

Streptamer[®] Manual

Antigen-specific staining and functional isolation of T-cells

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2 The *Streptamer*[®] Principle

Strep-tags[®], *Strep-Tactin*[®] and *Streptamers*[®]

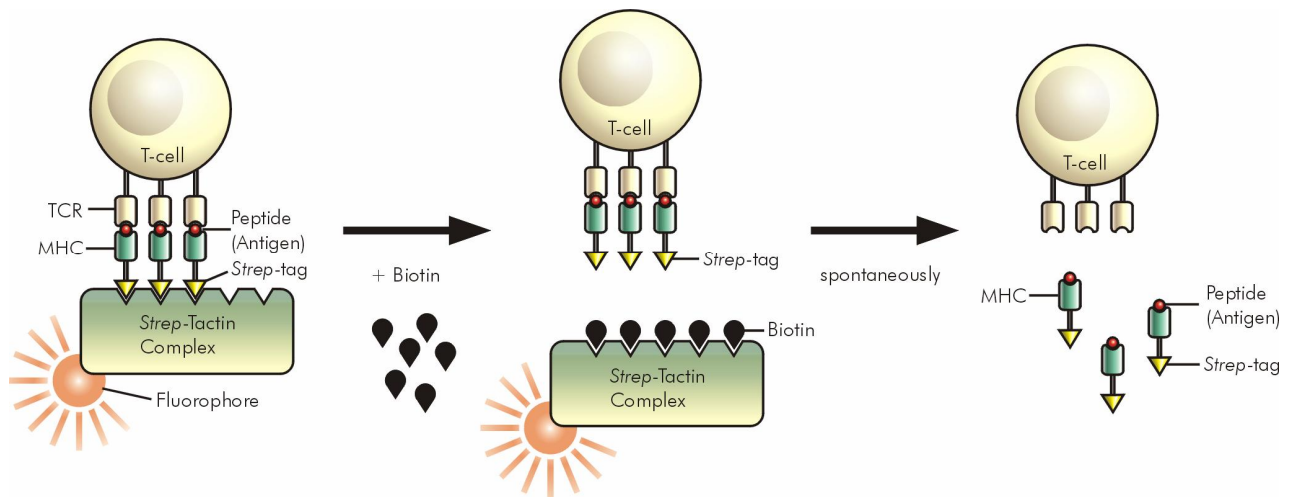
Strep-tags are short peptides with high binding selectivity for *Strep-Tactin*, an engineered streptavidin. The binding affinity of e.g. *Strep-tag II* to *Strep-Tactin* ($K_d = 1 \mu\text{M}$) is nearly 100 times higher than to streptavidin. *Strep-tags* may be fused to recombinant proteins which allows efficient one-step purification of such fusion proteins on immobilized *Strep-Tactin* under physiological conditions, thus preserving their bioactivity. As the *Strep-tag* binds to the biotin binding pocket of *Strep-Tactin*, purified proteins may be mildly eluted from the column by the addition of minute amounts of biotin. Further information is available at www.strep-tag.com.

A special application of the *Strep-tag:Strep-Tactin* technology is the oligomerization of MHC I-*Strep-tag* fusion proteins on *Strep-Tactin*. These complexes may be used for the efficient antigen specific staining of T-cells by using modified *Strep-Tactin* being labeled by a fluorescent probe or a magnetic particle. After separation of the stained T-cells from non-stained cells by FACS or by a magnetic field, respectively, the staining may be efficiently removed by the addition of biotin. This removal of the *Strep-Tactin* backbone leaves monomeric MHC I-*Strep-tag* fusion proteins on the surface of the T-cell. As the monovalent MHC I:T-cell receptor interaction is weak, MHC I-*Strep-tag* fusion proteins spontaneously dissociate from the T-cell receptor and may be removed from the T-cells simply by washing. Keeping stained cells at 4 °C together with rapid and complete dissociation of *Streptamers* from the T-cells after purification assures the isolation of fully functional, non-induced T-cells. Further information is available at www.streptamer.com.

3 Streptamer[®] Fluorescent T-cell Labeling and Isolation via FACS

3.1 Introduction

Scheme of a fluorescent Streptamer labeled T-cell and biotin induced removal of the complex to yield a functional, non-induced antigen specific T-cell preparation



3.2 Experimental procedure

Routinely approximately 5×10^6 cells are stained using $0.75 \mu\text{g}$ Strep-Tactin-PE ($5 \mu\text{l}$) and $1 \mu\text{g}$ MHC I ($4 \mu\text{l}$) in a final volume of $50 \mu\text{l}$.

Please note: All steps – the staining of the cells as well as the following dissociation of Streptamers – have to be performed **at 4°C**. Please make sure that all your reagents and the cells have reached the temperature before starting the protocol.

3.2.1 Titration (optional)

- If the staining protocol is not suitable for your application, a titration of the MHC should be performed. Our recommendation for the titration is:
- Test $0.75 \mu\text{g}$ Strep-Tactin-PE with $1 \mu\text{g}$, $2 \mu\text{g}$ and $5 \mu\text{g}$ MHC I, respectively.
- The assay can be conducted in a 96-well round bottom microplate.
- All incubations are carried out in the dark to protect PE from light.

3.2.2 Protocol for the staining of T-cells with Streptamers

1. Incubate 0.75 μg (5 μl) *Strep*-Tactin-PE and 1 μg (4 μl) MHC in a final volume of 50 μl Buffer IS for 45 minutes.
2. Add the pre-incubated *Strep*-Tactin-PE/MHC I preparation to the cell pellet.
3. Incubate for 45 minutes.
4. Wash cells twice with 200 μl Buffer IS.
5. Cells are ready for FACS-analysis or FACS-sorting.

Important:
All steps have to be performed at 4°C!

3.2.3 Protocol for the subsequent dissociation of Streptamers with D-biotin

1. After sorting wash cells twice with 200 μl Buffer IS.
2. Resuspend cells in 200 μl Buffer IS containing 1 mM D-biotin and incubate for 20 minutes.
3. Wash cells with 200 μl Buffer IS.
4. Incubate for further 20 minutes with Buffer IS containing 1 mM D-biotin.
5. Wash cells 4 times with 200 μl Buffer IS.
6. Transfer cells into the appropriate buffer or medium for further applications.

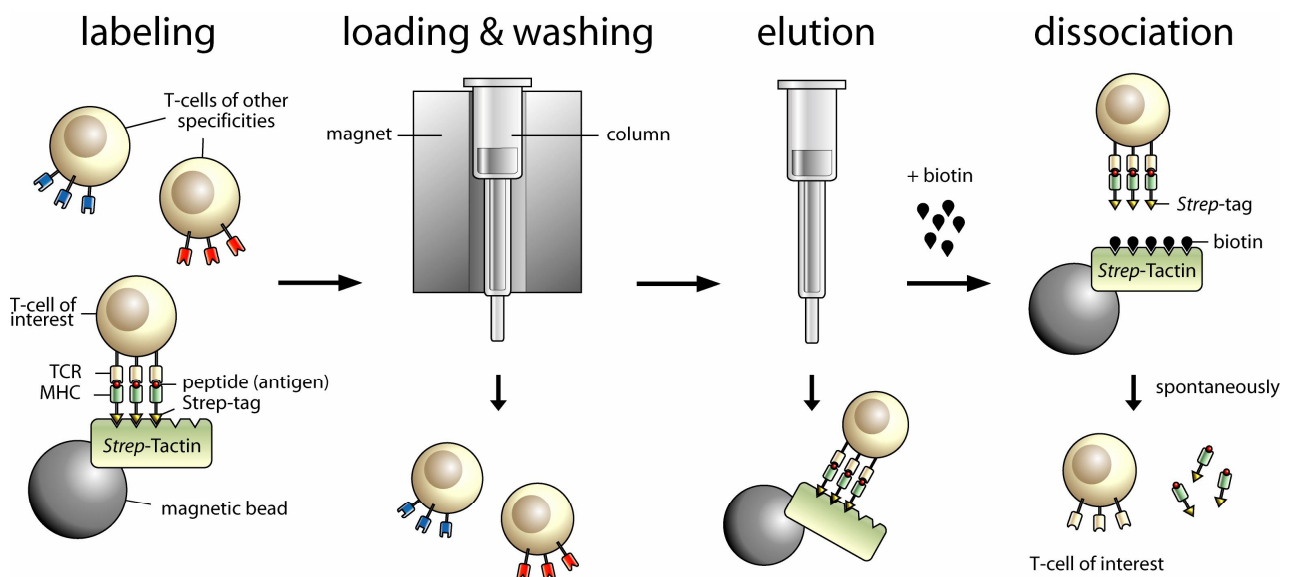
Important:
All steps have to be performed at 4°C!

4 Streptamer[®] Magnetic T-cell Labeling and Isolation via MACS

4.1 Introduction

4.1.1 Purification scheme

In a first step, T-cells are labeled with a magnetic *Streptamer* complex according to their antigen specificity, then stained T-cells are separated from other cells by a magnetic field and such purified T-cells are eluted and released from the *Streptamer* complex by the addition of biotin (vitamin H) to yield a functional, non-induced antigen specific T-cell preparation



4.1.2 Recommendations for isolating T-cells using *Strep-Tactin*[®] magnetic beads and recombinant MHC I proteins fused to *Strep-tag*[®]

Experimental procedure:

The procedure is optimized to isolate antigen-specific T-cells from 2×10^7 peripheral blood mononuclear cells (PBMC). Some cells like monocytes or natural killer cells may also be co-purified due to their ability to bind MHC and can be depleted before the actual T-cell isolation. The recommended procedure depends on species and source of cells.

For human blood:

- Anticoagulant treatment (4.2.1)
- Ficoll gradient (4.2.2)
- T-cell isolation (4.2.4)

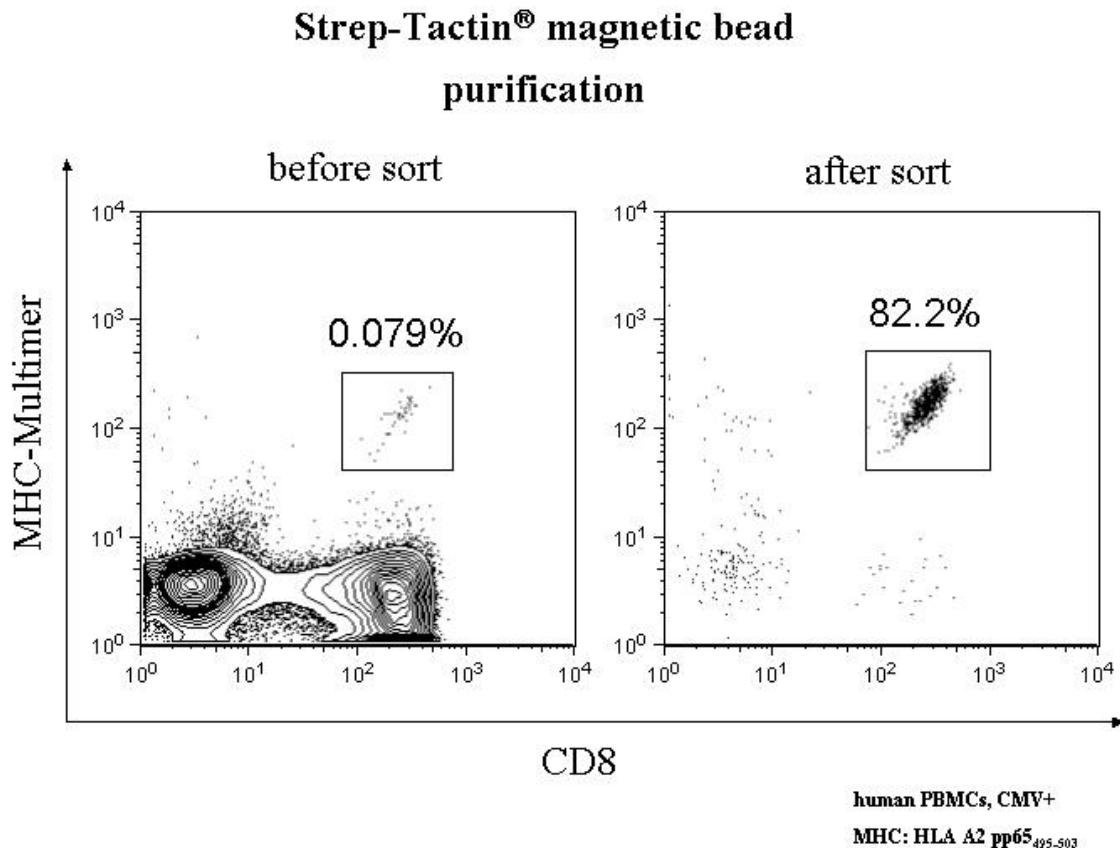
For mouse blood:

- Anticoagulant treatment (4.2.1)
- Ficoll gradient (4.2.2)
- CD8+ enrichment (4.2.3)
- Optional: NK-cell depletion
- Antigen-specific T-cell isolation (4.2.5)

Please note: All steps – the isolation of cells as well as the following dissociation of *Streptamers* – **have to be performed at 4°C**. Please make sure that all your reagents and the cells have reached the temperature before starting the protocol.

4.1.3 Example of an isolation of CMV-antigen specific T-cells

Isolation of antigen specific T-cells from PBMC using *Strep-Tactin*[®] magnetic beads (Cat. No. 6-5500-005) and recombinant MHC I proteins (HLA A2 CMV PP65; Cat. No. 6-7001-005) fused to *Strep-tag*[®].



F. Anderl et al., unpublished data

4.1.4 Set of reagents for isolation of antigen specific T-cells

Item	Cat. No.	Quantity
Streptamer Magnetic Beads	6-5500-005	250 μ l (suff. for $1 \cdot 10^8$ cells)
Streptamer Magnetic Beads	6-5500-025	1.25 ml (suff. for $5 \cdot 10^8$ cells)
MHC I- <i>Strep</i> -tag	6-70xx-005	200 μ l (suff. for pur. of $5 \cdot 10^8$ human or $2.5 \cdot 10^8$ mouse cells)
	6-70xx-015	600 μ l (suff. for pur. of $1.5 \cdot 10^9$ human or $7.5 \cdot 10^8$ mouse cells)
	6-70xx-050	2 ml (suff. for pur. of $5 \cdot 10^9$ human or $2.5 \cdot 10^9$ mouse cells)
Solution set for magnetic beads includes: <ul style="list-style-type: none"> • Buffer IS • Biotin stock solution • Nylon filter mesh (100 μm) 	6-5600-005	for 5 preps ($2 \cdot 10^7$ cells each)
Solution set for magnetic beads includes: <ul style="list-style-type: none"> • Buffer IS • Biotin stock solution • Nylon filter mesh (100 μm) 	6-5600-025	for 25 preps ($2 \cdot 10^7$ cells each)

4.1.5 Materials required but not provided

Blood or T-cell sample
Miltenyi columns and separators
Centrifuge
Test tubes

4.1.5.1 For optional staining and FACS analysis

EMA
Strep-Tactin PE and recombinant MHC I proteins fused *Strep*-tag
CD8-PE or CD8-FITC antibody
CD3-FITC or CD3-APC antibody
FACScan

4.1.5.2 For optional Ficoll gradient centrifugation

Ficoll
PBS or balanced salt solution
Pasteur pipettes
Syringe with needle
Silicone solution
Distilled water

4.1.5.3 For optional CD8+ enrichment

CD8+ T-cell Isolation Kit II (Miltenyi, #130-091-154)

4.2 Experimental procedure

The procedure is optimized to isolate antigen-specific T-cells from 2×10^7 peripheral blood mononuclear cells (PBMC). The first chapters describe the isolation of these cells and depletion of non-T-cell populations, respectively. When working with freshly isolated or frozen PBMC proceed to Chapter 4.2.4 (for human cells) or 4.2.5 (for mouse cells) respectively.

4.2.1 Anticoagulant treatment

1. EDTA is added to a final concentration of 20 mM

2. Same volume of PBS or balanced salt solution is added to the EDTA-blood

Other anticoagulants have been used like heparin, citrate, acid citrate dextrose, citrate phosphate dextrose.

4.2.2 Ficoll gradient centrifugation

Procedure for isolation of lymphocytes from blood samples.

Example: Amersham-Biosciences Ficoll-Paque[®] Plus (6 x 500 ml #17-1440-03)

For details please refer to the included protocol. Briefly:

1. The required volume of Ficoll (3 ml for 4 ml diluted anticoagulated blood sample) is aseptically withdrawn using a syringe

2. Ficoll-Paque Plus (3 ml) is added to a centrifuge tube

3. Carefully layer diluted blood sample (4 ml) on Ficoll-Paque[®] Plus

Important: When layering the sample do not mix Ficoll and diluted blood sample.

4. Centrifuge at 400x g for 30-40 minutes at 18-20°C

5. Draw off the upper layer using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface.

Care should be taken not to disturb the lymphocyte layer. The upper layer of plasma, which is essentially free of cells, may be saved for later use.

6. Using a clean pasteur pipette transfer the lymphocyte layer to a clean centrifuge tube.

It is critical to remove all of the interface but a minimum amount of Ficoll and supernatant. Removing excess Ficoll causes granulocyte contamination, removing excess supernatant results in platelet and plasma protein contamination.

7. Add at least 3 volumes of balanced salt solution to the lymphocytes in the test tube.

8. Suspend the lymphocytes by gently drawing them in and out of a the Pasteur pipette

9. Centrifuge at 10-100 x g and 18-20°C for 10 minutes.
10. Remove the supernatant
11. The lymphocytes should now be suspended in an appropriate medium and can be frozen

4.2.3 Optional: CD8+ enrichment

CD8+ enrichment is recommended for the isolation of **mouse** antigen specific T-cells only. A detailed protocol is available with the kit (i.e. Miltenyi #130-091-154). Here, a brief version.

4.2.3.1 Magnetic labeling

1. Determine cell number
2. Centrifuge cell suspension at 300 x g for 10 minutes. Pipette off supernatant completely
3. Resuspend cell pellet in 40 μ l of buffer per 10^7 total cells.
4. Add 10 μ l of biotin antibody cocktail per 10^7 total cells.
5. Mix well and incubate for 10 minutes at 4-8°C.
6. Add 30 μ l of buffer per 10^7 total cells.
7. Add 20 μ l of anti biotin MicroBeads per 10^7 total cells.
8. Mix well and incubate for an additional 15 minutes at 4-8°C.
9. Wash cells with buffer adding 10-20 x labeling volume and centrifuge at 300 x g for 10 minutes. Pipette off supernatant completely.
10. Resuspend cells in 500 μ l buffer.
Up to 10^8 total cells can be resuspended in 500 μ l, larger numbers require an accordingly larger volume of buffer.
11. Proceed to magnetic separation.

4.2.3.2 Magnetic separation with MS or LS columns

1. Place column in the magnetic field of a suitable MACS® separator.
2. Prepare column by rinsing with appropriate amount of buffer:

MS: 500 μ l LS: 3 ml
3. Apply cell suspension onto the column. Collect flow-through.

Allow cells to pass through and collect effluent as fraction with unlabeled cells, representing the enriched CD8+ T-cell fraction.

4. Wash column with appropriate amount of buffer.

To wash the column, buffer is added three times when column reservoir is empty:

MS: 500 μ l

LS: 3 ml

Collect entire effluent and pool with flow-through (step 3). This fraction represents the CD8+ T-cells.

5. Optional: Elute retained cells outside of the magnetic field.

This fraction represents the magnetically labeled non-CD8+ T-cells.

4.2.3.3 Magnetic separation with the autoMACS[®] separator

For detailed instructions on how to use the autoMACS[®] please refer to the corresponding user manual.

- 1. Prepare and prime the autoMACS[®]**
- 2. Place tube containing the magnetically labeled cells in the autoMACS[®] separator and choose program "Deplete".**
- 3. Collect negative fraction (outlet port "neg1"). This fraction represents the enriched CD8+ T-cells.**
- 4. Optional: Collect positive fraction (outlet port "pos1"). This fraction represents the magnetically labeled non-CD8+ T-cells.**

4.2.4 Isolation of antigen specific T-cells with Streptamer magnetic beads – protocol for human cells

When working with anti-coagulated peripheral blood or buffy coat, PBMC should be isolated by density gradient centrifugation first (see Protocol 4.2.2). This Protocol is adapted for 2×10^7 cells. Higher cell numbers require larger amounts of beads and MHC.

- 1. Thaw frozen cells in normal growth medium.**
 - Make sure concentration of DMSO is below 1%.
 - Cells grown in medium containing less than 10% FCS should be thawed in medium containing 10% FCS instead of their normal growth medium
- 2. Wash cells in buffer IS and resuspend in 10 ml (use 300 g for each centrifugation).**
- 3. Pass cells through enclosed 100 μ m nylon mesh**
 - This is necessary to remove cell clumps which may clog the columns.
- 4. Determine cell number, take sample ("before separation") and place cells on ice.**

5. Incubate 50 μ l magnetic beads, 8 μ l MHC, and 90 μ l buffer IS at least 45 minutes at 4°C (or over night).
6. Place MiniMACS® column in the magnetic field and prepare column by rinsing with 2 ml buffer IS.
7. Add 1 ml buffer IS to MHC/magnetic beads solution and load on MS column.
 - To wash away unbound MHC, magnetic beads are bound and washed on a MS column.
8. Wash with 2 ml buffer IS.
9. Add 250 μ l buffer IS and elute retained beads outside of the magnetic field into a fresh vial and firmly flush out the beads using the supplied plunger supplied with the column.
10. Centrifuge cell suspension (300 g) and resuspend in 250 μ l magnetic beads/MHC solution. Incubate 45 minutes on ice.
11. Add 1.5 ml buffer IS, centrifuge cell and beads mixture and wash carefully once with 2 ml buffer IS.
 - This is necessary to eliminate unbound magnetic beads which may trap cells on the column un-specifically.
12. Resuspend in 2 ml buffer IS. Proceed to magnetic separation.

4.2.5 Isolation of antigen specific T-cells with Streptamer magnetic beads – protocol for mouse cells

When working with cells from spleen or lymph node cells, be careful to resuspend cells completely. Other organ preparations may require protease digestion and/or gradient centrifugation. Mouse T-cell separation protocol is established for 2×10^7 cells. Higher cell numbers require larger amounts of beads and MHC.

1. Centrifuge cells twice 10 minutes at 300 g at 4°C and resuspend in 10 ml buffer IS, respectively.
2. Pass cells through enclosed 100 μ m nylon mesh
 - This is necessary to remove cell clumps which may clog the columns.
3. Determine cell number, take sample (“before separation”, approximately $1-5 \times 10^5$ cells are required per staining) and place cells on ice.
4. Incubate 50 μ l magnetic beads and 16 μ l MHC and 80 μ l buffer IS at least 45 minutes at 4°C (or over night).
5. Place MiniMACS® column in the magnetic field and prepare column by rinsing with 2 ml buffer IS.

6. Add 1 ml buffer IS to MHC/magnetic beads solution and load on MS column.

To wash away unbound MHC, magnetic beads are bound and washed on a MS column.

7. Wash with 2 ml buffer IS.

8. Add 250 μ l buffer IS and elute retained beads outside of the magnetic field into a fresh vial and firmly flush out the beads using the supplied plunger supplied with the column.

9. Centrifuge cell suspension and resuspend in 250 μ l magnetic beads/MHC solution. Incubate 45 minutes on ice.

10. Add 1.5 ml buffer IS, centrifuge cell and beads mixture 10 minutes at 300 x g at 4°C and wash once by resuspending in 2 ml buffer IS and centrifuging as above.

This is necessary to eliminate unbound magnetic beads which may trap cells on the column un-specifically.

11. Resuspend in 2 ml buffer IS. Proceed to magnetic separation.

4.2.6 Magnetic separation on Miltenyi LS and MS columns

For best results, the separation is repeated on a LS and MS column. For detailed description please refer to the protocol enclosed with the columns.

1. Place LS column in the magnetic field and prepare column by rinsing with 3 ml buffer IS.

2. Apply cell suspension (see Protocol 4.2.4 step 12 or 4.2.5 step 11, respectively) onto the column. Allow cells to pass through and collect effluent for later analysis ("Flow-through").

3. Wash column with 2 x 3 ml buffer IS.

4. Add 2 x 3 ml buffer IS and elute retained cells outside of the magnetic field into a fresh vial (optional: take sample for analysis).

5. Rinse MS column with 0.5 ml and apply cells eluted from LS column.

6. Wash column with 2 x 2 ml buffer IS.

7. Add 2 x 3 ml buffer IS and elute retained cells outside of the magnetic field into a fresh vial, take sample for analysis ("eluted fraction", should contain the isolated T-cells).

4.2.7 Magnetic separation with the autoMACS® separator

For detailed instructions on how to use the autoMACS® refer to the corresponding user manual.

- 1. Prepare and prime the autoMACS®**
- 2. Place tube containing the magnetically labeled cells (see Protocol 4.2.4 step 12 or 4.2.5 step 11, respectively) in the autoMACS® separator and choose program “Posseld”.**
- 3. Collect positive fraction (outlet port “pos2”). This fraction represents the magnetically labeled antigen specific T-cells.**
- 4. Optional: Collect negative fraction (outlet port “neg1”). This fraction represents mostly T-cells specific for other antigens and other cell types.**

4.2.8 Dissociation of Streptamers with D-biotin

- 1. Centrifuge eluted cells and resuspend in 2 ml buffer IS containing 1 mM D-biotin and incubate for 20 minutes.**
- 2. Centrifuge cells and resuspend in 2 ml buffer IS containing 1 mM D-biotin and incubate for another 20 minutes.**
- 3. Wash cells 4 x with 5 ml buffer IS.**

4.2.9 Staining of T-cells with Streptamers

To evaluate the purity of the enriched antigen-specific T-cells, fractions can be analyzed by flow cytometry. Live/dead stain can be analyzed with ethidium monazid bromide (EMA, Molecular Probes, E1374), CD8+ T-cells can be detected using a CD8-PE (CD8-FITC) antibody, and optionally a T-cell marker like CD3 can be detected with, e.g. CD3-FITC (CD3-APC) antibody. The antigen specific fraction of these cells can be detected by using (the same) MHC in combination with a *Strep*-Tactin-PE conjugate. Proceed as described under 3.2.2. When secondary staining of CD3 or CD8 is desired add the respective antibody 25 minutes after the addition of the *Strep*-Tactin-PE/MHC I complex to the cells so that its incubation will last 20 minutes (total incubation of the *Strep*-Tactin-PE/MHC I with the cells = 45 minutes).

5 Appendix: Buffer Composition

Phosphate buffered saline (PBS): 8.06 mM Na_2HPO_4 1.47 mM KH_2PO_4 137 mM NaCl pH 7.4	Buffer IS: 0.5 % BSA (w/v) in PBS pH 7.4
Biotin stock solution isotonic 100 mM biotin/NaCl pH 7.4	

6 References

Knabel M et. al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. Nature Medicine 6: 631-637 (2002).