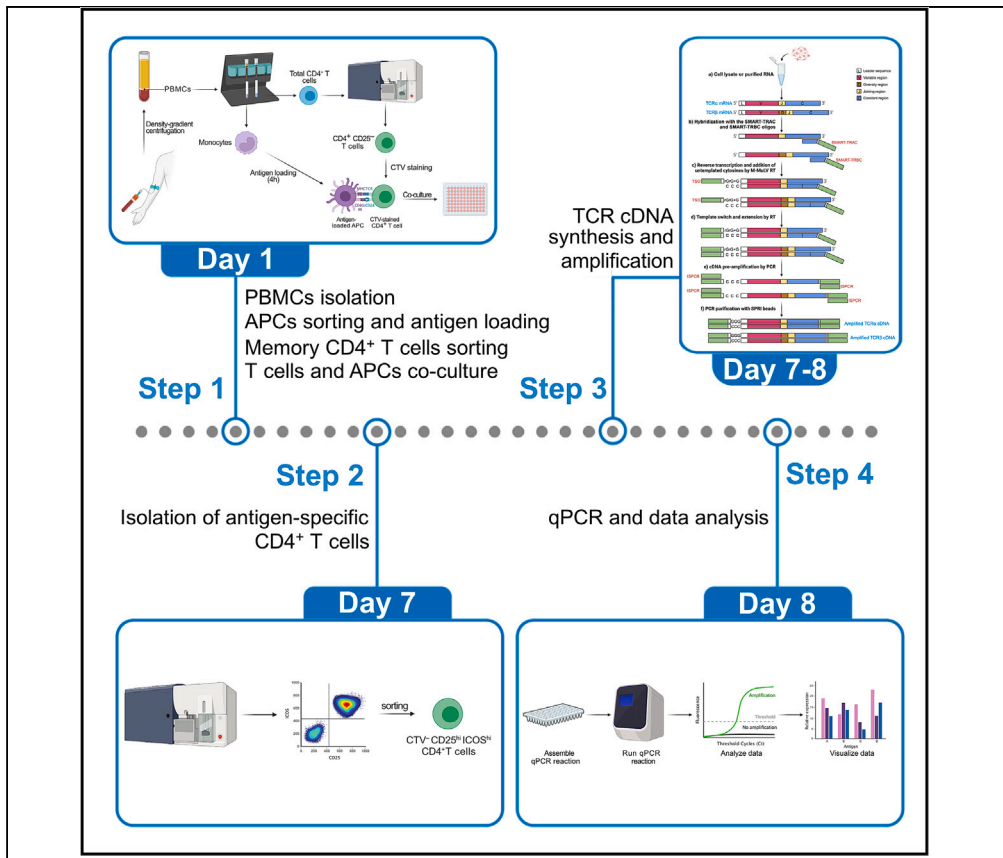


Protocol

Protocol for the detection of defined T cell clones in a heterogeneous cell population



Identifying defined T cell clones within a polyclonal population is key to clarifying their phenotype and function. Here, we present a protocol for detecting specified T cell clones in a heterogeneous cell population. We describe steps for stimulating human CD4⁺ T cells isolated from blood with a protein antigen, sorting antigen-specific cells by fluorescence-activated cell sorting, and detecting among these the presence of predefined T cell clones, based on their T cell receptor (TCR). TCR cDNA is amplified through 5'-RACE (TCR-SMART) and detected by qPCR.

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

Andrea Gobbini,
Alessandra Bandera,
Renata Grifantini,
Sergio Abrignani,
Samuele
Notarbartolo

samuele.notarbartolo@
policlinico.mi.it

Highlights

In vitro stimulation of human CD4⁺ T cells with a protein antigen

Antigen-specific human CD4⁺ T cells identification and isolation by FACS

Unbiased targeted amplification of TCR cDNA from low input samples

Detection of defined T cell clones within a heterogeneous population by qPCR

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Protocol

Protocol for the detection of defined T cell clones in a heterogeneous cell population

Andrea Gobbin¹, Alessandra Bandera^{2,3,4}, Renata Grifantini^{1,5}, Sergio Abrignani^{1,6} and Samuele Notarbartolo^{2,7,8,*}

¹INGM, Istituto Nazionale Genetica Molecolare "Romeo ed Enrica Invernizzi", 20122 Milan, Italy

²Infectious Diseases Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy

³Department of Pathophysiology and Transplantation, Università degli Studi di Milano, 20122 Milan, Italy

⁴Centre for Multidisciplinary Research in Health Science (MACH), Università degli Studi di Milano, 20122 Milan, Italy

⁵CheckmAb Srl, 20122 Milan, Italy

⁶Department of Clinical Sciences and Community Health, Università degli Studi di Milano, 20122 Milan, Italy

⁷Technical contact

⁸Lead contact

*Correspondence: samuele.notarbartolo@policlinico.mi.it
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SUMMARY

Identifying defined T cell clones within a polyclonal population is key to clarifying their phenotype and function. Here, we present a protocol for detecting specified T cell clones in a heterogeneous cell population. We describe steps for stimulating human CD4⁺ T cells isolated from blood with a protein antigen, sorting antigen-specific cells by fluorescence-activated cell sorting, and detecting among these the presence of predefined T cell clones, based on their T cell receptor (TCR). TCR cDNA is amplified through 5'-RACE (TCR-SMART) and detected by qPCR.

For complete details on the use and execution of this protocol, please refer to Notarbartolo et al. (2021).¹

BEFORE YOU BEGIN

This protocol describes the steps to isolate human CD4⁺ T cells from peripheral blood, stimulate them with a recombinant protein antigen, identify and FACS sort antigen-specific T cells based on cell proliferation and the upregulation of activation-induced markers, and detect the presence of pre-defined T cell clones in a population of interest, based on their T cell receptor (TCR) sequence.

Using this protocol, we demonstrated, for instance, that moderately expanded CD4⁺ T cell clones identified by scTCR-seq in PBMCs from patients with COVID-19 included clones specific to SARS-CoV-2 antigens.¹

Before starting, you need to identify the complementarity-determining region (CDR) sequence of the TCR(s) you want to detect and design target-specific qPCR primers. Also, verify to have all the reagents and tools described in the protocol.

The protocol is versatile and can be adapted to different experimental settings. Plan all the steps in advance and adjust them to your purpose.

Identification of TCR sequences in single-cell TCR-seq data

⌚ Timing: 3 h



This step describes how to extract information on T cell clone-specific TCR α and TCR β sequences from V(D)J-sequencing datasets generated with the Chromium Single Cell Immune Profiling kit (10 \times Genomics).

Note: the Cell Ranger software (10 \times Genomics) requires a workstation with a minimum of an 8-core processor, 64 GB RAM, and 1 TB of free storage space to function. The Python script, instead, is computationally not demanding and can be run on a personal computer. The timing indicated at the beginning of the paragraph refers to the analysis of a single V(D)J-sequencing dataset, where the Cell Ranger analysis was performed on a computer node equipped with a 64-core processor and 256 GB RAM, and the Python script was run in an interactive session on a machine with a 128-core processor and 2 TB RAM.

1. To extract the information about T cell clones TCR sequences from single-cell TCR-seq data follow the sub-steps described below.
 - a. Process the single-cell V(D)J-sequencing data with Cell Ranger using the following parameters: `cellranger vdj --id=<sample id> --fastqs=<path to fastqs folder> --sample=<sample name> --reference=/path/to/refdata-cellranger-vdj-GRCh38-alts-ensembl-4.0.0 --localcores=8 --localmem=64`.

Note: the Cellranger vdj script requires that fastq files have already been demultiplexed and that your computational platform meets the minimum hardware requirements previously described. For an accurate description of Cell Ranger use and output refer to the official guide at <https://support.10xgenomics.com/single-cell-vdj/software/pipelines/latest/using/vdj>.

△ CRITICAL: We recommend using Cell Ranger v6.0 or above as these versions automatically generate a file containing the required information on CDR1, CDR2, and CDR3 sequences leaving to the researcher only the need of filtering the sequences of interest. Use barcodes to filter the output files containing the annotated information.

- b. Among the Cell Ranger output files, select the “filtered_contig_annotation.csv” file, a tabular file of productive contig sequences for each cell with columns containing framework (fwr) and CDR annotation.

△ CRITICAL: use this file instead of the “clonotypes.csv” file, because the latter includes the clonotypes defined only based on CDR3 sequences, while the information on CDR1 and CDR2 sequences is not retained.

- c. Run the following scripts to select the T cell clones of interest and obtain their CDR sequences.

Note: execution of the Python scripts requires that the cell barcodes of interest have already been selected and stored in a text file. The script assumes that all files are in the same directory and the use of CDR nucleotide sequences instead of amino acidic sequences.

```
#!/usr/bin/env python
import pandas as pd
#Import the file containing cell contigs
contig_path="/cellranger/outs/filtered_contig_annotations.csv"
contig=pd.read_csv(contig_path)
#Import the file containing cell barcodes
```

```
barcodes = pd.read_csv("./barcodes.txt", header=None)

#Convert it in a list
barcodes = barcodes[0].tolist()

#Filter out csv lines not containing the desired barcodes
contig = contig[contig.barcode.isin(barcodes)].copy()

#Reconstruct all the nt sequences
contig['sequence_nt'] = (contig['fwr1_nt'] +
                        contig['cdr1_nt'] +
                        contig['fwr2_nt'] +
                        contig['cdr2_nt'] +
                        contig['fwr3_nt'] +
                        contig['cdr3_nt'] +
                        contig['fwr4_nt'])

#Reconstruct all the aa sequences
contig['sequence'] = (contig['fwr1'] +
                    contig['cdr1'] +
                    contig['fwr2'] +
                    contig['cdr2'] +
                    contig['fwr3'] +
                    contig['cdr3'] +
                    contig['fwr4'])

#Eliminate duplicates using nt sequences
deduplicated = contig[['barcode',
                    'chain',
                    'sequence_nt']].copy()

#Reshape data structure
deduplicated_pv = deduplicated.pivot(index='barcode',
                                    columns='chain',
                                    values='sequence_nt')

#Keep only one occurrence for duplicates with the same 'TRA' or 'TRB' values
deduplicated_df = (deduplicated_pv
                  .drop_duplicates(subset=['TRA', 'TRB'],
                                 keep='first'))

#Keep only TCR sequences of interest
contig = contig[contig.barcode.isin(deduplicated_df.index)]

#Write results
contig.to_csv('./unique_selected_contig.csv')
```

Note: examples of the file formats used in this step are reported in the [supplemental information \(Data S1\)](#).

qPCR primer design

⌚ Timing: 10 min per primer pair

- Design qPCR primers specific for the identification of the T cell clone(s) of interest. You can use Primer3 (v4.1.0)² with default parameters, except for the “Product Size Range” which is set at 80–150 bases to be used in combination with a fast qPCR protocol.

Note: other primer design tools or a manual design can be employed based on the user’s experience.

- Set the “Targets” parameter to design qPCR primers binding the CDR1 (forward primer) and CDR3 (reverse primer) regions of the TCR of interest to maximize their specificity.
- If Primer3 does not provide any primer as output, you can modify some parameters, such as “Primer size”, “Primer Tm”, and “Primer GC%”, to make them less stringent. As an alternative, try to partially shift the target region.

⚠ **CRITICAL:** validate the quality of designed qPCR primers by verifying they generate a unique PCR product. For instance, you can check that the dissociation curve after the qPCR run has a single peak or visualize the PCR product, which should appear as a single band, on an agarose gel.

Institutional permissions

The human blood samples analyzed in this protocol were collected according to the guidelines of a study protocol approved by the Institutional Review Board Milano Area 2. We remind readers to obtain all the necessary permissions from the relevant institutions before starting the experiment.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD4-BUV395 (clone SK3) (dilution used 1:100)	BD Biosciences	Cat. Num. 563550; RRID:AB_2738273
Anti-human CD8-FITC (clone RPA-T8) (dilution used 1:20)	BD Biosciences	Cat. Num. 561947; RRID: AB_10894003
Anti-human CD14-Alexa Fluor 488 (clone MφP9) (dilution used 1:100)	BD Biosciences	Cat. Num. 562689; RRID: AB_2737723
Anti-human CD19-BB515 (clone HIB19) (dilution used 1:100)	BD Biosciences	Cat. Num. 564456; RRID: AB_2744309
Anti-human CD56-BB515 (clone B159) (dilution used 1:100)	BD Biosciences	Cat. Num. 564488; RRID: AB_2744428
Anti-human CD45RA-Qdot655 (clone MEM-56) (dilution used 1:400)	Thermo Fisher Scientific	Cat. Num. Q10069; RRID: AB_2556451
Anti-human CCR7-BV711 (clone 150503) (dilution used 1:100)	BD Biosciences	Cat. Num. 566602; RRID: AB_2739758

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-human CD25-PE (clone BC96) (dilution used 1:200)	BioLegend	Cat. Num. 302605; RRID: AB_314275
Anti-human ICOS-PE-Cy7 (clone DX29) (dilution used 1:100)	BD Biosciences	Cat. Num. 567395; RRID: AB_2916579
Biological samples		
Human blood draws from patients with mild COVID-19	Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Infectious Diseases Unit, Milan, Italy. Notarbartolo et al.	N/A
Human serum	Swiss Blood Center Basel	N/A
Chemicals, peptides, and recombinant proteins		
Ficoll-Paque PLUS	Cytiva	Cat. Num. 17144003
DPBS 1X, no calcium, no magnesium	Thermo Fisher Scientific Gibco	Cat. Num. 14190094
RPMI 1640 medium, no glutamine	Thermo Fisher Scientific Gibco	Cat. Num. 31870025
RPMI 1640 medium, HEPES, no glutamine	Thermo Fisher Scientific Gibco	Cat. Num. 42401018
GlutaMAX supplement (100X)	Thermo Fisher Scientific Gibco	Cat. Num. 35050038
MEM non-essential amino acids (NEAA) solution (100X)	Thermo Fisher Scientific Gibco	Cat. Num. 11140035
Sodium pyruvate (100 mM)	Thermo Fisher Scientific Gibco	Cat. Num. 11360039
Penicillin-Streptomycin (5,000 U/mL)	Thermo Fisher Scientific Gibco	Cat. Num. 15070063
Kanamycin sulfate	Thermo Fisher Scientific Gibco	Cat. Num. 15160054
2-Mercaptoethanol (50 mM)	Thermo Fisher Scientific Gibco	Cat. Num. 31350010
Ethylenediaminetetraacetic acid (EDTA) disodium salt solution, 0.5 M	Merck Sigma-Aldrich	Cat. Num. E7889-100ML
Recombinant SARS-CoV-2 RBD and N proteins	Fondazione Istituto Nazionale Genetica Molecolare, Milan, Italy. Notarbartolo et al.	N/A
Recombinant human interleukin-2 (IL-2)	Miltenyi Biotec	Cat. Num. 130-097-744
Phytohemagglutinin (PHA)	Thermo Fisher Scientific Remel	Cat. Num. R30852801
BD Horizon brilliant stain buffer	BD Biosciences	Cat. Num. 566349
Nuclease-free water (not DEPC-treated)	Thermo Fisher Scientific Invitrogen	Cat. Num. AM9937
Triton X-100	Merck Sigma-Aldrich	Cat. Num. X100-100ML
RiboLock RNase inhibitor (40 U/μL)	Thermo Fisher Scientific	Cat. Num. EO0381
Ethanol, for molecular biology	Merck Sigma-Aldrich	Cat. Num. 51976-500ML-F
Tris buffer, 1.0 M, pH 8.0, molecular biology grade	Merck Millipore	Cat. Num. 648314-100ML
Critical commercial assays		
CD14 MicroBeads UltraPure, human	Miltenyi Biotec	Cat. Num. 130-118-906
CD4 MicroBeads, human	Miltenyi Biotec	Cat. Num. 130-045-101
MS columns	Miltenyi Biotec	Cat. Num. 130-042-201
CellTrace Violet Cell Proliferation Kit, for flow cytometry	Thermo Fisher Scientific	Cat. Num. C34557
Maxima H minus reverse transcriptase (200 U/μL)	Thermo Fisher Scientific	Cat. Num. EP0752
KAPA HiFi HotStart PCR Kit (250 U)	Roche	Cat. Num. 07958897001
SPRIselect beads	Beckman Coulter	Cat. Num. B23317
Qubit dsDNA high-sensitivity (HS) kit	Thermo Fisher Scientific Invitrogen	Cat. Num. Q32854
Oligonucleotides		
Oligo(dT) ₂₀ primer	Thermo Fisher Scientific Invitrogen	Cat. Num. 18418020
Template-switch oligo (TSO): 5'-AAGCAGTGG TATCAACGCAGAGTACATrGrG+G-3'. Note: at the 3' end of the oligo, there are two riboguanosines (rG) and one LNA-modified guanosine (+G) to facilitate template switching. The TSO bears the same adapter sequence present in the SMART-TRAC, SMART-TRBC, and ISPCR oligos.	QIAGEN; Picelli et al.	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SMART-TRAC oligo: AAGCAGTGG TATCAACGCAGAGTACACACATCA GAATCCTTACTTTG. Note: this oligo specifically anneals to the TRAC gene, which codes for the constant region of the TCR alpha chain. It also bears the same adapter sequence present in the TSO and the ISPCR oligos.	QIAGEN; This paper	N/A
SMART-TRBC oligo: AAGCAGTGGTAT CAACGCAGAGTACCAAGTATCTGG AGTCATTGA. Note: this oligo specifically anneals to the TRBC1 and TRBC2 genes, which code for the constant region of the TCR beta chain. It also bears the same adapter sequence present in the TSO and the ISPCR oligos.	QIAGEN; This paper	N/A
ISPCR oligo: 5'-AAGCAGTGGTAT CAACGCAGAGT-3'. Note: this oligo guides the unbiased amplification of the TCR cDNA. It works both as a forward and reverse PCR primer by binding to the adapter sequence present in the TSO, SMART-TRAC, and SMART-TRBC oligos.	Eurofins; Picelli et al.	N/A
qPCR primers. See Table 1.	Eurofins; This paper	N/A
Software and algorithms		
Cell Ranger v7.1	10x Genomics	https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger/ ; RRID: SCR_017344
Primer3web v4.1.0	Untergasser et al.	https://primer3.ut.ee/ ; RRID: SCR_003139
FlowJo v10.8	BD Biosciences	https://www.flowjo.com/solutions/flowjo/ ; RRID: SCR_008520
Prism v10	GraphPad Software	https://www.graphpad.com/ ; RRID: SCR_000306
Python v3.10.5	Python Software Foundation	https://www.python.org/psf-landing/ ; RRID: SCR_008394
Other		
MACS MultiStand	Miltenyi Biotec	Cat. Num. 130-042-303
MiniMACS separator	Miltenyi Biotec	Cat. Num. 130-090-312
MACSmix tube rotator	Miltenyi Biotec	Cat. Num. 130-090-753
40 µm cell strainer	Corning	Cat. Num. 352340
BD FACSAria III SORP cell sorter	BD Biosciences	RRID: SCR_016695
PCR thermal cycler (e.g., Biometra TRIO)	Analytik Jena GmbH	Cat. Num. 846-2-070-724
DNA LoBind tubes, 1.5 mL	Eppendorf	Cat. Num. 0030108051
DynaMag-2 magnet	Thermo Fisher Scientific Invitrogen	Cat. Num. 12321D
Qubit fluorometer (2.0 or later)	Thermo Fisher Scientific Invitrogen	Cat. Num. Q32866; RRID: SCR_020553
Axygen 96-well PCR microplates	Corning	Cat. Num. PCR-96-LP-AB-C
Axygen 70 µm ultra clear pressure sensitive sealing film for real-time PCR	Corning	Cat. Num. UC-500
Fast SYBR green master mix	Thermo Fisher Scientific Applied Biosystems	Cat. Num. 4385612
Real-time PCR system (e.g., QuantStudio 5 or StepOnePlus)	Thermo Fisher Scientific Applied Biosystems	RRID: SCR_020240; RRID: SCR_015805

MATERIALS AND EQUIPMENT

Alternatives: This protocol describes a validated custom procedure to isolate PBMCs from blood based on density-gradient centrifugation with Ficoll-Paque Plus. As an alternative, you can refer to

Table 1. qPCR primers

TRAC_Fwd	AGAACCCTGACCCTGCCG
TRAC_Rev	ATCAAATCGGTGAATAGGCAGA
TRBC_Fwd	AAGCAGAGATCTCCCACACC
TRBC_Rev	CTCCTTCCATTACCCACC
Clone_X_TCRa_Fwd	TGAGTATGTGATTGGTATCGACA
Clone_X_TCRa_Rev	TACCTCCTCCGACTCTGACG
Clone_X_TCRb_Fwd	GCCGTTCCCTGGACTTTCA
Clone_X_TCRb_Rev	AACTCCAGCACTGCAGATGT
Clone_Y_TCRa_Fwd	CAGTCTCTGGAAACCCTTATCTT
Clone_Y_TCRa_Rev	TGAAGCCTCCAGTGTGTCTC
Clone_Y_TCRb_Fwd	GCCGTTCCCTGGACTTTCA
Clone_Y_TCRb_Rev	ATTATCTCTAGCACTGCAGATGT

the Ficoll-Paque Plus manufacturer's protocol. (<https://cdn.cytivalifesciences.com/api/public/content/digi-16156-pdf>).

Alternatives: This protocol uses the Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific) for the full-length cDNA synthesis. You can use any other retrotranscriptase tested to be able to add untemplated cytosines at the 3' end of cDNA and to perform the template switch. For instance, the Superscript II reverse transcriptase (Thermo Fisher Scientific Invitrogen, cat. no. 18064–014) has been successfully used by others in similar applications.³

Alternatives: This protocol uses 1.5 mL DNA low-binding tubes to perform the SPRI-based purification of the amplified DNA. PCR plates or 0.2 mL tubes can also be used depending on the throughput of the experiment and the type of magnetic stand available.

Alternatives: This protocol uses the Fast SYBR Green Master Mix (Thermo Fisher Scientific Applied Biosystems) for the qPCR reaction. Any other qPCR kit based on a fluorescent DNA intercalating agent, such as SYBR Green, can be used paying attention to adjusting the thermocycler protocol according to the requirements of the kit used.

Alternatives: This protocol runs the qPCR reaction in a 96-well qPCR plate. If a qPCR machine with a 384-well block is available, the qPCR reaction can be run in this format to increase the throughput and cut the cost of the experiment.

Wash buffer: RPMI 1640 medium with HEPES supplemented with 0.5% human serum.

Filter through a 0.22 μ m PES membrane. Store at 4°C for up to 2 months.

FACS buffer: DPBS 1× supplemented with 0.5% human serum.

Filter through a 0.22 μ m PES membrane. Store at 4°C for up to 2 months.

MACS buffer

Reagent	Final concentration	Amount
DPBS	1×	241.5 mL
Human serum	3% (v/v)	7.5 mL

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Continued

Reagent	Final concentration	Amount
0.5 M EDTA	2 mM	1 mL
Total		250 mL

Filter through a 0.22 µm PES membrane. Store at 4°C for up to 2 months.

T cell medium

Reagent	Final concentration	Amount
RPMI 1640		224.75 mL
100 × GlutaMAX Supplement	1% (v/v)	2.5 mL
100 mM MEM NEAA	1 mM	2.5 mL
100 mM Sodium pyruvate	1 mM	2.5 mL
50 mM 2-Mercaptoethanol	50 µM	250 µL
5,000 U/mL Penicillin	50 U/mL	2.5 mL
5,000 µg/mL Streptomycin	50 µg/mL	
100 × Kanamycin	1% (v/v)	2.5 mL
Human serum	5% (v/v)	12.5 mL
Total		250 mL

Filter through a 0.22 µm PES membrane. Store at 4°C for up to 1 month.

Lysis buffer

Reagent	Final concentration	Amount
Nuclease-free H ₂ O		95.5 µL
10% (v/v) Triton X-100	0.2% (v/v)	2 µL
(40 U/µL) RiboLock RNase inhibitor	1 U	2.5 µL
Total		100 µL

Prepare a larger stock solution without the RNase inhibitor. Filter through a 0.22 µm PES membrane. Store at 4°C for up to 3 months. Add the RNase inhibitor to the working aliquot just before use.

Elution buffer

Reagent	Final concentration	Amount
Nuclease-free H ₂ O		9.9 mL
1 M Tris-Cl pH 8.0	10 mM	0.1 mL
Total		10 mL

Filter through a 0.22 µm PES membrane. Store at 4°C for up to 3 months.

⚠ **CRITICAL:** 2-Mercaptoethanol is toxic if swallowed or inhaled. It causes serious eye damage and skin irritation. Handle it with caution and use appropriate safety equipment.

⚠ **CRITICAL:** Triton X-100 is harmful if swallowed. It causes serious eye damage. Handle it with caution and use appropriate safety equipment.

STEP-BY-STEP METHOD DETAILS

Isolation of PMBCs

⌚ **Timing:** 1.5 h

- △ **CRITICAL:** perform all the steps from the isolation of PBMCs to the sorting of memory CD4⁺ T cells in sterile conditions, working under a UV-sterilized hood with laminar flow.

This step allows you to isolate PBMCs from blood.

1. Take the RPMI 1640 medium (with HEPES) and the wash buffer out from the fridge and let them reach 20°C–25°C.
2. Isolate PBMCs from blood by density-gradient centrifugation with Ficoll-Paque Plus.
 - a. Dilute blood with RPMI 1640 medium 1:2 (e.g., 20 mL blood with 40 mL RPMI 1640 medium) and mix by gently pipetting.
 - b. Aliquot 10 mL of Ficoll-Paque Plus in two 50 mL conical tubes.
 - c. Slowly add 30 mL of diluted blood on top of the Ficoll-Paque Plus in each tube by pipetting it at a 2–3 s/mL rate, while tilting the tube to maximize the Ficoll-Paque Plus surface.

- △ **CRITICAL:** too fast pipetting results in the mixing of blood and Ficoll-Paque Plus solution and impairs the efficiency of PBMC separation.

- d. Centrifuge at 800 × g for 30 min at 18°C–20°C in a swing-out rotor centrifuge with minimum acceleration and brake.

Note: only this centrifugation is performed with minimal acceleration and brake; use full acceleration and brake in the next steps. At the end of this centrifugation step, PBMCs form a ring (buffy-coat) in the middle of the tube, between the plasma and the semi-transparent Ficoll-Paque Plus layers.

- e. Collect the buffy-coat by slowly aspirating with a 1 mL pipette from the top, trying to minimize the carry-over of Ficoll-Paque Plus from the lower layer. Combine the buffy-coats from the two tubes in a new 50 mL conical tube.
 - f. Thoroughly wash the PBMCs by adding 20 mL of RPMI 1640 medium and pipetting up and down 3–4 times, while avoiding bubbling.

Note: this step serves to remove the residual Ficoll-Paque Plus.

- g. Centrifuge at 450 × g for 15 min at 18°C–20°C.
 - h. Aspirate the supernatant and resuspend the pellet in 10 mL of wash buffer.
 - i. Transfer the cell suspension into a 15 mL conical tube and centrifuge at 300 × g for 10 min at 18°C–20°C.
 - j. Aspirate the supernatant, resuspend the isolated PBMCs in 10 mL of wash buffer, and count them. You should get 1–2 × 10⁶ cells per mL of blood and, thus, 20–40 × 10⁶ PBMCs starting from a 20 mL blood draw. [Troubleshooting 1](#).

△ **CRITICAL:** when erythrocytes in whole blood are aggregated, some mononuclear cells are trapped in the clumps and sediment with the erythrocytes. This tendency to trap mononuclear cells is reduced by diluting the blood. Aggregation of erythrocytes is enhanced at higher temperatures (37°C), which consequently decreases the yield of mononuclear cells. At lower temperatures (4°C) the rate of aggregation is decreased but the time of separation is increased, which also decreases the yield of mononuclear cells. A compromise temperature of 18°C–20°C gives optimal results.

▮ **Pause point:** if you isolate PBMCs from a large blood donation, you can store the PBMCs in wash buffer for 12–16 h at 4°C.

Note: if you isolate PBMCs from a small blood draw, we recommend proceeding directly to the next steps.

Magnetic sorting of CD14⁺ monocytes

⌚ Timing: 70 min

This step enriches CD14⁺ monocytes by positive selection with magnetic CD14 MicroBeads.

Note: use cold MACS buffer and perform centrifugations at 4°C throughout this step.

3. Change the buffer of PBMC suspension from wash buffer to MACS buffer:
 - a. Centrifuge PBMCs at 300 × g for 10 min.
 - b. Resuspend cells in 5 mL of MACS buffer.
 - c. Centrifuge again at 300 × g for 10 min.
 - d. Resuspend 20–25 × 10⁶ PBMCs in 160 μL of MACS buffer.
4. Label PBMCs with CD14 MicroBeads:
 - a. Vortex CD14 MicroBeads for 20 s to resuspend them well.
 - b. Add 40 μL of CD14 MicroBeads and mix.
 - c. Incubate at 4°C–8°C for 20 min.
 - d. Add 4 mL of MACS buffer.
 - e. Centrifuge at 300 × g for 10 min.
 - f. Resuspend cells in 3 mL of MACS buffer.
5. Isolate CD14⁺ cells (monocytes):
 - a. Place an MS column on a MACS magnetic separator.
 - b. Prime the MS column with 1 mL of MACS buffer.
 - c. Apply the magnetically labeled cells on top of the MS Column.
 - d. Collect the flow-through (unlabeled CD14⁻ cells) into a 15 mL conical tube, do not discard.
 - e. Wash the MS column twice with 2 mL of MACS buffer.
 - f. Collect the effluent as CD14⁻ cell fraction into a 15 mL conical tube, as described in sub-step d.
 - g. Temporarily store the CD14⁻ cell fraction on ice.

Note: the CD14⁻ cell fraction will be used to isolate CD4⁺ cells (see step 8).

- h. Remove the MS column from the magnetic field of the MACS separator and place it on a suitable collection tube (e.g., a 15 mL conic tube).
- i. Pipette 3 mL of MACS buffer and firmly flush out the CD14⁺ cell fraction using the plunger supplied with the column.
- j. Remove the plunger, pipette an additional 3 mL of MACS buffer, and repeat the flushing to collect all the CD14⁺ cells.
- k. Count the CD14⁺ cells and proceed to the next step.

Note: monocytes are expected to be 10%–20% of the total PBMCs.

Irradiation of CD14⁺ cells and antigen loading

⌚ Timing: 75 min + 4 h of incubation

This step causes DNA damage that prevents cells from dividing and selectively kills residual lymphocytes, which are more sensitive than monocytes to ionizing radiations. The irradiated monocytes are still alive and can produce cytokines and other soluble factors. In addition, irradiation can induce the upregulation of some co-stimulatory molecules on antigen-presenting cells (APCs).^{4,5}

Note: use cold wash buffer and perform centrifugations at 4°C until step 6 g; then, use warm T cell medium and perform centrifugations at 20°C–23°C.

6. Irradiate CD14⁺ cells:
 - a. Centrifuge CD14⁺ cells at 300 × g for 10 min.
 - b. Resuspend cells in 5 mL of wash buffer.
 - c. Irradiate with 45 Gy of gamma-ray.

Note: it also works in the range of 30–60 Gy.

 - d. Centrifuge irradiated CD14⁺ cells at 300 × g for 10 min.
 - e. Resuspend cells in 5 mL of wash buffer.
 - f. Repeat the sub-steps d. and e. once again.
 - g. Centrifuge irradiated CD14⁺ cells at 300 × g for 10 min.
 - h. Resuspend cells in 2 mL of T cell medium and count them.
7. Load irradiated CD14⁺ cells with a recombinant protein antigen:
 - a. Centrifuge CD14⁺ cells at 300 × g for 10 min.
 - b. Resuspend cells at a concentration of 1 × 10⁶ cells/mL in warm T cell medium.
 - c. Add the recombinant protein antigen at a concentration of 5 µg/mL.

Note: leave an aliquot of monocytes unloaded as a negative control for the antigen-specific stimulation.

- d. Incubate at 37°C under slow rotation for 4 h.

Note: during this incubation step the recombinant protein is internalized and processed by APCs. The process of antigen uptake, processing, and presentation continues during the co-culture with T cells.

Optional: the antigen-loading incubation can be adjusted in a range from 2 h to 6 h depending on the experimental design and the antigen used. For instance, 6 h may be needed in the case you use microbial lysates as an antigen source and you want to wash away the unloaded antigen before the co-culture with T cells because it may be an undesired source of stimulatory pathogen-associated molecular patterns (PAMPs). On the contrary, 2 h is enough in the case of purified peptide antigens, which replace the peptides already exposed on the APCs' major histocompatibility complex class II (MHC-II).

Sorting of memory CD4⁺ T cells

⌚ **Timing:** 1.5 h + FACS sorting time

This step enriches, first, CD4⁺ T cells by positive selection with magnetic CD4 MicroBeads. Then, it enables the isolation of memory CD4⁺ T lymphocytes by fluorescence-activated cell sorting (FACS).

FACS sorting time may vary depending on the FACS facility.

Note: perform this step during the 4 h incubation time needed for antigen loading on APCs. Use cold MACS buffer and perform centrifugations at 4°C throughout this step.

Optional: You can pass the CD14⁺ cell fraction through a new MS column to remove residual CD14⁺ cells that were eventually not retained by the MACS separator magnetic field to avoid

possible contamination of the CD4⁺ cell fraction by residual CD14⁺ cells. This step is not necessary if you are sorting T cell subsets by FACS later.

8. Label CD14⁻ cells with CD4 MicroBeads:
 - a. Centrifuge the CD14⁻ cell fraction at 300 × g for 10 min.
 - b. Resuspend 20 × 10⁶ cells in 160 μL of MACS buffer.
 - c. Vortex CD4 MicroBeads for 20 s to resuspend them well.
 - d. Add 40 μL of CD4 MicroBeads and mix.
 - e. Incubate at 4°C–8°C for 20 min.
 - f. Add 4 mL of MACS buffer.
 - g. Centrifuge at 300 × g for 10 min.
 - h. Resuspend cells in 3 mL of MACS buffer.
9. Isolate CD4⁺ cells (T helper lymphocytes):
 - a. Place an MS column on a MACS magnetic separator.
 - b. Prime the MS column with 1 mL of MACS buffer.
 - c. Apply the magnetically labeled cells on top of the MS Column.
 - d. Collect the unlabeled CD14⁻ CD4⁻ cells into a 15 mL conical tube. Discard or use for other experiments.
 - e. Wash the MS column twice with 2 mL of MACS buffer.
 - f. Collect the effluent as CD14⁻ CD4⁻ cell fraction into a 15 mL conical tube, as described in sub-step d.
 - g. Remove the MS column from the magnetic field of the MACS separator and place it on a suitable collection tube (e.g., a 15 mL conic tube).
 - h. Pipette 3 mL of MACS buffer and firmly flush out the CD4⁺ cell fraction using the plunger supplied with the column.
 - i. Remove the plunger, pipette an additional 3 mL of MACS buffer, and repeat the flushing.
 - i. Count the CD4⁺ cells and proceed to the next step.

Note: CD4⁺ T cells are expected to be 30%–50% of the total PBMCs.

Optional: You can store the CD14⁻ CD4⁻ cell fraction in a freezing buffer at –150°C and use it for other experimental purposes.

Optional: If you are performing cell magnetic sorting for the first time, you may verify the purity of the sorted cells by flow cytometry after staining the sorted populations with the relevant antibody (e.g., CD14 for the CD14⁺ cell fraction and CD4 for the CD4⁺ cell fraction). The expected purity is > 95%.

10. Isolate memory CD4⁺ T lymphocytes by FACS:
 - a. Prepare the staining solution by mixing the following antibodies in 250 μL of MACS buffer: CD8-FITC (diluted 1:20), CD14-AF488 (1:100), CD19-BB515 (1:100), CD56-BB515 (1:100), CD25-PE (1:200), CD45RA-Qdot655 (1:400), and CCR7-BV711 (1:100) or other suitable antibodies.
 - b. Centrifuge the CD4⁺ cell fraction at 300 × g for 10 min.
 - c. Resuspend cells in the staining solution.
 - d. Incubate at 20°C–25°C for 25 min, then move the tube on ice for 5 min.

Note: some antibodies, such as CCR7, stain better at 37°C or 20°C–25°C than at 4°C. We found 20°C–25°C to be the best compromise for the indicated staining mix. The 5-minute incubation on ice is optional and may stabilize the antibody-antigen interaction.

- e. Wash the stained cells by adding 5 mL of MACS buffer.
- f. Centrifuge at 300 × g for 10 min.

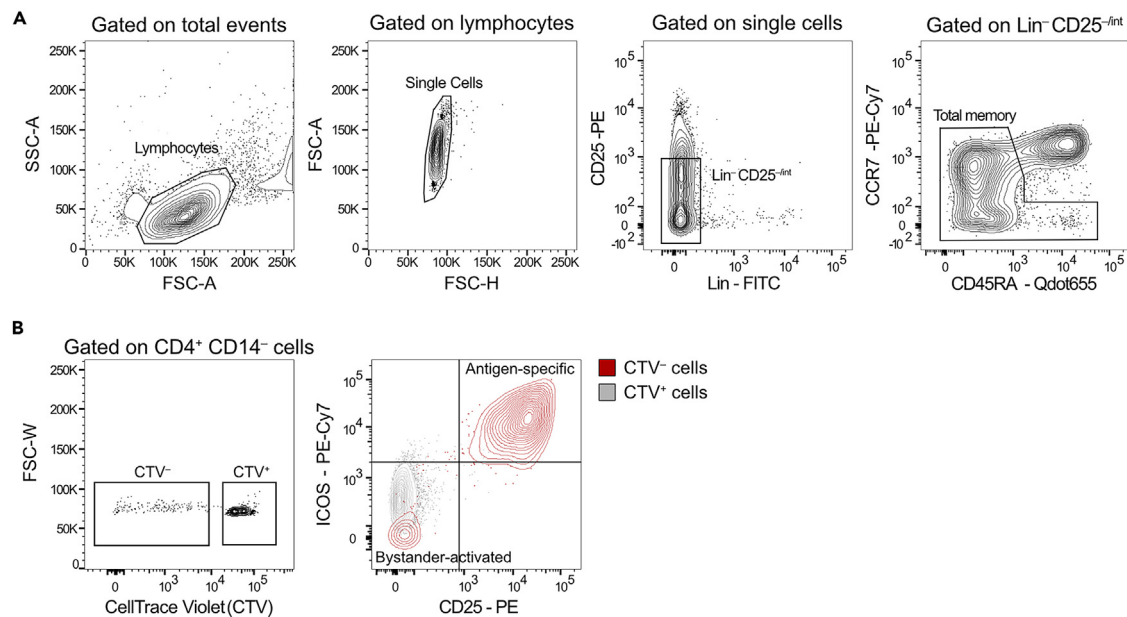


Figure 1. Gating strategies for T cell sorting

(A) Gating strategy for the identification and isolation of CD4⁺ total memory cells starting from CD4⁺ cells enriched by magnetic sorting.

(B) Gating strategy for the identification and isolation of *in-vitro* stimulated antigen-specific (CTV⁻ CD25^{hi} ICOS^{hi}), bystander-activated (CTV⁻ CD25⁻ ICOS⁻), and non-specific (CTV⁺ CD25⁻ ICOS⁻) CD4⁺ T cells.

- g. Carefully resuspend cells in 500 μ L of MACS buffer and filter them through a 40 μ m cell strainer to remove cell aggregates.

Note: MACS buffer contains EDTA which reduces cation-dependent cell adhesion and helps to obtain the single-cell suspension necessary for efficient cell sorting.

- h. Sort resting total memory CD4⁺ T lymphocytes by FACS based on the expression of CD45RA and CCR7, gating on lineage-negative (CD8⁻ CD14⁻ CD19⁻ CD56⁻) cells and removing T_{REG} and recently-activated T cells (CD25^{-/int}) (see the sorting strategy in [Figure 1A](#)).

Note: if possible, perform the cell sorting at 4°C.

△ CRITICAL: add 500 μ L of MACS buffer into the 5 mL sorting collection tube to avoid adhesion of cells to the plastic of the tube and the consequent cell loss or death.

Optional: if necessary, any other T cell subset of interest may be sorted based on the expression of established cell surface markers, as described by Cossarizza et al.⁶ in section III.1 on human conventional $\alpha\beta$ CD4⁺ T cells.

Optional: if a FACS cell sorter is not available at your institution, you can perform the experiment using magnetically sorted total CD4⁺ T cells or use an appropriate kit for the magnetic enrichment of memory T cells. In this case, we recommend depleting CD25⁺ (Treg) cells by negative magnetic selection before isolating CD4⁺ cells by positive selection.

CD4⁺ T cells and antigen-loaded monocytes co-culture

© Timing: 1 h + 6 days of incubation

This step is meant to label T cells to track their proliferation and stimulate antigen-specific T cells.

Note: use cold MACS buffer and DPBS 1× and perform centrifugations at 4°C until step 11 h; then, use warm DPBS 1× and T cell medium and perform centrifugations at 20°C–23°C.

11. CellTrace Violet (CTV) staining of CD4⁺ T cells:

- a. Pre-warm T cell medium and DPBS 1× at 37°C.
- b. Centrifuge the sorted CD4⁺ memory T cells at 300 × g for 10 min.
- c. Resuspend cells in 5 mL of MACS buffer.
- d. Centrifuge again CD4⁺ T cells at 300 × g for 10 min.
- e. Resuspend cells in a suitable amount of MACS buffer and count them.
- f. Centrifuge CD4⁺ T cells at 300 × g for 10 min.
- g. Resuspend cells in 5 mL of DPBS 1×.
- h. Centrifuge again CD4⁺ T cells at 300 × g for 10 min.
- i. Prepare a 10 μM CTV solution (2× concentrated) in warm DPBS 1× (500 μL per 1 × 10⁶ cells needed).
- j. Carefully resuspend cells in warm DPBS 1× at a concentration of 2 × 10⁶ cells/mL.

Note: it is important to have a single-cell suspension without cell aggregates to ensure good CTV staining.

- k. Add an equal volume of 2× concentrated CTV solution to the 2 × 10⁶ cells/mL cell suspension to have, in the end, a 1 × 10⁶ cells/mL cell suspension stained with a 5 μM CTV solution.
- l. Incubate cells at 37°C for 20 min under slow rotation in a CO₂ incubator.
- m. Add five volumes of warm T cell medium and incubate cells at 37°C for 5 min under slow rotation in a CO₂ incubator.
- n. Centrifuge stained CD4⁺ T cells at 300 × g for 10 min.
- o. Wash cells with 5 mL of T cell medium and centrifuge again as before.
- p. Resuspend cells in a suitable amount of T cell medium (e.g., at a concentration of 2 × 10⁶ cells/mL based on the previous count) and count them.

Note: a 20%–50% reduction in the number of cells from the previous count can be expected and may vary depending on cell fitness and the expertise of the operator.

- q. Adjust the cell suspension volume with T cell medium to have CTV-stained T cells at a final concentration of 1 × 10⁶ cells/mL.

12. Co-culture of CTV-stained CD4⁺ T cells with antigen-loaded monocytes:

- a. Plate 1 × 10⁵ cells CTV-stained CD4⁺ T cells with 5 × 10⁴ antigen-loaded monocytes (2:1 ratio) in a flat-bottom 96 well plate in 200 μL of T cell medium supplemented with IL-2 (20 U/mL). Make as many technical replicates as possible depending on the cell number.

Note: plate 1 × 10⁵ cells CTV-stained CD4⁺ T cells alone and 1 × 10⁵ cells CTV-stained CD4⁺ T cells with 5 × 10⁴ unloaded monocytes as negative controls.

- b. Quickly spin the plate (120 × g for 20 s) to let the cells settle down in the bottom of the well and incubate them at 37°C in a CO₂ incubator for 6 days.

Note: the incubation time is flexible in the 5–7 days range as the expression of the activation-induced markers described later is stable in this time window.

- c. After 48 h resuspend the cells by carefully pipetting and transfer them to a U-bottom 96 well plate.
- d. Incubate them at 37°C in a CO₂ incubator for the remaining 4 days.

Isolation of antigen-specific CD4⁺ T cells

⌚ Timing: 85 min + FACS sorting time

This step is aimed at isolating antigen-specific CD4⁺ T cells.

Note: use cold MACS buffer and perform centrifugations at 4°C throughout this step.

13. Activation-induced markers staining and CD4⁺ T cell sorting:
 - a. Resuspend co-cultured cells and pool all the technical replicates by collecting them in a 15 mL centrifuge tube.
 - b. Centrifuge cells at 300 × *g* for 10 min.
 - c. Resuspend cells in 5 mL of MACS buffer and centrifuge them as in b.
 - d. Repeat sub-step c once more.
 - e. Prepare an antibody mix of CD4-BUV395 (diluted 1:100), CD14-AF488 (1:100), CD25-PE (1:200), and ICOS-PE-Cy7 (1:100) in MACS buffer.

Note: the staining volume depends on the number of cells to stain; the antibody concentration depends on the antibody (e.g., clone and fluorochrome) used.

- f. Stain cells with the antibody mix for 20 min at 20°C–25°C, followed by 5 min on ice.

Note: CD4 and CD14 discriminate T cells from any residual monocytes while CD25 and ICOS are activation-induced markers.

- g. Add 5 mL of MACS buffer and centrifuge cells at 300 × *g* for 10 min.
- h. Resuspend cells in 5 mL of MACS buffer and centrifuge them as in g.
- i. Carefully resuspend cells in a suitable amount of MACS buffer and filter them through a 40 μm cell strainer to remove cell aggregates.
- j. Sort CD4⁺ CD14⁻ CTV⁻ CD25^{hi} ICOS^{hi} cells by FACS (see the sorting strategy in [Figure 1B](#)). These will be CD4⁺ T cells that proliferated and upregulated the expression of activation-induced markers in response to antigen stimulation and are, thus, antigen-specific cells.⁷ Also, sort non-antigen-specific cells (CD4⁺ CD14⁻ CTV⁺ CD25⁻ ICOS⁻) and keep a small aliquot of unsorted T cells as controls to evaluate the presence of the TCR(s) of interest by qPCR (see the [quantification and statistical analysis](#) section).

Note: if possible, perform the cell sorting at 4°C. The frequency of antigen-specific CD4⁺ T cells may vary depending on the antigen tested and the donor. For instance, in our experience, upon stimulation with SARS-CoV-2 Spike protein, CTV⁻ CD25^{hi} ICOS^{hi} cells were, on average, about 6.85% of the T cells harvested on Day 7, varying in a range between 1.83% and 12.65%.

⚠ **CRITICAL:** add 500 μL of MACS buffer into the 5 mL sorting collection tube to avoid cell adhesion to the plastic of the tube and the consequent cell loss or death.

- k. FACS-sorted antigen-specific CD4⁺ T cells and control samples can be directly subjected to the TCR-SMART protocol.

TCR-SMART core protocol

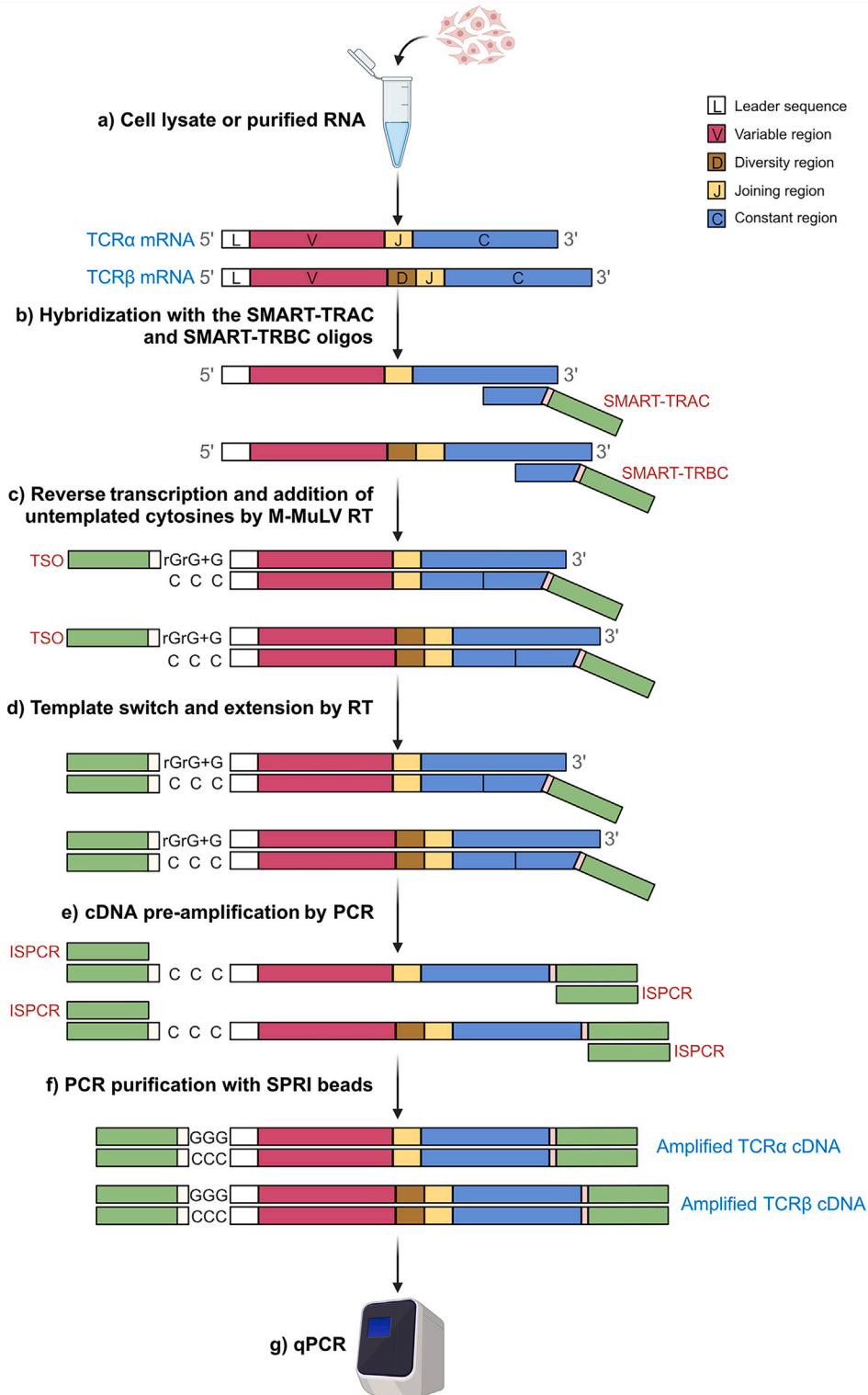


Figure 2. Schematic representation of the TCR-SMART core protocol

The cartoon visualizes the steps leading to the synthesis of amplified TCR α and TCR β DNA. These steps include cell lysis, cDNA synthesis, template switch, the addition of an adapter sequence at the cDNA 5' and 3' ends, and the TCR-targeted amplification of cDNA.

▮ **Pause point:** if necessary, sorted cells can be plated in a T cell medium supplemented with IL-2 (50 U/mL) and kept for 12–16 h at 37°C in a CO₂ incubator before proceeding to the next step.

Note: we recommend to start the TCR-SMART protocol with an input of 5,000–10,000 T cells. A lower number of T cells can be sufficient to obtain the result, but the experimental conditions haven't been thoroughly tested.

Optional: if needed, antigen-specific CD4⁺ T cells and control samples can be polyclonally expanded in a T cell medium supplemented with IL-2 (500 U/mL) for 5–6 days to increase the cell number.

Identification of defined CD4⁺ T cell clones by TCR-SMART

⌚ **Timing:** 4 h + 2 h per qPCR plate (including analysis)

This step is aimed at identifying pre-defined T cell clones by detecting their TCR sequences by RT-qPCR. The experimental approach is schematized in [Figure 2](#).

⚠ **CRITICAL:** it is critical to work in a DNA-free and RNase-free area to prevent potential contamination and RNA degradation. Before proceeding with this protocol section, clean the working surface and pipettes with an RNase-decontaminating solution or, if not available, with EtOH 70%, which reduces RNase activity. If your laboratory is not equipped with HEPA filters for the filtering of ambient air, the use of a UV-sterilized hood with laminar flow is highly recommended. Always use PCR-grade filter tips and tubes.

⚠ **CRITICAL:** thaw in advance all the reagents needed to prepare the RT and PCR mixes. Assemble the RT mixes during the 10-minute centrifugation after the cell lysis and keep them on ice.

⚠ **CRITICAL:** use a thermocycler with the lid temperature set to 105°C for all the incubations in this section to minimize sample evaporation and accumulation into the PCR tube cap.

14. Cell lysis, mRNA retrotranscription (RT), and enrichment of TCR cDNA:
 - a. Wash the sorted cells in 1 mL of FACS buffer and centrifuge at 300 × g for 10 min at 4°C.
 - b. Resuspend cells in a suitable amount of FACS buffer and count them.

Note: the resuspension volume can be calculated based on the cell count provided by the sorting cytometer to get a cell concentration compatible with a reliable count (e.g., in the range of 2.5 × 10⁵ to 2.5 × 10⁶ cells/mL in a Neubauer chamber). [Troubleshooting 2](#).

- c. Transfer the desired number of cells (in the range of 5,000–50,000) to a 0.5 mL PCR tube, wash with 200 μL of FACS buffer, and centrifuge at 300 × g for 10 min at 4°C.

Note: if you do not have a centrifuge adapter for 0.5 mL PCR tubes, you can make your own by using a 1.5 mL PCR tube with the lid cut.

d. Carefully aspirate the supernatant and proceed with the cell lysis.

△ **CRITICAL:** it is fundamental to remove all the supernatant after this centrifugation so as not to dilute the lysis buffer in the next step thus impairing the cell membrane lysis efficiency. To avoid disturbing the cell pellet, mark the orientation of the tube in the centrifuge and aspirate slowly moving along the tube wall opposite to the cell pellet. Keep in mind that using a fixed-angle rotor centrifuge the cell pellet will sit on the outer side of the tube bottom. First, aspirate the supernatant with a vacuum or with a 200 µL pipette (depending on your confidence level), leaving about 10 µL of residual supernatant. Then, aspirate the remaining volume with a 10 µL pipette using thin 0.1–10 µL filter tips. An eventual residual of 0.5–1 µL of supernatant will not impinge on the cell lysis. [Troubleshooting 3](#).

e. Lyse the sorted cells (5,000–10,000) in 8.5 µL of cold lysis buffer, gently pipetting 5 times, and incubating 5 min on ice.

Note: this protocol uses a relatively mild lysis buffer that lyses the cells without interfering with the RT reaction.

△ **CRITICAL:** add the RNase inhibitor to the lysis buffer just before performing the cell lysis step to ensure its activity.

f. Centrifuge at 1350 × *g* for 10 min 4°C to pellet nuclei, recover 7.5 µL of supernatant and move it into a 0.2 mL PCR tube.

Optional: depending on the starting number of cells, it is possible to isolate and purify RNA first and then proceed with the next steps of the protocol. For instance, you can isolate RNA using a silica membrane-based commercial kit, elute the purified RNA in nuclease-free H₂O, and keep it at –80°C for long-term storage. When starting from purified RNA, take 10 ng of purified RNA (or 1 ng of rRNA-depleted purified RNA), resuspend it in 7.5 µL of nuclease-free H₂O, and continue with the next steps.

g. Add 2.5 µL of RT Mix1.

Note: Mix1 contains primers specific for the *TRAC* (SMART-TRAC), *TRBC1*, and *TRBC2* genes (SMART-TRBC), which code for the constant region of the TCR α and β chains, linked to an adapter oligo that is exploited later for the unbiased cDNA amplification by PCR (primer sequences are listed in the [key resources table](#)). Mix1 also contains an oligo(dT)₂₀ primer to retrotranscribe all the other polyadenylated mRNA in addition to the TCR genes.

h. Heat to 72°C for 3 min in a PCR thermocycler, then move directly on ice. Keep on ice for 2–3 min.

RT Mix 1 (1 sample)		
Reagent	Final concentration	Amount
dNTPs (10 mM)	0.5 mM	1 µL
Oligo(dT) ₂₀ (10 µM)	0.25 µM	0.5 µL
SMART-TRAC oligo (10 µM)	0.25 µM	0.5 µL
SMART-TRBC oligo (10 µM)	0.25 µM	0.5 µL
Total volume		2.5 µL

i. Add 10 µL of RT Mix2.

Note: Mix2 contains the template-switch oligo (TSO) that has the same adapter sequence present in the SMART-TRAC and SMART-TRBC primers.

RT Mix 2 (1 sample)		
Reagent	Final concentration	Amount
Maxima RT buffer 5×	1×	4 μL
RiboLock RNase Inhibitor (40 U/μL)	1 U	0.5 μL
TSO (10 μM)	0.5 μM	1 μL
Maxima RT RNase H ⁻ (200 U/μL)	100 U	0.5 μL
Nuclease-free H ₂ O		4 μL
Total volume		10 μL

Note: the final concentration indicated in the RT Mix1 and Mix2 tables refers to the 20 μL final volume of the RT reaction.

j. Proceed with the RT protocol using the following cycling conditions.

Note: the 5 cycles after the initial step at 42°C are not critical, but they may increase the final yield. The 50°C step helps in unfolding the RNA secondary structures that may cause the termination of cDNA elongation due to steric hindrance. The 25°C step favors the annealing of the TSO to the untemplated cytosines and the consequent template switch. The annealing of the TSO is further facilitated by the presence of a locked nucleic acid (LNA)-modified guanosine present at its 3' end.³ The two steps at 42°C are aimed at completing the cDNA synthesis and the switched template extension.

RT cycling conditions			
Steps	Temperature	Time	Cycles
Retrotranscription and template-switch	42°C	45 min	1
Unfolding of RNA secondary structures	50°C	3 min	5 cycles
Completing retrotranscription	42°C	7 min	
Facilitating TSO annealing and template-switch	25°C	15 s	
Completing switched template extension	42°C	90 s	
Retrotranscriptase inactivation	85°C	5 min	1
Hold	4°C	Forever	

15. Unbiased PCR amplification of TCR cDNA and purification of the amplified DNA:

a. Prepare the PCR master mix as described in the table below. Assemble the PCR mix during the last 10 min of the RT reaction and keep it on ice.

PCR reaction master mix (1 sample)		
Reagent	Final concentration	Amount
KAPA Buffer HiFi 5×	1×	10 μL
dNTPs (10 mM)	0.3 mM	1.5 μL
ISPCR oligo (10 μM)	0.3 μM	1.5 μL
KAPA HiFi HotStart DNA Polymerase (1 U/μL)	0.5 U	0.5 μL
Nuclease-free H ₂ O		16.5 μL
Total volume		30 μL

b. Add 30 μL of the PCR master mix to the retrotranscribed cDNA. Amplify the cDNA by PCR using the cycling described in the table below.

Note: the ISPCR primer anneals to the adapter sequence present both in the SMART-TCR oligos and the TSO, making it possible to run the PCR using a single primer.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	98°C	20 s	13–15 cycles
Annealing	65°C	15 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

Note: the number of amplification cycles should be adjusted depending on the starting number of cells: 14 cycles are a good compromise in the range of 5,000–10,000 cells.

▮ **Pause point:** if necessary, you can keep the amplified DNA at 4°C up to 24 h.

c. Purify the PCR product with SPRI beads with size selection properties using the appropriate volume ratio to get rid of primers. For instance, add 60 µL of SPRIselect beads to 50 µL of PCR product (1:1.2 ratio).

△ **CRITICAL:** vortex the SPRI beads for 20 sec to resuspend them before use.

d. Mix the total reaction volume by pipetting 10 times, transfer the mix to a 1.5 mL DNA low-binding tube, and incubate at 20°C–25°C for 1 min.

△ **CRITICAL:** insufficient mixing of sample and SPRIselect beads will lead to inconsistent size selection results.

e. Place the reaction tube on an appropriate magnetic stand and wait 4 min to allow the SPRI beads to settle to the magnet. Remove and discard the clear supernatant.

△ **CRITICAL:** take care not to aspirate beads during this step, as the PCR product is associated with the beads. Bead loss will result in reduced yield.

f. Keeping the reaction tube on the magnet, add 180 µL of freshly prepared 85% ethanol solution and incubate at 20°C–25°C for 30 s. Aspirate and discard the ethanol supernatant.

Note: ethanol is hygroscopic; prepare it fresh for optimal results.

g. Repeat the ethanol washing once more.

h. Remove the reaction tube from the magnet and let the beads air-dry for 5 min or until the bead pellet shows little cracks.

△ **CRITICAL:** residual ethanol may impinge on the elution efficiency and downstream enzymatic reactions; on the opposite, beads over-drying may increase the elution time and reduce the yield. [Troubleshooting 4](#).

i. Add 44 µL of Elution Buffer, mix the total elution volume by pipetting 10 times to resuspend the beads, and incubate at 20°C–25°C for 2 min.

j. Place the reaction tube back on the magnetic stand and wait 4 min to allow the SPRI beads to settle into the magnet. Recover 42 µL of eluate and transfer it to a new tube.

Note: leaving 2 μL of eluate in the original reaction tube will avoid any carry-out of SPRI beads into the purified PCR product.

- k. Quantify the amplified DNA with the dsDNA High Sensitivity Assay on a Qubit fluorometer, using 2 μL of the eluate.
- l. Dilute the amplified DNA with the Elution Buffer to reach a concentration of 2 ng/mL.

△ CRITICAL: different SPRI beads kits may have different properties in terms of size selection capacity (e.g., they use different sample-to-SPRI beads ratios to select for the same DNA fragment size) and complementary reagents (e.g., ethanol solution for the washing steps may vary in the range of 70%–85%). We recommend always following the manufacturer's indications.

III Pause point: the purified amplified DNA can be frozen at -20°C for long-term storage.

16. Detection of pre-defined T cell clones by qPCR:
 - a. Perform the qPCR using the TCR-specific qPCR primers (Table 1) designed in advance and loading 4 ng of purified amplified DNA per reaction.
 - b. Prepare a 10 μM stock solution of forward and reverse TCR-specific primers by mixing 10 μL of 100 μM forward primer and 10 μL of 100 μM reverse primer with 80 μL of nuclease-free H_2O .
 - c. Assemble the qPCR reaction master mix as described in the table below. Prepare a master mix per each primer pair, including two mixes for the TRAC-specific and TRBC-specific primers (Table 1).

Note: these primers are designed to be nested compared to the ones used in the RT reaction and work as housekeeping controls providing a relative quantification of the TCR of interest.

qPCR reaction master mix		
Reagent	Final concentration/Amount	Amount
Fast SYBR Green Master Mix 2 \times	1 \times	10 μL
Fwd + Rev primer mix (10 μM)	0.1 μM	0.2 μL
Nuclease-free H_2O		7.8 μL
Total volume		18 μL

- d. Dispense 18 μL of the qPCR master mix per well in a 96-well qPCR microplate.
- e. Add 4 ng of the amplified DNA (2 μL of a 2 ng/ μL sample) in each well and pipette 2–3 times to mix.

Note: despite the SYBR Green master mix contains a hot start polymerase, we recommend assembling the reaction on ice.

- f. Seal the microplate with an optical clear sealing film and quickly spin it down (300 \times g for 30 s).
- g. Run the qPCR choosing the "Fast" protocol.

Note: run at least two technical replicates per each qPCR primer pair; three technical replicates may be better if you are approaching qPCR experiments for the first time or if you frequently experience delta- C_t s between replicates higher than 0.5.

- h. Analyze the data as described in the [quantification and statistical analysis](#) section.

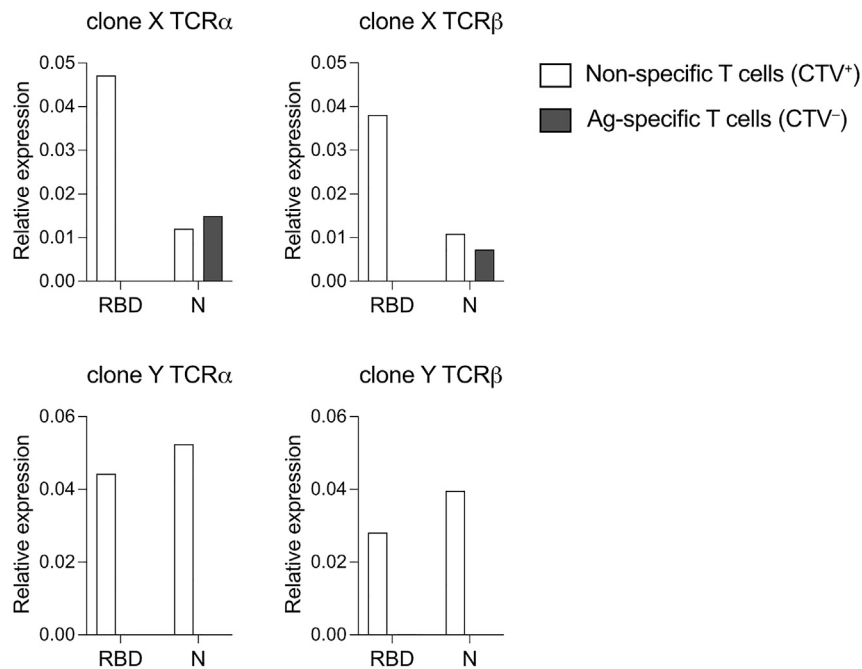


Figure 3. Identification of T cell clone-specific TCR sequences by qPCR

Bar plots show the expression of clone-specific TCR α and TCR β variable regions relative to the TCR α and TCR β constant regions in the same samples. Shown are the data from an N-specific T cell clone (upper panels) and from a non-specific T cell clone (lower panels). This figure partially uses some of the data shown in Figure 6a of Notarbartolo et al.¹

EXPECTED OUTCOMES

The TCR-SMART protocol describes how to detect the presence of T cells with a known TCR in a heterogeneous population of any kind. Here, we combined the protocol with a workflow leading to the identification of antigen-specific T cells, since in many immunological studies it is relevant to identify the T cells specific for a defined antigen of interest. Using the same workflow, it is also possible to isolate bystander-activated T cells, which are lymphocytes that get activated by the inflammatory microenvironment and proliferate independently of their antigen specificity. Representative gating strategies of flow cytometry data are shown in Figure 1. Usually, the frequency of T cells specific for a specific antigen or for the immunodominant antigens of a certain microbe is relatively low. Therefore, the number of sorted antigen-specific T cells can be limiting for the isolation and purification of RNA by commercial kits. Despite the T cells can be polyclonally expanded with allogeneic irradiated feeder cells and phytohemagglutinin in an IL-2-containing medium, the expansion may create clonal biases due to the differential proliferative potential of more or less differentiated T cell populations. For instance, central memory T cells have a higher capacity for proliferating compared with effector memory T cells.⁸ By performing the RT reaction directly on the cell lysate, the TCR-SMART protocol allows the identification of TCR sequences starting from a few thousand cells, thus bypassing the need for clonal expansion. Non-antigen-specific T cells from the same donor can be tested in parallel to verify the relative enrichment of the TCR of interest in the antigen-specific T cell population compared to the non-specific one (Figure 3).

The TCR-SMART protocol is scalable and versatile. As mentioned before, it can be performed starting from very limited amounts of cells, but also from purified RNA, when the cell number is sufficient for its isolation. It can be applied to any T cell-containing population and can be easily modified to detect simultaneously the other polyadenylated transcripts by adding a different adapter to the oligo-dT primer and increasing the extension time in the PCR amplification step.

Table 2. Example of qPCR data analysis

Target name	Sample name	Ct ₁	Ct ₂	Ct difference (Ct ₁ – Ct ₂)	Ct average	ΔCt (Ct _{clone-specific} – Ct _{constant region})	Relative expression (2 ^{-ΔCt})
TRAC (TCR-α constant region)	Nucleoprotein-specific T cells	23.211	23.402	-0.192	23.306		
Clone_X TCR-α (variable region)	Nucleoprotein-specific T cells	29.416	29.320	0.096	29.368	6.061	0.015
TRBC (TCR-β constant region)	Nucleoprotein-specific T cells	21.492	21.554	-0.062	21.523		
Clone_X TCR-β (variable region)	Nucleoprotein-specific T cells	28.428	28.531	-0.103	28.479	6.956	0.008

QUANTIFICATION AND STATISTICAL ANALYSIS

Analyze qPCR data following the example reported in Table 2. Calculate the difference between the threshold cycle (C_t) of technical replicates to evaluate the reproducibility of measurements. Then, calculate the average C_t between technical replicates for each target region. Subtract the constant region-specific (TRAC or TRBC) C_t to the clone-specific C_t. This calculation will provide the ΔC_t value. Elevate 2 to the power of -ΔC_t to obtain the expression of the clone-specific TCR relative to the alpha or beta TCR constant region. [Troubleshooting 5](#). Plot the obtained data in bar plots to visualize the presence of the T cell clone in the population of interest (Figure 3). We consider a T cell clone to be present in the sample when the qPCR reaction with clone-specific primers shows C_t values ≤ 30 in technical duplicates or ≤ 35 in at least three technical replicates. In addition, you should observe a reduction in the relative expression of the TCR in the non-specific T cell population compared with the same sample stimulated with an independent antigen (Figure 3) or stimulated with the same antigen but not sorted. The presence of the clone-specific TCR in the antigen-specific population and its depletion from the non-specific one ratify the specificity of the selected T cell clone.

Summary table with the time needed to perform each task of the TCR-SMART protocol

Task	Timing	Day
Identification of TCR sequences in single-cell TCR-seq data	3 h	Before you begin
qPCR primer design	10 min (per primer pair)	Before you begin
Isolation of PMBCs	1.5 h	Day 1
Magnetic sorting of CD14 ⁺ monocytes	70 min	Day 1
Irradiation of CD14 ⁺ cells and antigen loading	75 min hands-on + 4 h of incubation	Day 1
Sorting of memory CD4 ⁺ T cells	1.5 h + FACS sorting time	Day 1
CD4 ⁺ T cells and antigen-loaded monocytes co-culture	1 h + 6 days of incubation	Day 1
Isolation of antigen-specific CD4 ⁺ T cells	85 min + FACS sorting time	Day 7
Identification of defined CD4 ⁺ T cell clones by TCR-SMART	4 h + 2 h per qPCR plate	Day 7–8

LIMITATIONS

The TCR-SMART protocol detects the presence of α and β TCR sequences separately. When performed on polyclonal populations, it is not able to provide information on the matched α and β TCR sequences at the single-cell level. Nonetheless, since the β chain of the TCR is the main determinant of TCR diversity and specificity, the identification of a certain β TCR sequence can be considered a good approximation for the presence of a T cell clone, as usually done when performing bulk TCR-sequencing experiments. Currently, the TCR-SMART protocol remains a qualitative rather than a quantitative assay.

TROUBLESHOOTING

Problem 1

The isolated PMBCs are heavily contaminated by red blood cells and platelets (related to step 2).

Potential solution

You can remove the contaminating cells by performing a final washing step with 10 mL of wash buffer and centrifuging cells at 120 × g for 10 min. If this is not enough, you can lyse the red blood cells by osmotic shock, for instance using a commercial or home-made red blood cell lysis buffer.

Problem 2

The cell count is much lower than what is indicated by the FACS sorter or cells are lost (related to step 14 b).

Potential solution

To minimize post-sorting cell loss, pre-wet the collection tube by filling it with MACS buffer and incubating it at 4°C for 20 min. Then aspirate the buffer and add 500 μ L of MACS buffer. If you have a few hundred cells you can expand them as indicated in the optional notes at the end of Step 13. Alternatively, you can blindly continue the protocol, even if you cannot see a cell pellet, and verify the amount of amplified DNA at the end of the procedure.

Problem 3

The cell pellet gets disturbed during the aspiration step and looks loose (related to step 14 d).

Potential solution

Stop aspirating and repeat the centrifugation increasing the speed up to 500 \times g for 10 min. Slowly aspirate the supernatant with a 200 μ L pipette.

Problem 4

The SPRI beads get overdried and are difficult to resuspend in the Elution Buffer (related to step 15 h).

Potential solution

Increase the incubation time with the Elution Buffer to 5 min and pipette several times until the SPRI beads aggregates are dissolved. Calculate the 2 min of incubation for the elution starting from the moment the aggregates are dissolved and the SPRI beads are well resuspended.

Problem 5

The reaction with constant region-specific primers (*TRAC* or *TRBC*) reaches the threshold before the linear exponential phase of the qPCR (e.g., it has a C_t value ≤ 15) (related to [quantification and statistical analysis](#)).

Potential solution

Perform a serial dilution of the amplified cDNA (e.g., 8, 16, 32, and 64-fold dilutions) and repeat the qPCR reaction. Verify the linearity of the qPCR signal, namely that you observe an increase of one C_t for each 2-fold dilution, and choose a C_t value falling in the linear range. If the amplified cDNA sample was diluted only for the reaction with constant region-specific primers to avoid too high C_t values in the reaction with clone-specific primers, consider the sample dilution when calculating the relative expression of the clone-specific TCR.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Samuele Notarbartolo (samuele.notarbartolo@policlinico.mi.it).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Samuele Notarbartolo (samuele.notarbartolo@policlinico.mi.it).

Materials availability

The sequence of SMART and qPCR primers specific for the TCR alpha and beta chain constant genes generated in this study is readily available from the [key resources table](#) and [Table 1](#).

Data and code availability

This study did not generate datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2023.102787>.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.N.; methodology, S.N.; software, A.G.; investigation, S.N. and A.G.; resources, A.B.; writing – original draft, S.N.; writing – review and editing, S.N. and A.G.; visualization, S.N.; supervision, S.N., R.G., and S.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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