

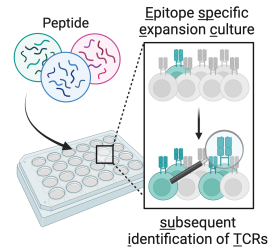


Aug 01, 2025

ESPEC-SUIT - A versatile and robust platform to identify and track antigen-specific T-cell receptors in cancer patients

DOI

<https://dx.doi.org/10.17504/protocols.io.rm7vzkq42vx1/v1>



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Protocol Citation: Isabel Poschke, Katharina Lindner, Saskia Stange, julian.hlawatsch hlawatsch 2025. ESPEC-SUIT - A versatile and robust platform to identify and track antigen-specific T-cell receptors in cancer patients. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.rm7vzkq42vx1/v1>

**Manuscript citation:**

ESPEC-SUIT - A versatile and robust platform to identify and track antigen-specific T-cell receptors in cancer patients; Journal for Immunotherapy of Cancer - in press

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Protocol status: Working

We use this protocol and it's working

Created: February 07, 2025

Last Modified: August 01, 2025

Protocol Integer ID: 119774

Keywords: T cell expansion, TCR sequencing, immunogenicity, TCR discovery, Cytokines, cell receptors in cancer patients espec suit, tcr repertoire analysis, out by tcr repertoire analysis, cancer patients espec suit, tcr candidate, candidate antigen, tcr cloning, epitope specific expansion cultures with subsequent identification, antigen, primed immune response, cell sequencing, immune response, espec, reactive tcr, cell receptor, espec suit, stream tcr, vaccine

Funders Acknowledgements:

German Cancer Research Center; Heidelberg, Germany

Grant ID: _

National Center For Tumor Diseases (NCT); Heidelberg, Germany

Grant ID: NCT IMPlatform

German Cancer Consortium (DKTK)

Grant ID: TCR Discovery Platform

Abstract

ESPEC SUIT - epitope specific expansion cultures with subsequent identification of (tumor-)specific TCRs:

ESPEC SUIT is a modular platform for identification of antigen-specific T cell clonotypes from peripheral blood. It makes use of in vitro expansion to amplify in vivo primed immune responses, which are then read-out by TCR repertoire analysis, paired with optional functional assays.

Candidate antigen-reactive TCRs can be traced in blood and tissue samples based on their sequence to gain deep insight into e.g. tumor-specific or vaccine-induced T cell responses. We found that >75% of TCR candidates identified in ESPEC SUIT can be functionally validated (if optional single-cell sequencing and TCR cloning, not described herein, are performed).

The supplied protocol describes the in vitro expansion, to be followed up by down-stream TCR repertoire analysis and discovery.

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Materials

GENERAL

- pipettes + tips
- serological pipettes + pipetting aid
- centrifuge tubes (conical, sterile) (15 mL, 50 mL)
- 1.5 mL tubes (sterile, safe-lock)
- cell counting equipment
- 24-well plates, cell culture grade
- cryotubes
- controlled rate freezing device
- sterile filters (0.22 µm, protein low-bind)
- DMSO
- EDTA
- Benzonase
- PBS (Ca²⁺, Mg²⁺)

OPTIONAL

reagents for magnetic-bead based cell enrichment, e.g. CD4⁺ T-cell isolation kit, human (Miltenyi, 130-096-533)/anti-human CD4 microbeads (Miltenyi, 130-045-101) or CD8⁺ T-cell isolation kit, human (Miltenyi, 130-096-495)/ anti-human CD8 microbeads (Miltenyi, 130-045-201)

CYTOKINES

	A	B	C	D	E
		Working conc (2x)	final conc per well	manufactur er	catalog no.
	IL-7	50 ng/mL	25 ng/mL	Miltenyi	130-095-362
	IL-15	50 ng/mL	25 ng/mL	Miltenyi	130-095-764
	IL-2	100 IU/mL	50 IU/mL	Novartis	Proleukin, Ch-B. 502519J

Cytokine concentrations for ESPEC culture media

CELL CULTURE MEDIUM

- X-Vivo20 (04-448Q, Lonza) with 2% human AB-Serum (e.g. H4522-100ML, Sigma-Aldrich; serum sterile filtered with 0.22 µm protein-low bind filter)

Before start

Starting material for this protocol are human peripheral blood mononuclear cells (PBMC), isolated from blood by density gradient centrifugation and cryopreserved. Fresh PBMC may also be used, omitting thawing and resting steps.



THAWING OF CELLS (day 0)

50m

- 1 **thaw PBMC** according to internal protocol. We recommend thawing in pre-warmed lymphocyte medium, such as X-Vivo20 (Lonza), and in presence of human serum

30m

Note

PBMC with known reactivity against the antigen-of-interest, e.g. previously determined by ex-vivo ELISpot, will usually result in the strongest expansion

- 2 determine **cell count** and **viability**

10m

Expected result

a viability >80% is expected

- 3 remove cells for **baseline pellet**

10m



- 3.1 transfer $1-2 \times 10^6$ PBMCs to a 1.5 mL tube (DNA-LoBind)

- 3.2 centrifugation:  1000 x g, 20°C, 00:05:00

- 3.3 completely remove supernatant, freeze/store pellet at -80°C for subsequent DNA or RNA extraction

RESTING

12h

- 4 depending on the cell number, place **$2-10 \times 10^6$ cells/mL** in **culture media** in a 15 or 50 mL conical tube with a slightly unscrewed lid

Note

to avoid cells settling too densely at the bottom of the tube, place tube in a tilted position



4.1 **incubate** PBMC overnight at **37°C, 5% CO₂**

CULTURE SETUP (day 1)

4h 55m

5 **centrifugation:**  430 x g, 20°C, 00:10:00

10m

6 remove supernatant, **resuspend** cell pellet in 2 mL culture media without cytokines

5m

7 determine **cell count** and **viability**

5m

8 **optional:** if an ex vivo antigen reactivity assay is planned, remove cell number needed for this assay

5m

*

9 adjust concentration to **2×10⁶ PBMC/mL**

5m

10 prepare two 24-well plates: one for 'antigen presenting cells' (APC), one for 'effectors', both will have the same layout

5m

10.1 add **250 µL culture medium per used well** on both plates

5m

required: For each assay, there has to be one culture expanded without antigen ('no peptide').

For each antigen and the 'no peptide' control, **at least one well** is set up on each plate.

Note

for optimal results, set up 2-4 wells/antigen or condition

**Note**

if a post-ESPEC readout is planned to verify expansion of T cells with the relevant specificity (QC assay), the number of 'no peptide' stimulated wells increases with the number of antigens used, as it is used to determine the background (unspecific expansion) for all antigens after expansion.

- 11 add **250 µL cell suspension/used well** on both plates 5m
- 12 **APC Plate:** add **4 µg/mL peptide of interest** to each well (total volume: 500 µL → 2 µg/well)
exception: 'no peptide' wells 5m
- 13 incubate both plates at **37°C** and **5% CO₂** for **4h** 4h
- 14 **after 4h** of incubation, thoroughly **resuspend wells of 'effectors' plate** and **transfer to corresponding well of 'APC' plate** (→ total volume 1 mL, peptide conc. 2 µg/mL)
Discard effectors plate. 5m
- 15 **incubate** at **37°C** and **5% CO₂**

FEEDING AND SPLITTING OF CULTURES (day 4-11)

10m

- 16 **feeding of cells** is performed **on day 4, 7, 9 and 11** by replacing half the medium volume with fresh, cytokine containing medium. No peptide is added! 10m
- 16.1 Carefully **remove half the medium volume** (500 µL) of each well without disturbing the cells
- 16.2 Replace with **500 µL of fresh cytokine-containing culture medium**

Note

see materials section for final cytokine concentrations. Prepare a working solution 2x the cytokine concentration needed/well, to achieve the correct final concentration after exchanging 50% of the medium



Note

Alternatively, splitting of cells is performed if wells have turned orange/yellow, as this indicates strong T cell proliferation. No peptide is added!
--> Carefully **resuspend** the well in question
--> **transfer half the medium volume (500 µL) to a new well**
--> **fill up both wells (old/new) with 500 µL of fresh medium containing 2x the cytokine concentration needed/well**

END OF ESPEC (day 14)

2h 30m

17 **harvest cells** by carefully resuspending all wells stimulated with the same peptide and pooling them in one 15 mL tube. Rinse well(s) with medium and add to tube.

10m

18 **centrifugation:**  430 x g, 20°C, 00:10:00

10m

19 remove supernatant and resuspend cells for counting

5m

20 determine **cell count** and **viability**

5m

21 **plan use of cells** (see note) **for TCR repertoire analysis** (required), **QC-assay** (recommended) and **SUIT** (if desired)

	A	B	C
	culture	viability [%]	total cell no.
	'no peptide'		
	'antigen a'		
	'antigen b'		
	...		

Cells available for down-stream analysis



Note

	A	B	C
	Assay	cells needed [x10e6]	comment
	TCR repertoire analysis by deep sequencing--> step 22-23	0.3-1	consider pre-enriching CD4/CD8 T cell subsets depending on antigen
	QC readout by ELISpot (performed after over night resting in cytokine-free media) --> step 24	0.15-1.2	25,000 - 125,000 cells/well depending on culture expansion and freq of ex vivo response; plan with duplicates or triplicates, include positive/negative controls; 'no peptide' culture should be tested against all antigens
	QC readout by intracellular cytokine stain (performed after over night resting in cytokine-free media) --> step 24	~0.9	300,000 cells/condition, 1 well/condition, include positive/negative controls, 'no peptide' culture should be tested against all antigens
	single cell sequencing for TCR retrieval --> step 23	0.3-0.5	to be frozen, scSeq after evaluation of QC readouts

Cell amounts needed (values represent general recommendations and will depend on your assay of choice and internal protocols used)

22 collect cells for **TCR repertoire sequencing** of antigen-expanded and control stimulated cultures.

in parallel: prepare cells for single cell sequencing (if planned, see step 23) and QC assay (if planned, see step 24)

22.1 **optional step**: Isolate CD4+ oder CD8+ T cell subsets using a method of your choice

1h 30m



**Note**

We recommend magnetic bead-based cell isolation procedures, which usually give significant enrichment of the cell type of interest, are easy to perform and less stressful on the cells than flow cytometric cell sorting. The advantage of T cell subset enrichment is improved signal-to-noise in repertoire analysis and more sequence information on the relevant subset, increasing sensitivity.

If antigen restriction is not known, e.g. after stimulating with long peptides with unknown binding to MHC I/II molecules, one can either analyze all T cells, or separate CD4 and CD8 T cells to gain insight into the main responding subset.

22.2 Transfer $0.3\text{--}1 \times 10^6$ (better 2×10^6) cells to a 1.5 mL tube (DNA-LoBind)

5m

22.3 **centrifugation:**  1000 x g, 20°C, 00:05:00

5m

22.4 completely remove the supernatant. **Freeze the pellet at -80°C** for subsequent **DNA/RNA extraction** and **TCR repertoire sequencing** by a method of your choice.

5m

Note

Various methods for TCR repertoire sequencing are available and will require different amounts of input material. We have performed post-ESPEC repertoire analysis using $\sim 0.5 \times 10^6$ expanded cells using DNA-based (Immunoseq, Adaptive Biotechnologies) as well as RNA-based (DriverMap AIR, Cellalecta) methods. TCR β sequencing is sufficient for selection of expanded, candidate antigen-reactive clonotypes.

We provide TCRSelect (<https://tcrselect.dkfz.de>), a tool to explore post-ESPEC repertoire data and select candidates for further analysis.

23 **freeze viable cells for single cell TCR sequencing** in DMSO-containing freezing medium.

10m

Note

Single cell TCR sequencing is required to retrieve paired TCR $\alpha\beta$ information, which is needed for TCR cloning/recombinant expression. Most platforms will accept $<20,000$ cells as input. However, since freezing very low cell numbers can lead to loss of viability upon thawing, we recommend to freeze $>300,000$ cells (optimally 1×10^6) per culture to be analyzed. In most cases, only post-ESPEC samples with clear antigen-specific clonal expansion will undergo single cell TCR sequencing to enable functional validation of candidate antigen-reactive TCRs.



- 24 **rest cells** needed for QC readout at $2 \times 10^6/\text{mL}$ in culture media without cytokine or peptide. For resting, cells can be returned to their original well on the 24-well plate or for volumes $>500 \mu\text{L}$ placed in a 15 mL tube (with partially unscrewed lid). Cells are incubated over night at **37°C** and **5% CO₂**. On the next day, perform a relevant QC assay according to your internal protocols.

Note

Consider cell loss during resting when planning the cell number needed for the QC assay

Note

For each culture, T cell reactivity against the antigen-of-interest is assessed.

Relevant controls include: no stimulation; strong, unspecific activation (induced e.g. by PMA/ionomycin, SEB, anti-CD3 beads); optimally: control antigens such as irrelevant peptides or wildtype-variants of neoepitopes)

Note**Recommended cell numbers:****ELISpot** as QC readout:

~50,000 cells/well for strongly expanding cultures and/or antigens to which a clearly measurable ex vivo T cell response was detected

~125,000 cells/well for weakly expanding cultures

--> if you are unsure about the strength of the response and require a quantitative readout, consider titrating the cell number or using flow cytometry as readout

Intracellular Cytokine Staining (Flow Cytometry, e.g. IFN γ or TNF α) as QC readout:

- 300,000 - 400,000 cells/condition