available such as: CUT&RUN Assay Kit #86652 (Cell Signaling) or ChIC/CUT&RUN kit (pG-MN), cat no: CHR101-EMD Millipore.

In addition, there are reports where researchers purify the enzyme and prepare all the reagents to perform the assay. Also, to permeabilize T-cells it has been reported to use the Permeabilization buffer from the Foxp3 transcription factor set (eBioscience).

**Alternatives:** For the CUT&RUN assays, in order to purify the DNA, we used the columns provided in the CUTANA kit. However, columns can retain DNA fragments of a minimum size of 50 bp. Thus, DNA isolation by phenol/chloroform is an alternative to be considered, especially if you anticipate that you will collect short fragments or if you are uncertain of the anticipated size of DNA fragments to be isolated.

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**T cell medium (TCM)**

| Reagent | Final concentration | Amount  |
|---------|---------------------|---------|
| DMEM    | N/A                 | 500 mL  |
| FBS     | 1% (v/v)            | 5 mL    |
| HEPES 1 M | 10 mM               | 5 mL    |
| Penicillin/ Streptomycin (10,000 units penicillin and 10 μg streptomycin per mL suitable for cell culture) | 50 U/mL Penicillin | 2.5 mL |
| Glutamax | 2 mM                | 5 mL    |
| 2-Mercaptoethanol (stock concentration 55 mM) | 55 μM | 0.5 mL |
| Na-Pyruvate (stock 100 mM, 100X) | 1 mM | 5 mL |

Store sterile TCM at 4°C up to 1 month.

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**FACS Buffer**

| Reagent | Final concentration | Amount  |
|---------|---------------------|---------|
| 1x PBS  | Not applicable      | 49 mL   |
| FBS     | 2%                  | 1 mL    |

Total volume 50 mL

Sterile FACS buffer can be stored at 4°C for months.

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**Surface staining antibody master mix**

| Reagent | Final concentration | Dilution factor |
|---------|---------------------|-----------------|
| Fixable viability dye (1000X) | 1 x | 1:1,000 |
| AF488 anti-mouse CD4 | 2.5 μg/mL | 1:200 |

(Continued on next page)
CRITICAL: Vortex and spin down. Protect from light until you are ready to use and keep the mix at 4°C. The precise amount of mix to be prepared depends on the number of cells after enrichment.

**Note:** We typically stain 3*10^6 cells in 100 μL master mix.

For the CUT&RUN assay prepare the following buffers (prepare fresh the day of the experiment):

| Wash Buffer | Reagent                 | Final concentration | Amount |
|-------------|-------------------------|---------------------|--------|
|             | Pre-Wash Buffer         | N/A                 | 1.8 mL |
|             | 1 M Spermidine          | 0.5 mM              | 0.9 μL |
|             | 25× Protease Inhibitor  | 1×                   | 72 μL  |

Store Wash buffer at room temperature, prepare fresh the day of the experiment.

**Note:** One tablet of the 25× Protease inhibitor is diluted in 2 mL molecular grade water (25×). The 25× protease inhibitor stock can be stored at −20°C for 12 weeks.

**Alternatives:** There are also commercially available liquid protease inhibitors. Check the compatibility with the CUTANA ChiC CUT&RUN kit and make sure that they are EDTA-free.

| Cell permeabilization buffer | Reagent            | Final concentration | Amount |
|-----------------------------|--------------------|---------------------|--------|
|                             | Wash Buffer        | N/A                 | 1.4 mL |
|                             | 5% Digitonin*      | 0.05%               | 14 μL  |

Keep on ice, prepare fresh the day of the experiment.

**Note:** We use digitonin to a final concentration of 0.05% which is optimal to permeabilize CD4 cells. For other cell types, it is recommended to perform a titration to determine the concentration of digitonin for optimal permeabilization.

| Antibody Buffer | Reagent                   | Final concentration | Amount |
|-----------------|---------------------------|---------------------|--------|
|                 | Cell Permeabilization Buffer | N/A                 | 100 μL |
|                 | 0.5 M EDTA                | 2 mM                | 0.4 μL |
|                 | Total                     |                     | 100.4 μL|

Keep on ice, prepare fresh the day of the experiment.
Enrichment of mature thymic subsets by negative selection

**Timing:** 1 h (Day 1)

Mature subsets CD4 SP, CD8 SP and iNKT cells are a small fraction of cells in the thymus, collectively comprising less than 20% of the cells in this organ (Figure 1). Thus, it is critical to deplete unwanted cells such as double positive (DP) cells that consist the vast majority of thymocytes. To enrich our sample for mature thymic subsets we employ the method of negative selection. This approach is based on labeling with biotinylated antibodies the unwanted cells. Subsequently, by incubating the mix of cells with streptavidin magnetic beads, only the cells labeled with biotinylated antibodies will be bound to the streptavidin coated magnetic beads. Following a brief incubation of the samples on a magnetic stand, unwanted cells will be attached to the walls of the tubes that are in touch with the magnetic stand. The desired cells, untouched, will be in the supernatant and can be isolated for downstream applications such as cell culture, protein isolation for western blots or DNA/RNA isolation for subsequent molecular analysis such as genome-wide assays.

1. Harvest the thymus from a euthanized mouse. Place the thymus into a 6-well plate (or a 15 mL tube) containing 3 mL TCM and put the 6-well plate on ice.
2. Obtain single cell suspension by dissociating the thymus:
   a. Transfer the thymus using clean forceps on a 70 μM cell strainer in a p-60 plate.
   b. Add 3 mL TCM and gently dissociate the organ by using the end of the plunger of a 1 mL syringe without a needle.
   c. Carefully remove the cell strainer to avoid discarding any medium that contains cells.

*Note:* After the complete dissociation of the organ the medium will appear cloudy.

d. Transfer the TCM containing the thymocytes to a polystyrene tube.

⚠️ **CRITICAL:** If you are processing simultaneously multiple samples make sure to clearly label the tubes to properly identify them.

e. Pipette the cells thoroughly (at least 10 times) in order to achieve single-cell suspension.
3. Count the cells.
   a. Transfer 10 µL of the sample to 90 µL PBS (1:10 dilution) and count in a Flow Cytometer (for instance Novocyte 3005, ACEA, Agilent).
   b. Alternatively, prepare a 1:10 dilution of the cells and add Trypan blue (to identify and exclude from our count dead cells). Count the cells using a Neubauer hemocytometer.

△ CRITICAL: If you are preparing single stain controls using cells make sure to save some cells at this step. 1–3 million of cells/ single staining control are sufficient. Also, save cells for the unstained control.

4. Stain cells with biotinylated antibodies that recognize proteins expressed on the surface of unwanted cells that you aim to deplete. In this case, we stained 150–200 million cells to deplete CD24+ cells and any contaminating erythrocytes.
   a. Centrifuge the cells at 453 g, 5 min, 4 °C. Carefully remove supernatant using an aspirator.
   b. Resuspend the cells in the appropriate volume of FACS buffer using a 5-mL pipette to achieve a concentration of 1*10^8 cells per mL.

Note: The volume that can be used ranges from 0.1–8 mL. For instance, If the starting number of cells is 150 million, resuspend the cells in 1.5 mL FACS buffer. If the starting amount is 200 million, resuspend the cells in 2 mL FACS buffer. Transfer the cells to a polystyrene round-bottom tube.

Note: The total amount of cells that can be processed depends on the magnet.

If you are using a magnet that can hold only a standard 12 × 75 mm (5 mL) polystyrene tube such as the Easysep Magnet (STEMCELL Technologies, cat. no.: 18000) you can process from 10^7 up to 2 × 10^8 cells, resuspended cells in a volume of 0.1–2 mL of buffer respectively. However, if you have access to a magnet that can hold 15 mL tubes such as the Big Easy magnet (STEMCELL Technologies, cat. no. 18001) or the EasyEights EasySep magnet (STEMCELL Technologies, cat. no. 18103) you can process per tube up to 8.5 × 10^8 cells resuspended in 8.5 mL of buffer.

c. Add 50 µL of Rat Serum that is thawed and has reached a temperature of 22 °C in 1 mL of FACS buffer containing 100 million cells.
   i. For instance, in 1.5 mL FACS buffer containing 150 million thymocytes, add 75 µL Rat serum.
   ii. Carefully mix.

d. Add 3 µg/mL of the biotinylated anti-CD24 antibody and 1 µg /mL anti-Ter119 (to label erythrocytes).
   i. Mix well the samples.

Note: The amount of the biotinylated antibodies to use is determined by the abundance of cells that we aim to deplete. As a general guideline, consider the following:

| Frequency of cells to deplete | Final concentration of biotinylated antibody |
|-----------------------------|---------------------------------------------|
| <10%                        | 0.3–0.5 µg/mL                               |
| 10%–40%                     | 0.5–1.0 µg/mL                               |
| 40%–60%                     | 1–2 µg/mL                                   |
| >60%                        | 2–3 µg/mL                                   |

It is recommended to titrate the antibody/ antibodies of interest to ensure optimal enrichment of the target population and depletion of unwanted cells.

e. Incubate the sample at 22 °C for 10 min.

Note: Optionally, to ensure optimal mixing, one can incubate the samples with gentle shaking.
5. Bind the labeled cells with biotinylated antibodies to magnetic streptavidin beads (e.g., mouse streptavidin beads EASYSEP RapidSpheres, from STEMCELL Technologies).
   a. Add 100 μL RapidSpheres per mL of sample. Mix and incubate at 22°C for 2.5 min.

   △ CRITICAL: Beads will settle gradually. Just before use, resuspend thoroughly the magnetic streptavidin RapidSpheres by vortexing them for 30 s. Ensure that the solution is homogeneous before aspirating the desired volume of beads.

   Note: The incubation can be performed with gentle shaking to ensure optimal mixing.

   Note: The amount of magnetic streptavidin RapidSpheres is determined based on the abundance of cells that we aim to deplete. Per the manufacturer, when the unwanted cells to be depleted are more than 30% of the total cells in the sample, the quantity of beads to be used is within the range of 75–125 μL of RapidSpheres/mL.

6. Attach the beads and the bound cells to the magnet.
   a. Add FACS buffer up to 2.5 mL to each tube. Mix by pipetting 2–3 times.
   b. Place the tube on the magnet (without the cap) and incubate for 2.5 min.
      i. At this point you should be able to see the brown, magnetic streptavidin beads (RapidSpheres) retained on the wall of the tube that is in touch with the magnet.
      ii. For our experimental purpose, the cells that we aim to deplete are the vast majority of the sample. Thus, the supernatant becomes substantially less cloudy at this point. This is an indication that the enrichment has worked efficiently.

7. Collect the supernatant that contains unbound, enriched CD24- mature thymic subsets.
   a. Count the cells. Typically, at this step we obtain 4–7 million cells that consist of approximately 50%–60% CD4 SP cells.
   b. Keep on ice while you prepare the next steps.

   **Isolation of pure thymic subsets by fluorescence activated cell sorting (FACS)**

   © Timing: 1 h to prepare samples (Day 1)

   While in the previous step we enriched for mature thymic subsets, our sample is still heterogeneous, containing distinct T cell lineages with unique molecular and functional characteristics. By combining antibodies conjugated with distinct fluorophores we can distinguish and isolate these subsets by FACS sorting and use the pure populations in downstream applications of our choice.

   Sorting time varies. Estimate around 40 min for 10 million cells starting material.

8. Stain the cells using the desired cell surface staining master mix.
   a. Make sure to include a compatible viability dye, such as fixable viability dye conjugated with eFluor780, to exclude dead cells during sorting.

   **Surface staining antibody master mix**

   | Reagent                  | Dilution |
   |--------------------------|----------|
   | Fixable viability dye    | 1:1,000  |
   | AF488 anti-mouse CD4     | 1:200    |
   | APC anti-mouse CD8a      | 1:200    |
   | PE PBS57 loaded tetramer | 1:400    |
   | PERCPeCy5.5 anti-mouse TCRb| 1:200  |

   Prepare the master mix in FACS buffer before use.

   Stain up to 3 million cells in 100 μL of antibody master mix in a 1.5 mL tube.
b. Incubate cells at 4°C for 30 min.
c. Wash two times with ice cold FACS buffer.
   i. Centrifuge the cells for 5 min at 453 × g at 4°C.
   ii. Carefully discard the supernatant using a P1000 pipette, making sure not to disturb the pellet.
   iii. Resuspend cells in 500 μL FACS buffer.
   iv. Centrifuge the cells for 5 min at 453 × g at 4°C. Repeat the steps i-iv once more.
d. Resuspend in appropriate volume to achieve the recommended concentration suggested by your Flow Cytometry core.

**Note:** For our experiments we resuspend 10 × 10⁶ cells/mL FACS buffer.

e. Filter cells using a round bottom tube with cell strainer cap.

⚠️ CRITICAL: Filtering the cells is crucial prior to FACS-sorting in order to remove aggregates that might end up clogging the machine. In addition, running samples that contain single-cell suspension is instrumental for acquiring optimal signal.

9. Proceed with the FACS sorting using a 70 μm nozzle.
   a. Prepare collection tubes containing FBS (or other collection medium of choice).

   **Note:** Adding serum optimizes viability of the sorted cells.

   b. Make sure that the adaptor for the collection tubes is pre-chilled to make sure that the sorted cells are continuously kept at 4°C throughout the process.
   c. During sorting, record approximately 5,000 events from the sample.
      i. Set up the gates to exclude doublets and dead cells (Figure 2A).
      ii. Set the gates to sort for the desired mature thymic subsets e.g., iNKT cells, CD4 SP or CD8 SP cells (Figure 2A).
   d. After sorting, run a small amount of your sample (record 5,000 events) to assess purity and cell viability.

   **Note:** Typically, for our samples we get a minimum of 98% purity (Figure 2B).

   **Note:** If cell numbers are limited or if you are sorting at single-cell level this is not applicable.

**Assessing gene expression by RNA sequencing**

⏱️ Timing: 2 days total

⏱️ Timing: 45 min (Day 1) for step 10

⏱️ Timing: 30–45 min if you run an entire chip in the Bioanalyzer, can be performed at Day 2 or later (for step 11)

⏱️ Timing: 2 h for step 12

⏱️ Timing: 50–60 min for step 13

⏱️ Timing: 1 h for step 14
The goal of this step is to isolate total RNA from the sorted subsets and generate cDNA using the SMART-seq v4 Ultra Low Input RNA kit protocol for sequence. The advantage of this approach is that one can start with only 10 ng of total RNA. To prepare our libraries we utilized the Switching Mechanism at 5’ End of RNA Template (SMART)-seq v4 Ultra Low Input RNA kit for sequencing.
(Takara, Clontech) that offers a straightforward approach to prepare RNAseq libraries when starting with low amount of RNA (we started with 10 ng of material but the protocol can work with as little as 10 pg of starting RNA). Depending on the starting RNA material it is critical to optimize the number of PCR amplification cycles. Too few will compromise the yield, whereas over-amplification will compromise the complexity of the library, resulting in an excess of duplicate reads that will be discarded from the subsequent analysis.

10. Isolate total RNA.
   a. Once the cells are sorted and their purity is confirmed, count the cells to obtain a precise quantification of the retrieved material.

   **Note:** Based on the number of retrieved cells one can determine the optimal RNA isolation method. For starting material equal or larger than 1 million cells we utilize the RNA plus mini kit (QIAGEN). For lower amount of starting material, one can use the RNA plus micro kit (QIAGEN). Typically, we have used mainly the RNA plus micro kit (QIAGEN).

   △ **CRITICAL:** Typically, after sorting, the core facilities provide a cell count for each cell sample. While this count is most of the times an accurate estimate, it is important for downstream molecular applications to measure the cells just before the experimental procedure to get a precise number and design the best isolation strategy.

   **Note:** If the desired cell population is limited, one can directly sort the cells in lysis buffer.

   **Note:** The QIAGEN RNA plus kits provide columns that eliminate DNA in a fast and efficient manner. Alternatively, if a kit that is not providing these columns is used, DNase can be used to eliminate DNA.

   b. Pellet the cells by centrifugation at 200 × g at 4°C for 5 min.
   c. Wash once with ice-cold PBS and centrifuge the cells at 200 x g at 4°C. We will describe the RNA isolation process for 500,000 cells or less (using the micro RNA kit).

   △ **CRITICAL:** Ideally, one can work on a dedicated clean bench or a clean room. If that option is not available, make sure to clean the bench and the pipettes with RNase ZAP.

   d. Resuspend thoroughly the cell pellet in 350 μL RLT plus (provided in the RNA plus kit) containing 10 μL of β mercaptoethanol that was added just before the RNA isolation.
   e. Transfer the material to a DNA eliminating column (provided by the manufacturer).
   f. Centrifuge at speed higher than 8,000 × g (we typically perform this step at 9,600 × g) for 30 s at 22°C.
   g. Discard the column (contains the bound DNA) and save the flow-through.
   h. Add equal volume of 70% ethanol and carefully mix using the 1 mL pipette.
   i. Then transfer the mix (700 μL) to a new RNA isolation column (easily identifiable by pink color).

   **Note:** The RNA isolation columns of the RNA plus micro kit are stored at 4°C until the they are used.

   j. Centrifuge at 9,600 × g for 30 s. Discard the flow through.
   k. Wash the column with 700 μL RW1 buffer. Centrifuge at 9,600 × g for 30 s. Discard the flow-through.
   l. Wash with 500 μL ERP buffer. Centrifuge at 9,600 × g for 30 s. Discard the flow-through.
   m. Repeat the wash with 500 μL 80% Ethanol. Discard the flow-through.
n. Place the column in a new, clean collection tube (provided) and centrifuge at maximum speed for 5 min.

⚠ CRITICAL: This step is described as optional by the manufacturer, but we highly recommend to dry the membrane before eluting the RNA, to ensure that you remove any residual ethanol that might interfere with downstream enzymatic reactions.

o. Place the dried column to a new 1.5 mL certified DNase/ RNase free tube (provided).

Note: Alternatively, if preferred, one might use low binding tubes at this step to ensure minimal loss of the desired material.

p. Apply directly to the membrane 13 μL of RNase free water (provided). Incubate for 1 min.

q. Then centrifuge at maximum speed for 1 min. Discard the column.

r. Store the RNA at −80°C until you perform further analysis.

Note: At this point, it is recommended to aliquot around 3 μL of RNA to perform quality control (step 11) to avoid multiple freeze/thaw cycles of the RNA.

|| Pause Point: RNA can be stored at −80°C indefinitely.

11. Assess quality and quantity of total RNA.
   a. Thaw the RNA samples. Quantify using Qubit RNA High Sensitivity (HS) Assay kit. Prepare and quantify in a Qubit Fluorometer 4.
   b. Assess RNA integrity using an Agilent High Sensitivity RNA screen tape and reagents if you are having access to a Tapestation (Agilent) (Figure 3) or alternatively use an Agilent RNA 6000 pico kit if you are using a Bioanalyzer. Follow carefully the instructions.

Note: Especially if you are using Bioanalyzer, timing is critical for the successful outcome of the experiment. Make sure to follow with precision the incubation times recommended by the manufacturer.

⚠ CRITICAL: For our assays we are using RNA samples with a minimum RNA integrity number (RIN) value equal to at least 9. However, some samples are more prone to degradation. In that case you might consider starting with RNA with less RIN value. The higher the RIN value is the higher the success rate and the quality of the prepared libraries are.

12. First strand cDNA synthesis.

Note: We used SMART-seq v4 Ultra Low Input RNA kit for sequencing (Clontech) to prepare the libraries. The first part is First strand cDNA synthesis.

⚠ CRITICAL: Perform the described reactions in a dedicated RNA bench that we clean with RNaseZap before we start the procedures. We recommend using dedicated pipettes and tube racks that we are thoroughly cleaned before the experiments.

a. Thaw the 5× Ultra Low First-Strand Buffer at 22°C. Precipitates might form.

Note: Vortex the buffer to ensure that all the components are properly mixed and a homogeneous solution is formed. Nuclease free water can also be thawed at room temperature. The rest of the materials required for the first strand cDNA synthesis must be gradually thawed on ice. Specifically, in a container with ice, place the following: 10× lysis buffer, 10× reaction buffer, 3′ SMART-Seq CDS Primer II A, SMART-Seq v4 Oligonucleotide. The
RNAse inhibitor and the SMARTScribe Reverse Transcriptase are to be stored at the –20°C until they are used.

**Note:** We recommend placing the SMARTScribe Reverse Transcriptase in a –20°C portable cooler (e.g., Nalgene) and use the enzyme directly from there to minimize exposure to room temperature that may compromise the enzymatic activity.

b. Prepare a 10× Reaction buffer by mixing 19 μL of 10× Lysis buffer with 1 μL of RNase Inhibitor to a total of 20 μL 10× Reaction buffer.

c. Mix gently to avoid forming bubbles and spin down. Store on ice.

d. Prepare your samples in 0.2 mL pcr tubes (nuclease free certified) as follows:

e. Place the tubes on a rack on ice.

f. Add 2 μL of the thawed 3’ SMART-Seq CDS Primer II A (12 μM).

g. Vortex gently to ensure a homogeneous solution. Spin down the tubes.

**Note:** At this step the total volume of the reaction is 12.5 μL.

h. Incubate the tubes at a thermocycler at 72°C for 3 min.

△ **CRITICAL:** Make sure that the lid has been preheated so that the reaction starts immediately.
i. While the tubes are incubating at 72°C prepare the mix for the cDNA synthesis reaction.

△ CRITICAL: Make sure to calculate enough volume for all your samples. As a rule of thumb, prepare 10% more of the total volume needed to ensure you will have sufficient mix for all your tubes.

The volume needed per reaction is:

| Reagent                          | Final concentration (in 20 µL of cDNA synthesis reaction) | Amount  |
|----------------------------------|----------------------------------------------------------|---------|
| 5X Ultra Low First-Strand Buffer | 1x                                                       | 4 µL    |
| SMART-Seq v4 Oligonucleotide     | 2.4 µM                                                   | 1 µL    |
| (48 µM)                          |                                                          |         |
| RNase Inhibitor (40 U/µL)        | 2 U/µL                                                   | 0.5 µL  |
| Total volume                     |                                                          | 5.5 µL  |

j. Immediately after the completion of the 3 min incubation at 72°C, place the tubes on ice. Incubate for 2 min.

k. During the 2 min incubation period, add to the master mix 2 µL per reaction, plus 10%, of the SMARTScribe Reverse Transcriptase.

Note: The volume would be 2.2 µL.

△ CRITICAL: Add the enzyme immediately before use. Gently mix the reverse transcriptase enzyme, just before you add this to the master mix. Then spin down.

l. Transfer 7.5 µL of the mix to each tube. Carefully mix the content of each tube by pipetting. Spin the tubes briefly. The total volume of the cDNA synthesis reaction is 20 ul.
m. Transfer tubes to a thermal cycle with the pre-heated lid set at 42°C. Use the following program:

| Steps               | Temperature | Time    |
|---------------------|-------------|---------|
| cDNA synthesis      | 42°C        | 90 min  |
| Heat inactivation of the enzyme | 70°C        | 10 min  |
| Hold                | 4°C         | Forever |

Pause point: The tubes, containing cDNA, can be safely stored at 4°C or at −20°C overnight.

13. Next, the cDNA will be amplified using PCR Primer II A. The Primer II A identifies and amplifies SMART sequences that were introduced in the previous steps by 3’ SMART-seq CDS primer IIA and the SMARTseq V4 oligonucleotide.

△ CRITICAL: Determining the number of PCR cycles depends on the amount of starting RNA. As we started with 10 ng of RNA we performed 8 cycles of PCR amplification. It is crucial not to overamplify the cDNA at this step to ensure complexity of the libraries. It is highly recommended to check the optimal yield for the cells that you use before determining the exact amount of PCR amplification samples to perform.

a. Prepare the master mix for the PCR amplification reaction.
   i. Calculate the amount of reactions needed and prepare an additional 10% to ensure that there will be sufficient amount of mix for all the samples.
   ii. Mix the following reagents/ reaction:
△ CRITICAL: Thaw the reagents on ice. Add the SeqAmp polymerase just before the reaction.

b. Add 30 µL of the master mix to the tube containing first strand cDNA (from step 12m) for a total volume of 50 µL.
c. Mix the content of the tubes and spin down.
d. Run the following program in a thermocycler with heated lid:

| PCR cycling conditions | Temperature | Time  | Cycles |
|------------------------|-------------|-------|--------|
| Initial Denaturation   | 95°C        | 1 min | 1      |
| Denaturation           | 98°C        | 10 s  | 8 cycles |
| Annealing              | 65°C        | 30 s  |        |
| Extension              | 68°C        | 3 min |        |
| Final Extension        | 72°C        | 10 min|        |
| Hold                   | 4°C         | Forever|       |

|| Pause point: The tubes can be safely stored at 4°C overnight.

△ CRITICAL: The precise number of PCR amplification cycles is determined by the amount of starting total RNA and also the quality (evaluated as integrity at the quality control step) of the isolated RNA. For our purposes, we start with 10 ng of total RNA and high integrity (RIN value above 9). If the quantity is limited and the starting material is less or if obtaining RNA of high integrity is challenging then the amount of cycles should be increased.

We recommend to carefully define the optimal amount of cycles for your type of cells and the quality of total RNA that you can obtain before you proceed with large scale experiments. Determining the optimal amount of PCR amplification cycles is instrumental for the success of the experiment. While too few cycles might result in low yield and insufficient amount of library for sequencing, overamplification compromises the complexity of the library and results in duplicate reads that will be discarded at the analysis step.

14. Purify the amplified cDNA.
   a. Add 1 µL of 10x lysis buffer to each reaction.
   b. Purify the cDNA using the Agencourt AMPure XP beads (Beckman).

△ CRITICAL: Make sure to bring the beads (stored at 4°C) at room temperature for a minimum of 30 min before use. It is important to vortex the beads well to ensure optimal dispersion just before use.

c. Add 50 µL of vortexed AMPure XP beads/ sample.
d. Mix thoroughly by pipetting up and down the entire volume of the reaction. The beads are viscous.
e. Let the samples stand at room temperature for 8 min to allow binding of the DNA to the beads.
f. Spin down the tubes and place them in a magnetic rack.
g. Incubate for 5 min to allow binding of the beads to the wall of the tube that is in touch with the magnet.

Note: The supernatant should appear clear and no beads should be visible. The beads have a bright brown color and are easy to detect.

h. While the tubes are still on the magnetic stand carefully remove the supernatant and avoid touching the beads.

i. Wash the beads with 200 μL of freshly prepared 80% ethanol.

j. Let the ethanol stand for around 30 s and proceed to carefully remove the ethanol, making sure not to touch the beads.

k. Repeat one more time the wash with 80% ethanol keeping the tubes to the magnetic rack and making sure not to touch the beads.

l. After the last wash, quickly spin down the tubes and place them back to the magnetic rack.

Note: That way the residual ethanol will be in the bottom of the tube.

△ CRITICAL: Using a 20 μL tip carefully remove the residual ethanol without touching the beads.

m. After the last wash quickly spin down the tubes and place them back to the magnetic rack.

n. Wait for the beads to dry for around 2 min.

△ CRITICAL: It is essential to dry the beads but at the same time you should make sure not to overdry these, as this may reduce the yield of the eluted DNA. Overdried beads appear cracked.

o. Remove the beads from the magnetic stand and add 17 μL elution buffer.

p. Resuspend the beads in the elution buffer by pipetting up and down for 10 times.

q. Incubate for 2 min at 22°C to allow rehydration of beads.

r. Spin down.

s. Place on magnet and allow to stand for 2 min.

Note: Make sure that the supernatant appears clear.

t. Collect the supernatant (that contains the amplified DNA) and transfer to a low binding tube.

Pause point: The DNA can be stored at −20°C until quality control and sequencing.

15. Quantify the libraries and perform quality control check.
   a. Proceed to quantification and quality control. Measure the DNA by using High Sensitivity Qubit 1x DS DNA assay using a Qubit Fluorometer.
   b. To assess the size distribution and the overall quality of your library run a small amount using Agilent D5000 Assay for Tapestation (Figure 4).

16. We submit the libraries to the Sequencing core facility for pooling.

17. The libraries were sequenced using the Hiseq 4000 platform (Illumina) and we used paired-end (PE) 75 bp read lengths.

Note: Alternative sequencing platforms can be used based on availability and cost.
This protocol describes a method to study the genome-wide binding of a transcription factor in FACS sorted, highly pure, murine CD4 SP cells. CUT&RUN allows to start with limited number of cells, making it an ideal technique for studies with primary, less abundant populations such as mature thymic subsets. We used the CUTANA ChiC CUT&RUN kit provided by Epicypher and we modified the instructions provided in the CUTANA ChiC CUT&RUN kit manual in order to be applicable for sorted T cells. Briefly, in this method, the antibody of interest is added to intact cells, which have been previously immobilized in 96 well plates. Following the antibody incubation, cells are incubated with a fusion of Protein A and Protein G to Microccocal Nuclease (pAG-MNase), which
bind on the antibody of interest and cleave the chromatin around it. The cleaved DNA is released and purified using DNA Cleanup Columns. Since the quantity of the cleaved DNA is low, library preparation is modified for a low amount of initial DNA input as well as to retain very small DNA fragments (50–70 bp).\textsuperscript{5,7} However, CUT&RUN can be also used to map histone modifications. In that case, the goal is to isolate larger fragments since the distribution of histones across the genome is broader, compared to the focal binding to the DNA of transcription factors.

For these experiments, we used $5 \times 10^5$ CD4-SP cells as starting material. However, if the starting material is limited, fewer cells can be used. Alternatively, cells from more than one mouse can be pooled. To assess the non-specific signal of our antibody of interest, we used a non-specific antibody that is in the same isotype as our antibody. For each experiment, the antibody of interest and the control antibody were added to samples from the same mouse donor. For each experiment, we used a minimum of two biological replicates for the antibody of interest. Increasing the number of biological replicates can increase power and confidence of the statistical analysis.

18. Prepare the Wash, the Permeabilization, and the Antibody buffers as described.

19. Mobilizing the cells into 96-well plates.
   a. Count the CD4 SP cells after sorting to confirm the precise number of starting material.
   b. Transfer the required volume of cell suspension corresponding to $5 \times 10^5$ cells per sample to a nuclease-free 1.5 mL tube.
   c. Centrifuge at $200 \times g$ for 3 min at RT. Carefully remove the supernatant.
   d. Wash with 100 $\mu$L Wash Buffer per sample by resuspending the cell pellet thoroughly.
   e. Centrifuge the tube at $200 \times g$ for 3 min at RT. Carefully remove the supernatant.
   f. Repeat the steps d, e once.
   g. Add 105 $\mu$L Wash Buffer per sample to the tube and resuspend the cells thoroughly.
   h. Split the samples into separate wells.
   i. Aliquot 100 $\mu$L of washed cells into the wells of a 96-well U-bottom plate. Each well for each antibody that will be used.

20. Binding of the antibody in intact cells.
   a. Centrifuge the plate at 453 $\times g$ for 5 min at 4°C. Discard the supernatant.
   b. Immediately, resuspend the cells within the wells using 50 $\mu$L cold Antibody Buffer.
   c. Capture the protein of interest and the bound DNA by adding 0.5 $\mu$g of GATA3 (clone: D13C9) XP Rabbit mAb (antibody of interest) to the corresponding sample.

   \textbf{Note:} For the GATA3 antibody, check the precise concentration of the antibody listed in the “Certificate of analysis” on the right side of the product’s webpage. If you are using a previous lot, contact the Technical support to ensure the precise concentration.

   d. 0.5 $\mu$g of the IgG control antibody is added to the other sample.

   \textbf{Note:} For the IgG control, if you are using the CUTANA rabbit IgG CUT&RUN negative control antibody (Cat # 13-0042) prepare 1 mg/mL working stock dilution in antibody buffer. Add 0.5 $\mu$L of antibody in 50 $\mu$L antibody buffer.

   If you are using the CUTANA ChIC/CUT&RUN Kit, the rabbit IgG antibody (negative control) is provided at a concentration of 0.5 mg/mL so you can add 1 $\mu$L of antibody in 50 $\mu$L antibody buffer.

   \textbf{Note:} Mix each sample by gently pipetting. Use a P200 pipette by setting it to 40 $\mu$L.

   \textbf{Note:} It is important to use CUT&RUN validated antibodies if available. Keep in mind that an antibody that is validated for ChIP-seq experiments may not work well for CUT&RUN. We
used 0.5 μg as suggested by the EpiCypher. However, for other antibodies, one might need to titrate the quantity of the antibody to determine the optimal concentration.

△ CRITICAL: The control antibody must be in the same concentration as the antibody of interest.

e. Incubate the samples for 1 h at 4°C.

Note: We have also tested overnight incubation. However, when we sequenced the libraries the enrichment for GATA3 peaks compared to the IgG control was very low. Thus, different time points of incubation time should be evaluated for different cell types and different antibodies to achieve optimal results.

f. After the incubation, centrifuge the plate at 453 x g for 5 min at 4°C. Discard the supernatant.
g. Permeabilize the membranes of the cells by adding 200 μL cold Cell Permeabilization Buffer.
h. Centrifuge the plate at 453 x g for 5 min at 4°C. Discard the supernatant.
i. Repeat the steps f, g once.
j. Resuspend the samples in 50 μL cold Cell Permeabilization Buffer.

21. Binding and Activation of pAG-MNase.
a. Mix 2.5 μL pAG-MNase to each sample. Mix by gently pipetting.

Note: Use a P200 pipette to mix the sample by setting it at 40 μL.

b. Incubate the plate at 22°C for 10 min.
c. Centrifuge the plate at 453 x g for 5 min, 4°C. Discard the supernatant.
d. Wash the samples by adding 200 μL cold Cell Permeabilization Buffer. Centrifuge the plate at 453 x g for 4 min, 4°C. Remove supernatant.
e. Repeat the previous step.
f. Resuspend the cells to 50 μL cold Cell Permeabilization Buffer. Mix well by gently pipetting.

22. Targeted chromatin digestion and release.
a. Place the plate on ice. Activate the tethered pAG-MNase by adding 1 μL Chromatin Digest Additive (provided) to each sample.
b. Mix gently by pipetting. Use a P200 pipette by setting it to 40 μL.
c. Incubate the plate for 2 h at 4°C.
d. Terminate the activity of the pAG-MNase by adding 33 μL Stop buffer to each sample. Mix gently by pipetting.
e. At this step, you can add a Spike-in DNA that will be used for the normalization of the CU-T&RUN data. The CUTANA kit provides a E. coli Spike-in DNA. You can add 1 μL of this Spike-in DNA, which corresponds to 0.5 ng, to each sample. Mix by gently pipetting.
f. Transfer the samples to 0.2 mL PCR tubes. Spin briefly the samples to collect all the residuals liquids.
g. Place the tubes in a thermocycler and incubate them for 10 min at 37°C.
h. At the end of incubation, transfer the samples to nuclease-free 1.5 mL tubes.
i. Centrifuge the tubes at 600 x g for 4 min at 22°C. Transfer the supernatant (which contains the released DNA) to a new 1.5 mL tubes.

Note: You can transfer the first 60 μL of the supernatant with a P200 pipette and the rest with a P20 or P10 pipette.

23. DNA purification.
Note: The CUTANA kit provides columns (DNA cleanup columns) that retain DNA fragments larger than 50 bp. For each DNA cleanup column, a DNA collection tube is provided too.

a. Place a DNA cleanup column into a DNA collection tube.
b. Mix each sample with 420 μL DNA Binding Buffer. Vortex well to mix. Then, transfer the sample onto the DNA cleanup column placed in a DNA collection tube.

▲ CRITICAL: These DNA Cleanup Columns can retain fragments > 50 bp. For shorter DNA fragments, phenol/chloroform extraction should be performed.

c. Centrifuge the column at 16,000 × g for 30 s at 22°C. Discard the flow-through.
d. Wash the column with 200 μL DNA Wash Buffer. Centrifuge for 30 s, at 16,000 × g at 22°C. Discard the flow-through.
e. Repeat the previous step twice. Discard the flow-through.
f. Centrifuge the samples at 16,000 × g for 30 s at 22°C to completely dry the column.

Note: This is required to avoid carry over of any residual wash buffer that might inhibit downstream enzymatic reactions.

g. Carefully place the column in a new nuclease-free 1.5 mL tube.
h. Add 12 μL DNA Elution Buffer to the center of the column.
i. Incubate the samples for 5 min at 22°C.
j. Centrifuge at 16,000 × g, for 1 min at 22°C. The eluted material contains the CUT&RUN DNA.

Note: According to the instructions of the kit, DNA can be eluted in a range of 6–20 μL elution buffer regarding how concentrated you prefer the final sample.

■ Pause point: CUT&RUN DNA can be stored at −20°C for future processing.

24. Determine the concentration of small fractions of CUT&RUN DNA.
a. Use 1 μL to measure the concentration (C) of the eluted DNA by using the Qubit™ fluorometer 4.
b. Use 1 μL (or less if it is applicable) to prepare a 0.5–1 ng/μL dilution of each sample in molecular grade water. Analyze the diluted samples in the High Sensitivity D1000 ScreenTape assay and determine the percentage of the large fractions of chromatin within each sample (Figure 5).

Note: Fragments over 1,000 kb are considered large fragments.

c. Calculate the concentration of the small fraction of chromatin (Csf) by using the formula below:

\[ C_{sf} = C \times (100\% - \text{percentage of the large fragment}) \]

Where C was defined in step 24a.

Next, we proceed with the preparation of the libraries. For this purpose we use reagents that are provided at the NEBNext Ultra II DNA library prep kit for Illumina. To this end, we follow the steps described in the protocol with modifications.6,7

Note: Before you start, clean thoroughly the bench and the pipettes. All mixes are prepared on ice.
25. End Repair.
The goal of this step is to end repair the fragmented DNA so that we can proceed with the next steps of library preparation.

   a. Prepare a 10 mM Tris pH 8.0 buffer. This buffer will be used to dilute the samples.

   *Note:* Dilute the 1 M Tris pH 8.0 to 1:100 in Molecular Grade Water.

   b. Transfer 3 ng corresponding to the small fraction of DNA to a sterile nuclease-free 0.2 mL PCR tube.

   *Note:* If the starting number of cells is limited, the protocol works well with 1 ng of a small fraction of DNA.

   ▲ CRITICAL: Make sure to use less than 30 ng of total DNA in each reaction. Excessive quantity of DNA may compromise enzymatic activity.

   c. Bring the volume of the tubes to 25 µL by adding the appropriate amount of 10 mM Tris pH 8.0.

   d. Prepare the end repair reaction by adding the following components to the tubes containing the 25 µL DNA:

   | Component                          | Volume  |
   |------------------------------------|---------|
   | NEBNext Ultra II End Prep Enzyme Mix| 1.5 µL  |
   | NEBNext Ultra II End Prep Reaction  | 3.5 µL  |
   | Total Volume in the tube           | 30 µL   |

   e. Set a P200 pipette to 25 µL and then mix the sample thoroughly (by pipetting at least 10 times). Spin briefly the tube to collect all the residual liquids.

   f. Place the tube in a thermocycler. Set the heated lid to ≥ 60°C, and run the following program:

![Small and large fragments CUT&RUN](image)
26. Adaptor Ligation.
   a. Dilute the Adaptor (stock 15 μM) to 1:12.5 fold in 10 mM Tris pH 8.0, to achieve 1.2 μM.
   b. In the tube containing the End Prep Mixture, add the following components:

   **Note:** Combine the above components with the order shown. You can also prepare a premix containing the Ligation Master Mix and the Ligation Enhancer. This premix is stable for at least 8 h, at 4°C. Do not add the adaptor to this premix. Add the adaptor last in the Ligation Mixture.

   Mix well the Ultra II Ligation Master Mix by pipetting prior to adding it to the reaction.
   c. Set a P200 pipette to 25 μL and then mix the sample by thoroughly pipetting (at least 10 times). Spin briefly the tube.
   d. Place the tube in a thermocycler. Set the heated lid off and incubate the sample at 20°C for 15 min.
   e. Add 1.5 μL of USER enzyme to the ligation mixture. Mix well by pipetting.

   **Note:** USER enzyme is added if NEBNext adaptors are used.

   f. Place the tube in the thermocycler. Set the heated lid to ≥ 47°C. Incubate at 37°C for 15 min.

27. Cleanup of Adaptor Ligated DNA.
   a. The adaptor ligated DNA will be purified using the AMPure XP beads.

   **△ CRITICAL:** Fully resuspend AMPure XP beads by vortexing and equilibrate them at 22°C for at least 30 min.

   b. Prepare fresh 80% ethanol.
   c. Add 80 μL (~1.75×) AMPure XP beads to the adaptor ligation mixture. Mix thoroughly by pipetting (at least 10 times).

   **Note:** Adding 1.75× AMPure beads compared to the sample volume ensures the binding to the beads of DNA molecules larger than 150 bp and the efficient cleanup of non-ligated adaptors.

   d. Incubate the samples at 22°C for at least 5 min.
   e. Place the tubes on an appropriate magnetic stand to separate the beads from the supernatant.
   f. After 5 min (or when the solution is clear), carefully remove and discard the supernatant by pipetting without disturbing the beads.
g. Keep the tubes on the magnetic stand. Wash the beads by adding 200 μL of 80% of freshly prepared ethanol and incubate for 30 s at 22°C.
h. Carefully remove and discard the supernatant by pipetting.

**Note:** Avoid disturbing the beads.

i. Repeat the wash with ethanol.

⚠ CRITICAL: After the last wash, it is important to remove any residual ethanol. For this purpose, briefly spin the tubes, place them back on the magnet and, once the beads are attached to the side of the tubes, remove any residual ethanol with a P10 pipette, making sure not to disturb the beads.

j. Air-dry the beads for 5 min to ensure ethanol evaporation while the tubes are on the magnetic stand with the lid open.

⚠ CRITICAL: Do not over dry the beads. Resuspend them while there is no apparent cracking of the beads.

k. Remove the tubes from the magnetic stand. Elute the DNA target by adding 15 μL of 10 mM Tris pH 8.0. Mix well by pipetting (at least 10 times) or by vortexing.
l. Incubate the samples for at least 2 min at 22°C.

**Note:** You can perform a quick spin to collect all the residual liquids.

m. Place the tubes on a magnetic stand. After 5 min (or when the solution is clear), carefully transfer 13 μL to a new sterile 0.2 mL PCR tube.

|| Pause point: At this point, the sample can be stored safely at −20°C.

28. PCR enrichment of adaptor-ligated DNA.
   a. Prepare the PCR reaction for the enrichment of the adaptor-ligated DNA by adding the components below:

| PCR reaction | Component | Volume |
|--------------|-----------|--------|
|              | Adaptor Ligated Fragments | 13 μL |
|              | (blue) NEBNext Ultra II Q5 Master Mix | 15 μL |
|              | (blue) Index Primer/i7 Primer | 1 μL |
|              | (blue) Universal PCR Primer/i5 Primer | 1 μL |
|              | Total Volume in the tube | 20 μL |

b. Set a P200 pipette to 25 μL to mix the sample thoroughly (by pipetting at least 10 times). Spin briefly the tubes.
c. Place the tubes in the thermocycler. Run the following PCR program:

| PCR cycling conditions | Cycle steps | Temperature | Time | Cycles |
|------------------------|-------------|-------------|------|--------|
| Initial Denaturation   | 98°C        | 45 s        | 1    |
| Denaturation           | 98°C        | 15 s        | 12   |
| Annealing/ Extension   | 60°C        | 10 s        |      |
| Final Extension        | 65°C        | 5 min       | 1    |
| Hold                   | 4°C         | Forever     |      |
△ CRITICAL: If the starting material is 1 ng of DNA, you can increase the number of cycles to 14.

29. Cleanup of PCR amplification reaction using Agencourt AMPure beads.
   a. Vortex AMPure XP beads to resuspend and incubate them at 22°C for at least 30 min.
   △ CRITICAL: Make sure to fully resuspend the beads to create a homogeneous solution.
   b. Prepare fresh 80% ethanol.
   c. Add 30 μL (1.0 x ratio) AMPure XP beads to the adaptor ligation mixture. Mix well by pipetting (at least 10 times).
   d. Incubate the samples at RT for at least 5 min.
   e. Place the tubes on an appropriate magnetic stand to separate the beads from the supernatant.
   f. After 5 min (or when the solution is clear), carefully remove and discard the supernatant by pipetting without touching the beads.
   g. Keep the tubes on the magnetic stand. Add 200 μL of 80% of freshly prepared ethanol to wash the beads and incubate for 30 s at 22°C. Then, carefully remove and discard the supernatant by pipetting without disturbing the beads.
   △ CRITICAL: Do not leave the beads with ethanol for prolonged time.
   h. Repeat the wash with 80% ethanol twice.
   △ CRITICAL: After the last wash, remove any residual ethanol. Briefly spin the tubes, place them back on the magnet and remove ethanol residues with a P10 pipette.
   i. Air-dry the beads for 5 min while the tubes are on the magnetic stand with the lid open to ensure ethanol evaporation.
   △ CRITICAL: Do not over dry the beads. Resuspend them while there is no apparent cracking of the beads.
   j. Remove the tubes from the magnetic stand.
   k. Elute the DNA target by adding 15 μL of 10 mM Tris pH 8.0.

   Note: Thoroughly resuspend the beads by pipetting (at least 10 times) to ensure efficient elution of the DNA from the beads.
   l. Let the samples stand for at least 2 min at 22°C.

   Note: Perform a quick spin to collect at the bottom of the tube the beads.
   m. Place the tubes on a magnetic stand. After 5 min (or when the solution is clear), carefully transfer 13 μL to a new sterile 0.2 mL PCR tube.
   △ CRITICAL: At this step avoid touching the beads.
   ■ Pause point: Samples can be stored safely at −20°C.

30. Size distribution of libraries and quality control.
   a. Use 1 μL to quantify the library by using the Qubit 1x HS DNA assay in a Qubit™ fluorometer.
b. Use 1 μL (or less if it is applicable) to prepare a 0.5–1 ng/μL dilution of each library in molecular grade water.

c. Use the High Sensitivity D1000 ScreenTape assay to determine the size distribution of each library using an Agilent Tapestation (Figure 6).

31. The libraries were pooled and subsequently sequenced at the Duke Center for Genomic and Computational Biology.

32. Libraries were sequenced using the Illumina Novaseq 6000 platform. We used PE reads, with 50 bp length.

EXPECTED OUTCOMES

A thymus from a young (3–5 weeks old) wild type mouse (C57/J background) typically after dissociation will yield 150–200 million thymocytes. The vast majority of cells in the thymus are DP cells (Figure 1). After the initial enrichment step, where we deplete CD24+ cells, we typically retrieve 4–7 million cells that consist of approximately 50%–60% CD4 SP cells (Figure 2A). Post sorting the cells are live and consist mainly of CD4 SP cells at a purity that reaches at least 98% (Figure 2B). We typically retrieve between 500.000–1 million CD4 SP cells at this step. The variability depends on the initial starting material of thymocytes. However, some populations are more limited compared to CD4 SP cells. For instance, when we collect iNKT cells we retrieve around 100.000 cells from the thymus.

Regarding RNA isolation when we start with 400.000–500.000 cells we typically retrieve from 13 ng/μL to 22 ng/μL in a total volume of 13 μL of RNase free water. This is plenty material that allows to assess integrity and proceed with the SMARTseq protocol to assess gene expression. In addition, it allows to use the same RNA to perform TCR sequencing analysis if needed using for instance SMARTer mouse TCR a/b profiling kit (Clontech). We evaluate the RNA integrity and we consistently obtain RNA with RNA integrity number RIN>9. We provide an example where we have analyzed our samples using Tapestation (Figure 3).
For our RNAseq libraries, when we start with 10 ng of total RNA (RIN>9) after 8 rounds of total amplification we typically obtain libraries with a concentration ranging from 15–18 ng/µL in a total volume of 17 µL elution buffer and average size of 550 bp. It is critical to have enough volume to perform quantification and quality control check. We provide an example of the size distribution of our libraries as assessed in a Tapestation using a D5000 DNA Tape and reagents (Figure 4).

For our CUT&RUN libraries we started with 500,000 cells. After DNA elution our samples typically consist of large fragments of DNA and the smaller fragments cannot be detected using the HS tape of the Tapestation (Figure 5). For the GATA3 CUT&RUN experiments after the elution step (step:23) we typically retrieved (quantified as C in step 24a): 9.5 ng/µL-11.8 ng/µL in a total volume of 11 µL for GATA3 and for the IgG we typically retrieved 1.5 ng/µL-3.38 ng/µL in a total volume of 11 µL for the 1 h incubation. For the 16 h incubation, we retrieved 17 ng/µL-18 ng/µL in 11 µL total volume for GATA3 and 5.5 ng/µL for IgG in a total volume of 11 µL. For the libraries, after 12 rounds of amplification, we obtained for GATA3 CUT&RUN libraries 8.8 ng/µL-15.1 ng/µL in total volume 12 µL. For the IgG CUT&RUN libraries we got around 17 ng/µL in a total volume of 12 µL (quantified in step 30a). For the samples that were incubated for 16 h, the concentration of the GATA3 CUT&RUN libraries was around 20 ng/µL in a total volume of 12 µL and for the IgG CUT&RUN libraries the concentration was around 32 ng/µL in a total volume of 12 µL (quantified in step 30a). Note that for the CUT&RUN library for GATA3 we obtain a different profile of peak structure compared to the IgG control CUT&RUN library when we run our samples in Tapestation (Figure 6).

After sequencing and data analysis a typical example of visualization of RNA-seq and CUT&RUN data for selected genes is depicted in Figure 7.

QUANTIFICATION AND STATISTICAL ANALYSIS

We did not generate new code for RNAseq data analysis. We followed the steps described at Åijö et al., Frontiers in Immunology, 2022. Briefly:

1. Adapter trimming and quality filtering of the sequencing libraries was performed using fastp (0.21.0) with the default parameters.
2. Libraries were mapped against mm10 and the GRCm38.100 transcriptome using STAR (2.7.5a) using the following parameter values: –quantMode GeneCounts.
3. To perform differential expression analysis DESeq2 was used based on the read counts per gene produced by STAR. The used threshold for adjusted p-value was 0.01."

We did not generate new code for CUT&RUN data analysis. We followed the steps described at Åijö et al., Frontiers in Immunology, 2022. Briefly:

4. Adapter trimming and quality filtering of the sequencing libraries was done using fastp (0.21.0) with the default parameters.
5. The sequencing libraries were mapped against mm10 using Bowtie 2 (2.4.1) (–very-sensitive -X 2000).
   a. Mitochondrial reads were removed after alignment.
   b. Additional filtering was done using samtools (1.12) using the following parameter values: -q 30 -h -b -F 1804 -f 2.
   c. Reads with identical sequences were filtered and only one was retained for subsequent analysis.
6. The coverage tracks were generated from the samples obtained by pooling the biological replicates using HOMER (4.10) (makeBigWig.pl -norm 1e6).
   a. The peaks were identified from the pooled samples against controls using HOMER (4.10) (findPeaks -style factor).
b. The coverage at the transcription sites was quantified using HOMER (4.10) (annotatePeaks.pl tss mm10 -size 1000 -hist 5 -ghist).13

c. The coverage at the peak sites was quantified using HOMER (4.10) (annotatePeaks.pl peaks.txt mm10 -size 1000 -hist 5 -ghist).13"
Obtaining too many cells at this step reflects a poor enrichment and will impact the efficiency of FACS sorting since more cells require increased sorting time.

**Potential solution**

- Carefully quantify the starting number of cells (total thymocytes) to allow for efficient depletion of unwanted cells.
- It is recommended to establish a successful enrichment protocol before you perform this experiment. Work on titrating the optimal concentration of biotinylated antibodies and amount of magnetic streptavidin beads to achieve consistent and successful enrichment.
- Always vortex the magnetic beads immediately before use to ensure full resuspension.

**Problem 2**
Low yield of FACS sorting murine subsets (Isolation of pure thymic subsets by FACS sorting).

**Potential solution**

- Make sure to filter the cells and provide a single cell suspension in optimal concentration. The doublets will be excluded (gated out) and this might result in reduced yield. If cells tend to form aggregates it is helpful to add 0.02 mg/mL DNase I (Sigma, cat# D4513-VL) in the cell preparation steps to remove free DNA from broken cells that can result in cell clumps.

⚠️ CRITICAL: EDTA should not be added in the buffers, because cations must be present in the buffer for the DNase to function.

- Determine the best diameter nozzle for sorting to avoid stressing the cells and compromising viability.
- Collecting the cells on a tube placed in a pre-chilled adaptor and determining the optimal collection medium (for instance serum or medium containing serum instead of PBS) will also increase the chances of obtaining cells with high viability. Use a collection buffer that is suitable for optimal viability of the cells.

**Problem 3**
Insufficient yield of RNA-seq library (Step: assessing gene expression by RNA sequencing).

**Potential solution**

- RNA degradation might lead to this. To avoid RNA degradation work fast, clean thoroughly the bench and the pipettes. Change gloves frequently. Use reagents and materials that are certified RNase free.
- Insufficient PCR amplification cycles. Increase the number of PCR amplification cycles.
- Low recovery during library cleanup. Avoid overdrying the beads after ethanol washes since this may reduce the yield. Using elution buffer that has been preheated at 37°C may improve the efficiency of elution.

**Problem 4**
Low yield of DNA after CUT&RUN (step: cleavage under targets & release using nuclease (CUT&RUN) strategy to assess the binding of transcription factors across the genome of murine CD4 SP cells).

After the antibody incubation, pAG-MNase digestion and cleanup the retrieved DNA might be insufficient.
Potential solution
Potential causes and solutions are described below:

- The selected antibody is not working optimally for CUT&RUN. If available, purchase antibodies that have been validated to perform optimally for CUT&RUN. If an antibody against the protein of interest is not available and if it is possible to genetically manipulate the cells of interest one might consider adding a tag such as HA or FLAG or biotin that will allow to specifically target the tagged protein of interest.
- For the vast majority of targets, CUT&RUN is performed under native conditions. However, for some epigenetic modifiers it has been reported that slight fixation (ranging from light to moderate crosslinking: using 0.1%–1% formaldehyde respectively for 1 min) can improve the efficiency of the method. Increasing the cross-linking may compromise the yield of DNA recovery and thus should be avoided.

Problem 5
Low yield of CUT&RUN library (step: cleavage under targets & release using nuclease (CUT&RUN) strategy to assess the binding of transcription factors across the genome of murine CD4 SP cells).

Potential solution
- Insufficient PCR amplification cycles. Increase the number of PCR amplification cycles.
- Low recovery during library cleanup. Avoid over-drying the beads after ethanol washes since this may reduce the yield. Using elution buffer that has been preheated at 37°C may improve the efficiency of elution.

Problem 6
Data analysis of CUT&RUN libraries reveals poor enrichment of peaks for the antibody of interest (step: cleavage under targets & release using nuclease (CUT&RUN) strategy to assess the binding of transcription factors across the genome of murine CD4 SP cells).

Potential solution
- Identifying optimal incubation time with the antibody is critical. In our hands, reducing the incubation to 1 h increased significantly the number of identified peaks.
- Identifying antibodies that are suitable for CUT&RUN.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ageliki Tsagaratou (ageliki_tsagaratou@med.unc.edu).

Materials availability
We have not generated new materials for this study.

Data and code availability
We have not generated any new genome-wide datasets or code for this study. We have used datasets from our published article1 (Äijö et al., 2022) that are available at GEO: GSE206450.

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AUTHOR CONTRIBUTIONS
D.T. wrote the first draft of the manuscript and generated figures. T.Ä. wrote the section describing data analysis for RNA-Seq and CUT&RUN and generated relevant figures. A.T. wrote the final version of the manuscript, generated figures, supervised the study, and secured funding. All the authors read the final version of the manuscript and approve its content.

DECLARATION OF INTERESTS
The authors have no competing interests to declare.

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