Cell Selection & Expansion

For research use only

Protocol

Murine T cell expansion with CD3/CD28 Streptamer®

Protocols - from cell preparation to analysis

OVERVIEW

The following protocols are included in this document:

1. Preparation of splenocytes
2. Pre-enrichment of T cells (optional)
3. CFSE staining (optional)
4. Cell stimulation with CD3/CD28 Streptamer®
5. Cell activation analysis
6. Cell count analysis
7. CFSE dilution analysis

1. PREPARATION OF SPLENOCYTES

1.1. Required reagents and material:

Product	Cat. No.	Provider
Cell Strainer, 40 µm	431750	Corning®
10x Buffer CI	6-6320-085	IBA Lifesciences
Dulbecco's PBS	PBS-1A	Capricorn
RBC Lysis Buffer	00-4333-57	Thermo Fisher Scientific
Sterile H_2O		

Further required lab equipment 5 ml syringe, 15 ml conical tube, Petri dish, sterile tweezers

1.2. Procedure

1.2.1. Aseptically remove murine spleen and transfer into sterile PBS. Keep on ice until further processing.

1.2.2. Place the cell strainer in a Petri dish and add around **5 ml** cold PBS. Transfer the spleen into the cell strainer by using sterile tweezers.

1.2.3. Pull out the plunger from a 5 ml syringe. Mash the spleen inside the cell strainer to obtain a homogeneous cell suspension.

Work under a safety cabinet to avoid contamination of your samples.

1.2.4. Transfer the cell suspension to a 15 ml conical tube and fill up the volume to **15 ml** with sterile PBS.

1.2.5. Centrifuge the cell suspension for **6 min** at **300 x g** at **4°C**. Discard the supernatant.

1.2.6. Resuspend the cell pellet in **5 ml** RBC Lysis Buffer. Incubate for **5 min** at room temperature. Invert the tube 2-3x during the incubation period.

1.2.7. Fill up the tube with ice-cold PBS and centrifuge for **6 min** at **300 x g**. Discard the supernatant.

1.2.8. Resuspend the cell pellet in **5 ml** 1x Buffer CI (dilute 10x stock with sterile H_2O). Filter the suspension through a 40 µm cell strainer. Determine the cell number (e.g. by using counting beads see **Protocol 6**).

Cells are now ready for starting stimulation (see **Protocol 4**.). Optionally, T cells can be pre-enriched (see **Protocol 2**.) and/or stained with CFSE to monitor proliferation (see **Protocol 3**.)

2. PRE-ENRICHMENT OF T CELLS (OPTIONAL)

2.1. Required reagents and material:

Product	Cat. No.	Provider
Strep-Tactin® TACS Agarose Column, 0.3 ml	6-6310-300	IBA Lifesciences
CD3 Fab-Strep, mouse, lyophilized	6-8504-150	IBA Lifesciences
Biotin stock solution, 100 mM	6-6325-001	IBA Lifesciences
10x Buffer CI	6-6320-085	IBA Lifesciences
TACS Column Adapter (0.3 ml column)	6-3333-001	IBA Lifesciences
Sterile H_2O		

2.2. Reagent preparation

Allow the reagents to equilibrate to room temperature (RT) prior to use. For a sterile isolation, work under a safety cabinet. **The following volumes will be sufficient for one selection process**.

2.2.1. Prepare 1x Buffer CI from 10x stock by diluting with ddH_2O. Degas buffer before use, as air bubbles could block the column.

2.2.2. Dissolve **one vial** of lyophilized Fab-Strep (**50 µg**) in **1 ml** Buffer CI by carefully pipetting up and down (avoid foam formation). **Do not vortex!**

Required per column: **20 µg** Fab-Strep in **400 µl** Buffer CI. Store remaining Fab-Strep solution at -80 °C (stable for 6 months) if not required immediately

2.2.3.

Prepare 1 mM Biotin Elution Buffer by adding **60 µl** of the 100 mM Biotin stock solution to **6 ml** Buffer CI. Mix thoroughly.

2.3. Sample preparation

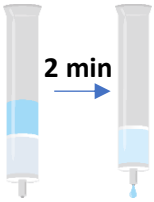
2.3.1. Prepare splenocytes in Buffer CI (see **Protocol 1.**).

2.3.2. Adjust the concentration of the splenocytes to up to 5 x 10^7 total cells per ml. To remove clumps and to prevent aggregates, pass splenocytes through a 40 µm nylon mesh before isolation

2.4. Column preparation

2.4.1. Remove the caps at the top and at the bottom of the column. Allow the storage solution to drain. Place the Strep-Tactin® TACS Agarose Column into the TACS Column Adapter.

2.4.2. Wash the Strep-Tactin® TACS Agarose Column by applying **1 ml** Buffer CI and allow the buffer solution to enter the packed bed completely.

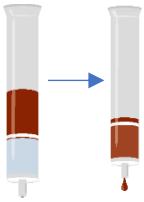


2.4.3. Load the **400 µl** Fab-Strep solution (2.2.2.) onto the Strep-Tactin® TACS Agarose Column. Let the Fab-Strep solution enter the packed bed completely. Incubate for **2 min**.

2.4.4. Wash the Strep-Tactin® TACS Agarose Column with **600 µl** Buffer CI. Discard effluent and change collection tube. Strep-Tactin® TACS Agarose Column is now ready for cell isolation.

Do not interrupt the procedure for more than 60 min.

2.5. Procedure



2.4.1. Load
Apply diluted splenocytes (2.3.2.) in steps of **max. 3 ml**. Collect flow-through containing unlabeled cells.

2.4.2. Wash
Apply **3x 3 ml** Buffer CI. (In each step: Let the buffer solution enter the gel bed completely). The agarose bed should now be white again.

2.4.3. Elute
From this step on your effluent contains your target cells. Use a **new collection tube**. Apply **400 µl** Biotin Elution Buffer (2.2.3.) and incubate for **5 min**. Elute target cells by applying **2.6 ml** Biotin Elution Buffer. Elute a second time with additional **3 ml** Biotin Elution Buffer.

2.6. Further procedure

Centrifuge your eluted cell suspension for **10 min** at **300 x g**. Discard the supernatant and dissolve cell pellet in your desired buffer.

T cell activation with CD3/CD28 Streptamers is a biotin-sensitive assay. Please remove biotin by washing with **50 ml** Buffer CI twice. Discard supernatant **completely.**

3. CFSE STAINING (OPTIONAL)

3.1. Required reagents and material:

Product	Cat. No.	Provider
Carboxyfluorescein succinimidyl ester (CFSE)	C34570	Thermo Fisher Scientific
Fetal Bovine Serum, qualified	26140079	Gibco
Dulbecco's PBS	PBS-1A	Capricorn

3.2. Procedure

3.2.1. Resuspend cells (either whole splenocytes or pre-enriched T cells) in PBS at a concentration of 5 x 10^6 cells/ml in a 15 ml Falcon tube.

3.2.2. Add CFSE at a final concentration of **1.5 µM**.

3.2.3. Vortex the cell suspension gently and keep in the dark for **5 min** at room temperature.

3.2.4. Wash the cells 3x with **10 ml** PBS + 5% FCS.

Cells are now ready for starting stimulation (see **Protocol 4**.).

4. CELL STIMULATION WITH CD3/CD28 STREPTAMER®

4.1. Required reagents and material:

Product	Cat. No.	Provider
CD3/CD28 Streptamer® Kit, mouse	6-8920-050	IBA Lifesciences
Recombinant murine IL-2	212-12-20	PeproTech
RPMI Medium 1640	21875034	Life Technologies
Fetal Bovine Serum, qualified	26140079	Gibco
Penicillin/Streptomycin	15140122	Life Technologies
Dulbecco's PBS	PBS-1A	Capricorn

4.2. Reagent preparation

Volumes are suitable for stimulation and expansion of **5 x 10^5** T cells. For different cell numbers, adapt volumes according to **Table 1**.

4.2.1. Dissolve each Fab-Strep in **500 µl** buffer. Store Fab-Strep suspensions in aliquots at -80 °C.

4.2.2. Combine **5 µl** CD3 Fab-Strep with **5 µl** CD28 Fab-Strep and **5 µl** of Strep-Tactin® Multimer in a 0.5 ml tube. Incubate the mixture for at least **20 min at 4 °C** under constant agitation (e.g. using a roller mixer) to generate CD3/CD28 Streptamer® complexes.

We recommend preparing CD3/CD28 Streptamer® complexes freshly for each experiment. If necessary, store pre-mixed components at 4 °C. **Do not freeze!**

4.3. Cell preparation

4.3.1. Isolate primary T cells or subsets of interest from murine splenocytes and resuspend in a buffer suitable for cells (see **Protocol 1. and 2.**).

Alternatively, whole splenocytes can be used (see **Protocol 1.**).

4.3.2. Determine the number of T cells (either in pre-isolated population or within whole splenocyte population)

4.3.3. Resuspend cells in cell culture medium (recommended: RPMI + 10% FCS + 1% Pen-Strep + 50 U/ml IL-2) at a concentration of **5 x 10^5 T cells** per 1 ml (optimal conditions should be titrated).

Table 4.1.: Recommended volumes for different T cell numbers

	96-well	48-well	24-well
T cell number	5 - 8 x 10^4	2 - 5 x 10^5	0.5 - 1 x 10^6
Volume culture medium [ml]	0.1 - 0.2	0.5 - 1.0	1.0 - 2.0
CD3/CD28 Streptamer® premix [µl]	3	15	30
Biotin [µl]	1 - 2	5 - 10	10 - 20

Biotin is required if you want to terminate the stimulation at a certain point during the expansion period or remove CD3/CD28 Streptamers from the cells. See **4.6.** for details.

4.4. T cell activation

4.4.1. Seed **2 – 5 x 10^5** purified T cells in **0.5 – 1 ml** cell culture medium in a 48-well plate.

4.4.2. Add **15 µl** CD3/CD28 Streptamer® premix to the cells and mix gently. Incubate cell suspension in a humidified CO_2 incubator at 37 °C, according to your experimental setup.

4.4.3. Harvest activated T cells and use directly for further analysis.

Activation markers CD25 and CD69 should be upregulated after **48 h**.

4.5. T cell expansion

4.5.1. Examine culture daily regarding cell size, shape and cluster formation (using a microscope). Count the cells (at least every two or three days) to evaluate cell density (should not exceed 1 x 10^6 cells/ml). If cell medium turns yellow or cell density is too high, split cultures back into a new plate of appropriate size (see Table 4.1. for recommended cell densities).

4.5.2. Restimulation of the cells might be necessary after a couple of days in culture (signs of exhaustion typically after 10 - 14 days). Repeat therefore from step 4.4.2.

4.6. Termination of stimulation/removal of CD3/CD28 Streptamers

4.6.1. Add biotin directly to the culture medium containing the activated T cells (volumes see Table 4.1.). The final concentration of biotin should be **1 mM**.

4.6.2. Incubate cell suspension for **30 min** at **room temperature**.

4.6.3. Harvest cells by resuspension and transfer them to a 15 ml reaction tube. Add **10 ml** of culture medium for dissociation of CD3/CD28 Streptamers. Collect cells by centrifugation at **300 x g** for **6 – 10 min**.

4.6.4. **Discard supernatant completely** and repeat washing with **10 ml** cell culture medium. Cells are now ready for further downstream analyses

For T cell expansion, cells should be stimulated at least for **24 h**.

5. CELL ACTIVATION ANALYSIS

5.1. Required reagents and material:

Product	Cat. No.	Provider
Anti-mouse CD3 APC	B301543	BioLegend
Anti-mouse CD25 Violet 605	B305285	BioLegend
Anti-mouse PD-1 PeCy7	B260172	BioLegend
Anti-mouse CD69 AF700	B279259	BioLegend
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	D1306	Invitrogen
10x Buffer CI	6-6320-085	IBA Lifesciences
Sterile H_2O		

5.2. Cell staining

5.2.1. Resuspend 5 x 10^6 cells in **50 µl** Buffer CI and transfer cell suspension to a FACS tube or a 96-well plate. Keep cells at **4 °C**.

5.2.2. Add all antibodies at a **1:100** dilution to the cell suspension and mix thoroughly.

5.2.3. Incubate cells for **20 min** at **4 °C** in the dark.

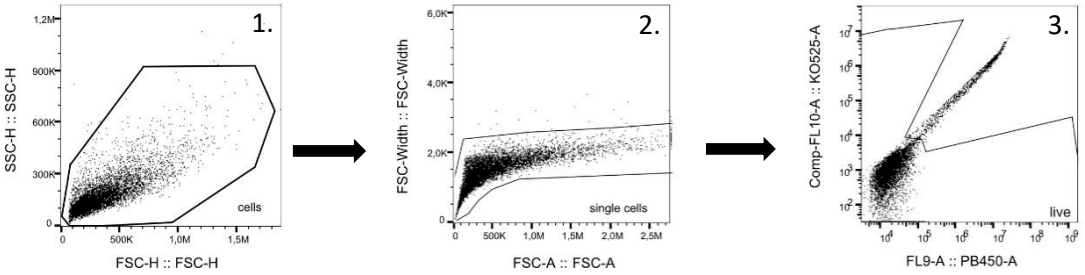
5.2.4. Add **1 ml** (FACS tube) or **200 µl** (96-well plate) Buffer CI to the cells. Centrifuge for **5 min** at **300 x g** at **4 °C**. Discard supernatant.

5.2.5. Resuspend cells in **200 µl** Buffer CI. Shortly before starting the measurement, add DAPI (**1:500** dilution) to the cells.

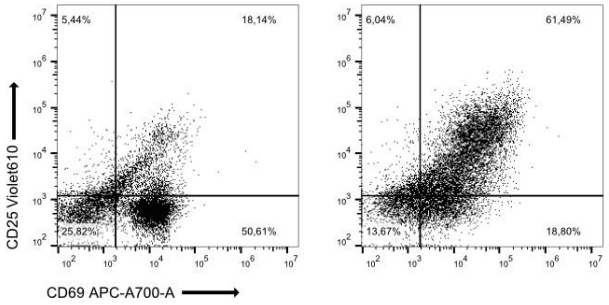
DAPI is added to discriminate living and dead cells. Other options are Propidium iodide (PI), 7-amino actinomycin D (7-AAD) or fixable viability dyes.

5.3. Cell analysis example

5.3.1. Gating strategy: 1. choose cell-sized particles using FSC vs. SSC. 2. exclude doublets using FSC-A vs. FSC-Width. 3. exclude dead cells (for DAPI staining: KO525^{+}- PB450^{+} double positive cells).



5.3.2. Choose activation markers of interest, e.g. CD25 vs. CD69 and compare experimental conditions such as day 0 vs day 4 to observe an increase in expression.



Day 0 Day 4

6. CELL COUNT ANALYSIS

6.1. Required reagents and material:

Product	Cat. No.	Provider
123count eBeads	01-1234-42	Thermo Fisher Scientific
Propidium Iodide (PI)	P1304MP	Thermo Fisher Scientific
10x Buffer CI	6-6320-085	IBA Lifesciences
Sterile H_2O		

6.2. Cell preparation

If only part of the cells are counted, volume taken out of the original sample and total volume of the original sample has to be known.

6.2.1. *Optional*: Perform staining with antibodies (see **5.2.**) to count specific subpopulations.

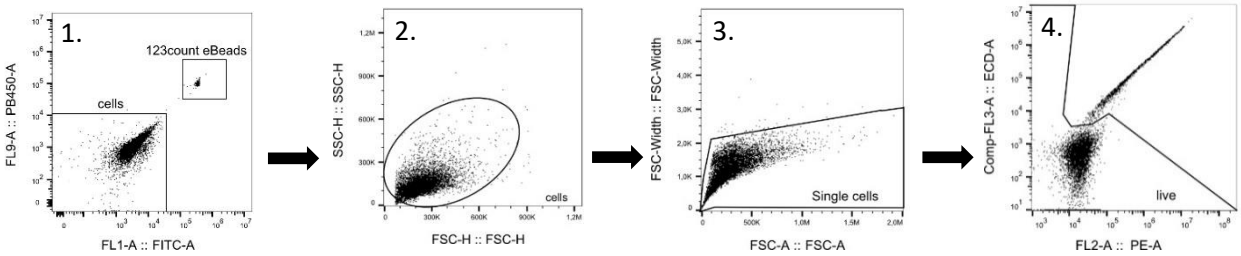
CD3 on T cells is downregulated during the first days of activation and therefore not detectable by flow cytometry.

6.2.2. Add PI (**1:500** dilution) and **10.000** counting beads to the (stained) cells. Vortex thoroughly before starting the measurement.

6.2.3. Measure a defined amount of beads (recommended: at least 1000 beads = 10% of the sample).

6.3. Cell analysis example

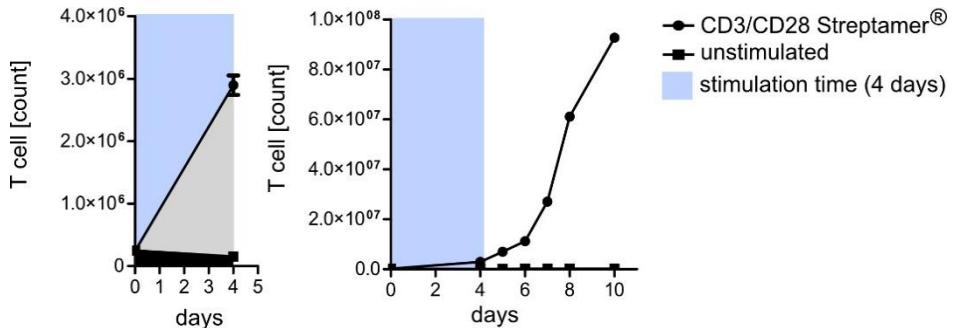
6.3.1. Gating strategy: 1. separate cells from beads using FITC vs. PB450 channel. 2. choose cell-sized particles using FSC vs. SSC. 3. exclude doublets using FSC-A vs. FSC-Width. 4. exclude dead cells (for PI staining: ECD$^+$- PE$^+$ double positive cells).



6.3.2. Determine the number of events of single, living cells. Calculate the amount of cells in your sample. Calculation example:

- Number of single, living cell events: 5.000
- 1.000 out of 10.000 beads acquired, which equals 10% of the sample
- 10 x 5.000 = 50.000 cells were in the FACS tube
- For measurement, 100 µl of sample were taken out of 2 ml total volume: 2000 µl/100 µl = 20
- 50.000 x 20 = 1.000.000 cells were in the starting sample

6.3.3. Example data: A 10- to 15-fold expansion can already be observed during the first 4 days of expansion (left graph). Up to 1 x 10^8 T cells can be yielded from initially 2 x 10^5 T cells after 10 days of expansion (right graph).



7. CFSE DILUTION ANALYSIS

7.1. Required reagents and material:

Product	Cat. No.	Provider
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	D1306	Invitrogen
10x Buffer CI	6-6320-085	IBA Lifesciences
Sterile H_2O		

7.2. Cell preparation

7.2.1. *Optional*: Perform staining with antibodies (see **5.2**.) to pre-gate on T cells.

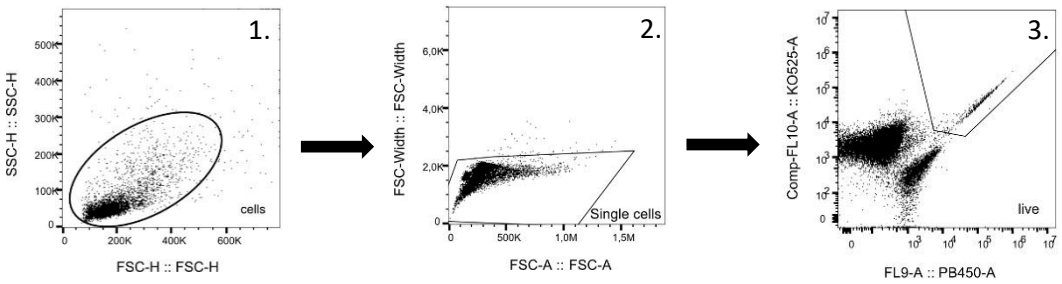
CD3 on T cells is downregulated during the first days of activation and therefore not detectable by flow cytometry.

7.2.2. Add DAPI (**1:500** dilution) to the (stained) cells. Vortex thoroughly before starting the measurement.

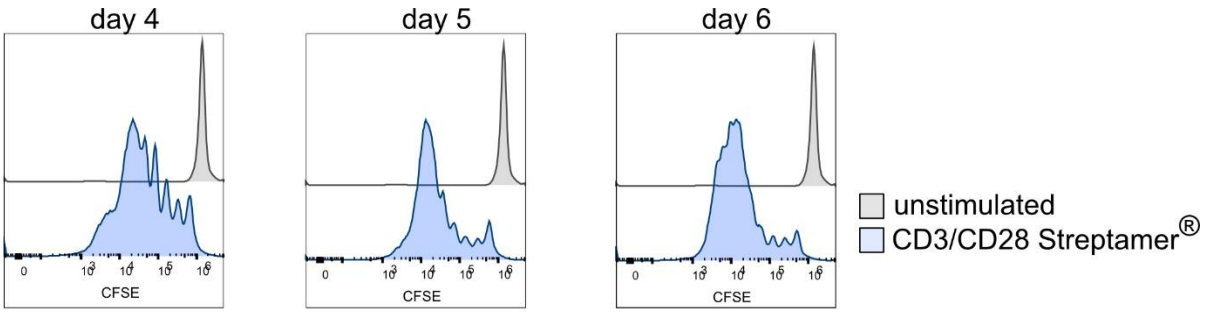
We recommend DAPI staining for discriminating living from dead cells to minimize spectral overlap with the CFSE staining.

7.3. Cell analysis example

7.3.1. Gating strategy: 1. choose cell-sized particles using FSC vs. SSC. 2. exclude doublets using FSC-A vs. FSC-Width. 3. exclude dead cells (for DAPI staining: $KO525^+$- $PB450^+$ double positive cells).



7.3.2. Example data: Typical CFSE dilution peaks (see example below) should be visible on day 4 after starting the stimulation.



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strep-tag@iba-lifesciences.com

We are here to help!

IBA Lifesciences GmbH · Rudolf-Wissell-Str. 28 · 37079 Goettingen · Germany · Phone + 49 551 50672-0 · info@iba-lifesciences.com