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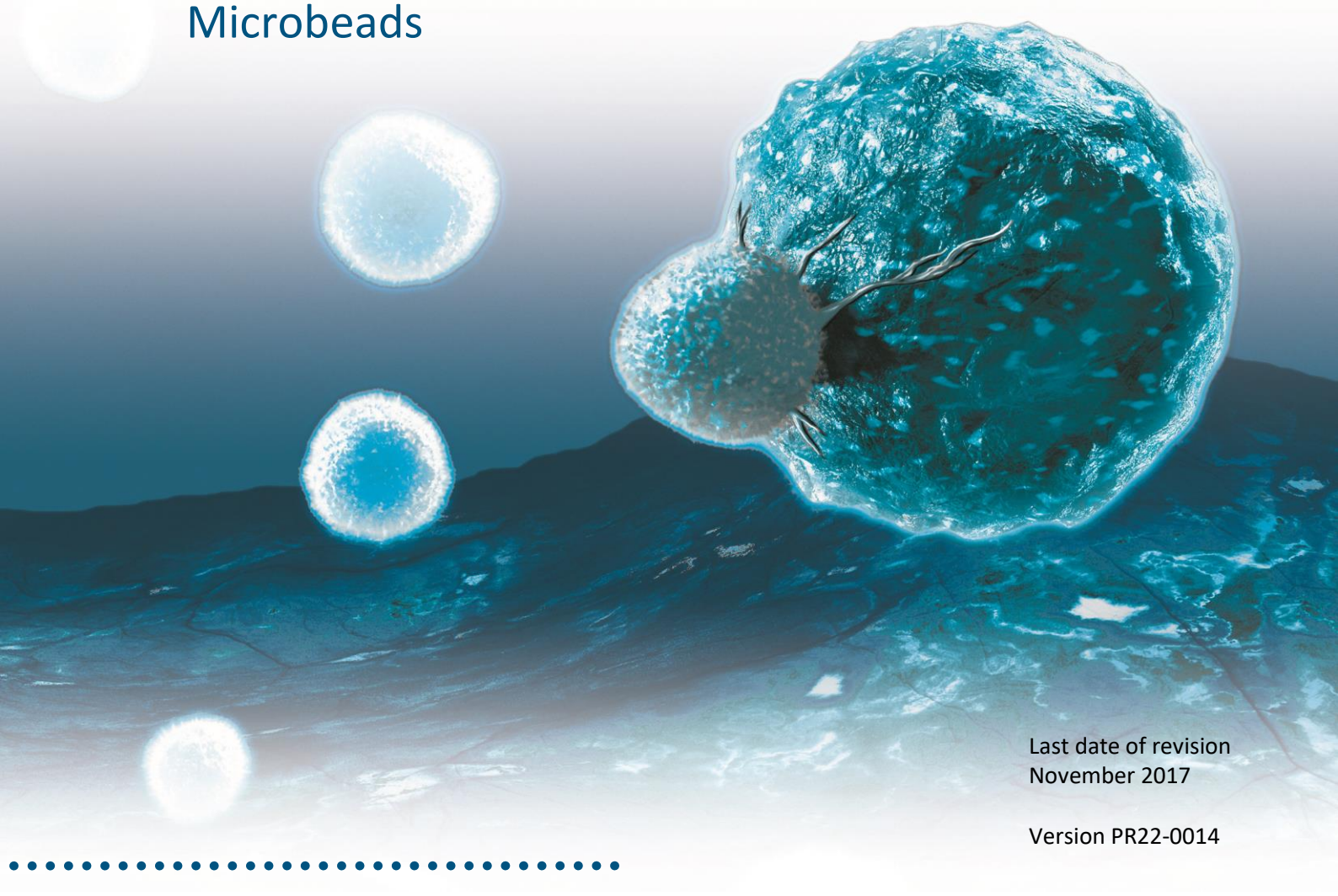
Solutions
For Life Sciences

MHC I Streptamer® Manual

I) Staining of antigen-specific CD8$^+$ T cells with reversible MHC I Streptamers® and FACS isolation

II) Isolation of antigen-specific CD8$^+$ T cells with reversible MHC I Streps and Strep-Tactin® Magnetic Microbeads



Last date of revision
November 2017

Version PR22-0014

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Content

1 The Streptamer® Principle

Strep-tag®, Strep-Tactin® and Streptamer®

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® (kD = 1 µM) 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.

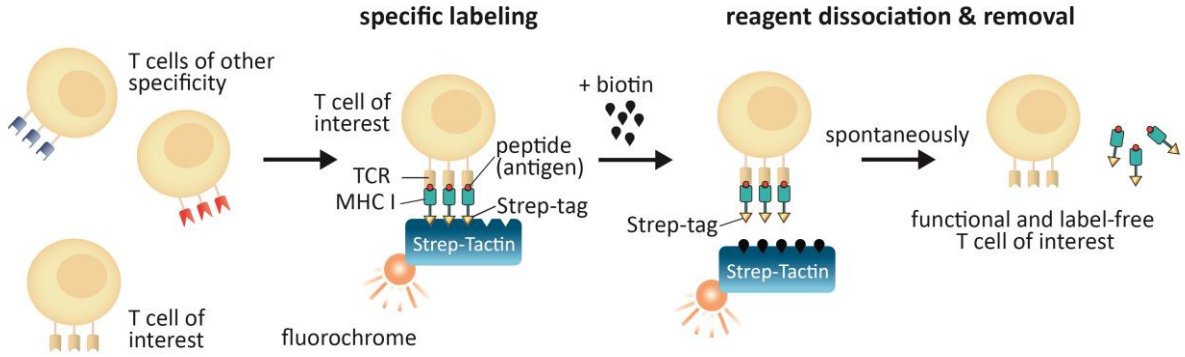
A special application of the Strep-tag®:Strep-Tactin® technology is the oligomerization of MHC I-Strep-tag® fusion proteins (MHC I-Strep proteins) on Strep-Tactin®. Multimers of MHC I-Streps complexed with either fluorescently or magnetically labeled Strep-Tactin®, so-called Streptamers, are used for efficient staining or isolation of antigen-specific T cells. After separation of the labeled T cells from non-labeled cells by flow-cytometric or magnetic cell isolation, the Streptamers are efficiently disrupted on the cell by addition of biotin. Subsequently, the dissociation and removal of the Strep-Tactin® backbone leaves monomeric MHC I-Strep proteins on the surface of the T cell. As the interaction of monovalent MHC I:T cell receptor is weak, MHC I-Strep proteins spontaneously dissociate from the T cell receptor and may be removed from the T cells simply by washing. Keeping cells at cooled conditions as well as performing the rapid and complete removal of the Streptamers® from the T cells assures the isolation of fully functional, non-induced T cells.

Further information is available at www.streptamer.com.

2 Reversible staining of antigen-specific CD8+ T cells with MHC I Streptamers® and FACS isolation

2.1 Introduction: cell staining and removal of staining reagents

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



2.2 Required reagents and materials

2.2.1 MHC I-Streps and Strep-Tactin® PE or APC

Cat.no	Product Name	Staining of	Size
6-7XXX-001	MHC I-Strep	$1x10^6$ to $2x10^6$ cells	40 µl
6-5000-001	Strep-Tactin® PE for MHC I Streptamers®	$1x10^6$ to $2x10^6$ cells	50 µl
6-5010-001	Strep-Tactin® APC for MHC I Streptamers®	$1x10^6$ to $2x10^6$ cells	50 µl

2.2.2 Streptamer® Solution Set and pre-separation filters

Cat.no.	Product Name	Content
6-5603-005	Streptamer® Solution Set Standard	Buffer IS, D-Biotin

The Streptamer® Solution Set Standard contains 50 ml Buffer IS as 10x concentrate for washing, and 1 ml of a D-Biotin stock solution (100 mM) for dissociation of the Streptamers® from the isolated cells. **Buffer IS has to be diluted with 9 volumes of water prior to use.** We recommend to add EDTA at a final concentration of 1 mM. Degas buffer before use. **The 100 mM Biotin stock solution has to be diluted with 99 volumes of Buffer IS prior to use (Biotin working solution is 1 mM; see 2.6.).**

Pre-separation filters (IBA GmbH, cat.-no.: 6-5601-010) are recommended for removal of cell clumps.

2.3 Use and storage of MHC I-Streps and fluorescent Strep-Tactin®

MHC I-Streps are shipped on dry ice and then stored at -80°C until use. After initial thawing prepare aliquots for long-term storage at -80°C. Aliqouts for immediate use should be kept permanently on ice. **Aliquotation is mandatory to avoid freeze thaw cycles which denature the MHC I-Streps**.
Strep-Tactin® PE or APC for MHC I Streptamers® is shipped on blue ice and stored at 4°C.

2.4 Staining procedure

The procedure is optimized for staining of antigen-specific CD8+ T cells from fresh or frozen peripheral blood mononuclear cells (PBMCs). When working with anti-coagulated peripheral blood or buffy coats, PBMCs should first be isolated by density gradient centrifugation and separated from platelets.
Please adjust cell density to 10^7 cells / 100 µl before starting the protocol.

Important: All steps 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. Protect labeled cells and fluorochrome reagents from light by incubating in the dark.

Protocol for staining of ca. 1 - 2x10^6 cells (1 test):

1. Prepare ca. 3 ml Buffer IS from 10 x stock.
2. Incubate 1 µl Strep-Tactin® PE or APC and 0.8 µl MHC I-Strep in a final volume of 10 µl Buffer IS for 45 minutes.
3. Add the pre-incubated Streptamers® (complex from Strep-Tactin® PE (or APC) and MHC I-Strep, step 2) to the cell pellet.
4. Incubate for 45 minutes.
5. Wash cells twice with 200 µl Buffer IS.
6. Cells are ready for FACS-analysis or FACS-sorting.

Dead cell exclusion is strongly recommended (e.g. propidium iodide, 7-AAD, etc.)

2.5 Titration (optional)

If the staining protocol is not suitable for your application, a titration of the Streptamers® should be performed. Our recommendation for the titration is:
Keep the cell concentration of 10^7 cells / 100 µl constant and increase the amount of Streptamers® stepwise (2-, 3- and 4-fold). Add the following volumes of pre-incubated (45 min) Streptamers® to your cell pellet:

- 2-fold increase (1.6 µl MHC I-Strep + 2 µl Strep-Tactin® PE in 20 µl buffer IS)
- 3-fold increase (2.4 µl MHC I-Strep + 3 µl Strep-Tactin® PE in 30 µl buffer IS)
- 4-fold increase (3.2 µl MHC I-Strep + 4 µl Strep-Tactin® PE in 40 µl buffer IS)

The assay can be conducted in a 96-well round bottom microplate.

2.6 Dissociation of Streptamers® with D-Biotin

Important: All steps 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.

1. Collect cells by centrifugation.
2. Prepare ca. 1 ml Buffer IS containing 1 mM D-Biotin (1 mM Biotin working solution).
3. Resuspend cells in 200 µl Biotin working solution and incubate for 10 minutes.
4. Collect cells by centrifugation.
5. Repeat step 3 and 4.
6. Wash cells 4 times with 200 µl Buffer IS.
7. Transfer cells into the appropriate buffer or medium for further applications.

2.7 Short Protocol

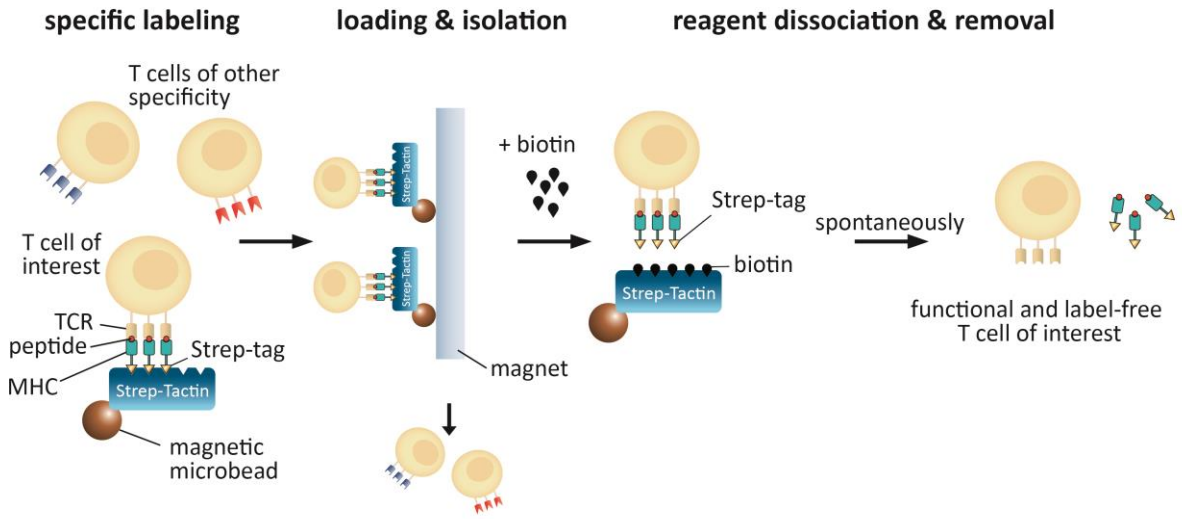
Please request a copy of our Short Protocol PR38 for MHC I Streptamer® Staining at info@streptamer.com or download it from www.streptamer.com

3 MHC I Streptamer® Magnetic Cell Labeling and Cell Isolation with Strep-Tactin® Magnetic Microbeads on a permanent magnet

3.1 Introduction

3.1.1 Purification Scheme

$CD8^+$ T cells are labeled according to their antigen specificity with Strep-Tactin® Magnetic Microbeads coupled to the specific MHC I-Strep. Labeled cells are separated from other cells by a magnetic field. All Streptamer® reagents are then released from the target cells by the addition of biotin (vitamin H) to yield a functional, non-induced antigen specific $CD8^+$ T cell preparation.



3.2 Streptamer® reagents

3.2.1 Buffers

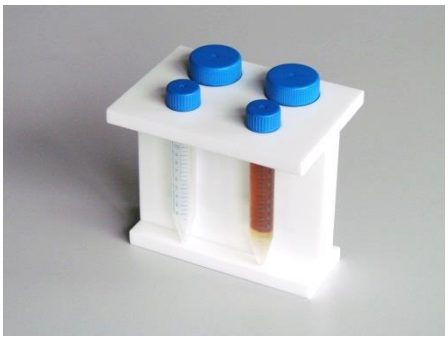
Buffer IS has to be diluted with 9 volumes of water prior to use. We recommend adding EDTA at a final concentration of 1 mM.
The 100 mM Biotin stock solution has to be diluted with 99 volumes of Buffer IS prior to use (Biotin working solution is 1 mM; see 3.4.2)**.**

3.2.2 Pre-separation filters

We recommend our pre-separation nylon filters for removal of cell clumps
(cat.-no.: 6-5601-010; includes 10 filters).

3.2.3 StrepMan Magnet

Cat.no.	Product Name	Size
6-5650-065	StrepMan Magnet	for 2 x 15 ml and 2 x 50 ml tubes



3.3 Use and storage of MHC I-Streps and magnetic beads

MHC I-Streps are shipped on dry ice and then stored at -80°C until use. After initial thawing prepare aliquots for long-term storage at -80°C. Aliquotation is mandatory to avoid freeze thaw cycles, which denature the MHC I-Streps.

Strep-Tactin® Magnetic Microbeads are shipped on blue ice and stored at 4°C (do not freeze). The storage buffer of the beads contains 0.05 % sodium azide. We therefore recommend washing the beads with 1 volume of Buffer IS (or at least 1 ml) on the magnet prior to use.

3.4 Experimental procedure

The procedure is optimized for T cell isolation from $2x10^7$ PBMCs. For cell numbers higher than $2x10^7$ we suggest a linear upscale of beads and MHC I-Streps (example shown in Table 1).
Some cells like monocytes or natural killer cells may be co-purified due to their ability to bind MHC I; they can be depleted by $CD8^+$ pre-selection prior to T cell isolation.

Important: 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.

Avoid foaming, which interferes with proper bead retention on the magnet!

3.4.1 Preparation of cells and Streptamers®

Human cells: The procedure is optimized to isolate antigen-specific $CD8^+$ T cells from 2 $x10^7$ freshly isolated or frozen peripheral blood mononuclear cells (PBMCs). When working with anti-coagulated peripheral blood or buffy coat, PBMCs should be isolated by density gradient centrifugation first.

MHC I Streptamer® preparation for human cells (step 1-2):

Optional: Wash magnetic microbeads before use to remove sodium azide. Therefor transfer the appropriate amount of homogeneously resuspended Strep-Tactin® Magnetic Microbeads to a new tube. Add 1 ml of buffer IS and resuspend carefully by pipetting up and down. Hold the tube on the magnet for at least 1 min and discard the supernatant. Remove the tube from the magnet and resuspend the Strep-Tactin® Magnetic Microbeads in the appropriate initial volume of fresh Buffer IS.

1. Mix 8 µl MHC I-Strep with 2 µl Buffer IS in a fresh reaction tube.

2. Add 30 µl from the homogeneously resuspended Strep-Tactin® Magnetic Microbeads to the MHC I-Strep solution above. Ensure proper mixing to achieve a homogeneous MHC I-Strep distribution on the Strep-Tactin® Magnetic Microbeads. Incubate under gentle constant agitation, e.g. on a roller mixer, for 30 minutes (overnight incubation might be suitable).

Table 1: MHC I Streptamer® labeling
Suggestions for adaptation of reagent amounts to different cell numbers

Number of PBMCs	MHC I-Strep	Buffer IS	micro-beads	tube size	cell volume	final volume	washing (StrepMan Magnet)
	Step 1	**Step 1**	**Step 2**	**Step 3**	**Step 3**	**Step 4**	**Step 5**
$2x10^7$	8 µl	2 µl	30 µl	15 ml	210 µl	250 µl	5 ml
$2x10^8$	80 µl	20 µl	300 µl	15 ml	2.100 µl	2500 µl	10 ml

Cell labeling with MHC I Streptamers® (step 3-4):

Optional: Wash pre-cooled cells with 10 ml Buffer IS in a 15 ml reaction tube to remove potentially interfering components (e.g. Biotin). Pass cells through a nylon filter tube to remove cell clumps and place cells on ice.

3. Resuspend cells in 210 µl Buffer IS and add the pre-incubated MHC I-Strep Microbead preparation (magnetic Streptamers®) from step 2 to the cells. Mix thoroughly by gentle pipetting.

4. Incubate for 20 minutes under gentle constant agitation, e.g. on a roller mixer, to prevent cells from sedimentation.

Magnetic separation (step 5-8):

5. Add 5 ml of buffer IS to the cell:bead preparation from step 4.

6. Separate magnetically labeled cells by placing the tube for at least 3 min firmly onto the StrepMan Magnet. Remove the supernatant containing the negative cell fraction by careful pipetting while keeping the reaction tube on the StrepMan Magnet.

7. Remove the reaction tube from the StrepMan Magnet and carefully flush labeled cells (positive cell fraction) off the tube wall by resuspending in 5 ml Buffer IS.

8. Repeat magnetic selection (steps 6 and 7) twice.

3.4.2 Dissociation of MHC Streptamers® from the isolated cell population

Dissociation and removal of Strep-Tactin® Magnetic Microbeads (step 1-6)

1. Prepare a 1 mM biotin working solution by mixing 10 ml buffer IS and 100 µl 100 mM biotin stock solution.

2. Centrifuge eluted cells from step 8, 2.4.1. and resuspend final cell fraction in 5 ml 1 mM biotin working solution. Incubate for 10 minutes.

3. Place the reaction tube on the StrepMan Magnet for at least 3 minutes. Carefully pipet off the supernatant containing the target cell fraction and transfer the supernatant to a fresh reaction tube.

4. Remove the reaction tube from the StrepMan Magnet and thoroughly resuspend residual cells and magnetic beads with 5 ml 1 mM biotin working solution. Incubate for 10 minutes.

5. Repeat step 3.

6. Pool the supernatants and collect cells by centrifugation (400 x g, 6-10 min).

Removal of MHC-Streps (step 7-11)

7. Carefully remove supernatant and discard. Resuspend cell pellet in 5 ml Buffer IS and incubate for 10 minutes.

8. Place tube back on the magnet (to remove any potential residual beads) and incubate for 3 minutes.

9. After incubation, transfer supernatant to a new tube and collect cells in this tube by centrifugation (400 x g, 6-10 min).

10. Repeat step 7 and 8 and pool cell fractions. Collect cells by centrifugation (400 x g, 6-10 min).

11. Remove supernatant and resuspend cells in the appropriate buffer or medium for further applications.

3.4.3 Titration (optional)

If the protocol does not provide satisfactory results in a given application, especially for obtaining high yields of very abundant target cell populations, a titration of the ratio between the Streptamers® (MHC I-Strep microbead preparation) and the cell number should be performed.

4 References

Knabel, M., Franz, T.J., Schiemann, M., Wulf, A., Villmow, B., Schmidt., B., Bernhard, H., Wagner, H. and Busch, D. *(2002)* Reversible MHC multimer staining for functional isolation of T cell populations and effective adoptive transfer. *Nature Medicine* **8** (6), 631-637.

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For more references please visit www.streptamer.com

5 Warranty

The product sold hereunder is warranted only to conform to the quantity and contents stated on the label at the time of delivery to the customer. There are no warranties, expressed or implied, that extend beyond the description on the label of the product. IBA's sole liability is limited to either replacement of the products or refund of the purchase price. IBA GmbH is not liable for property damage, personal injury, or economic loss caused by the product.

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