Spot-Tag: a Nanobody-based Peptide-Tag System for Protein Detection, Purification and Imaging

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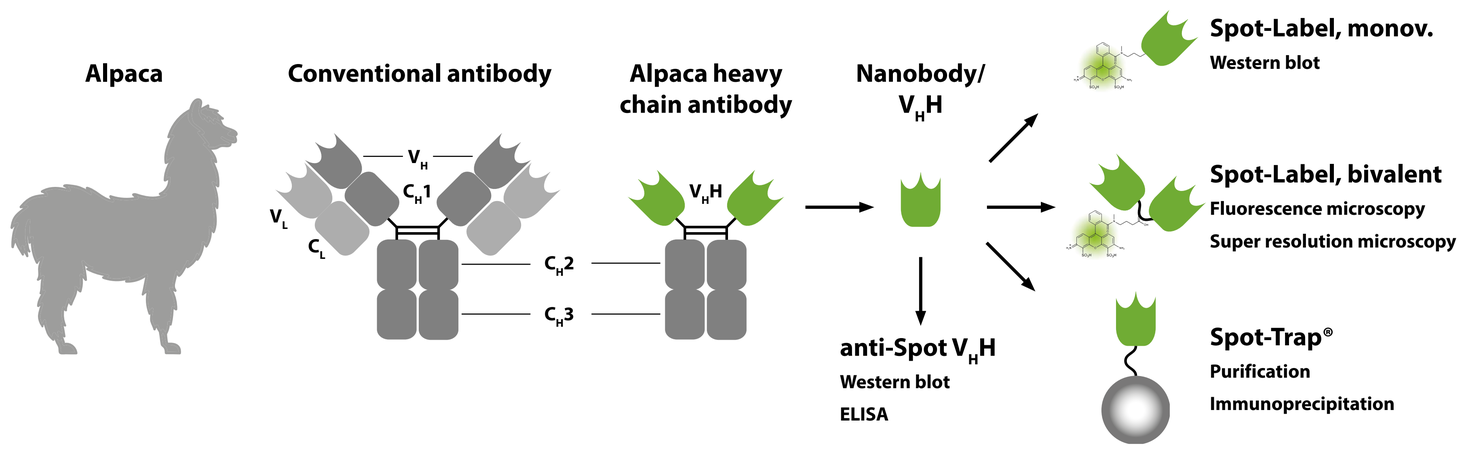
We have developed a new epitope tag system based on a V_HH (also called nanobody®), i.e. a small and highly stable alpaca single-domain antibody. This V_HH binds with high affinity to the Spot-Tag®, an engineered 12-amino acid sequence (PDRVRAVSHWSS) derived from a linear epitope within β-catenin. Owing to the unique properties of the anti-Spot-Tag-V_HH, the Spot-Tag capture and detection system is universally applicable.



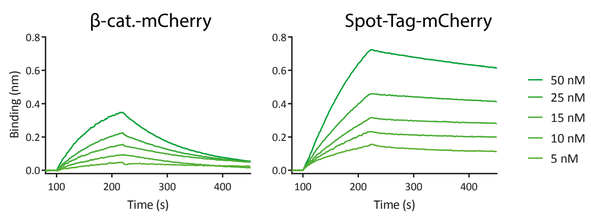
The Spot-Tag

Origin of the Spot-Tag

Immunisation of alpacas with β-catenin yields nanobody that binds unstructured N-terminus[1] (amino acids 16-27).

Further screening of 30 variants identifies tag sequence optimised for high affinity: the Spot-Tag.

High affinity of the Spot-Tag



	K_D	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
β-cat. 16-27 C-term.	45 nM	1.5×10^5	6.7×10^{-3}
β-cat. 16-27 N-term.	56 nM	1.4×10^5	8.0×10^{-3}
Spot-Tag C-term.	7 nM	1.8×10^5	1.3×10^{-3}
Spot-Tag N-term.	6 nM	1.3×10^5	7.4×10^{-4}

Biolayer interferometry (BLI) kinetics analysis of binding of Spot-Tag nanobody to mCherry tagged with β-catenin 16-27 or Spot-Tag.

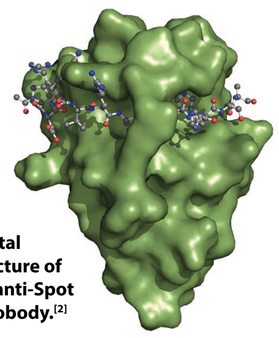
→ Engineering of original epitope leads to significantly decreased dissociation and thus improved affinity for Spot-Tag.

→ High affinity binding of Spot-Tag to anti-Spot nanobody

The anti-Spot nanobody

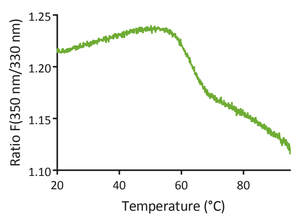
Characteristics

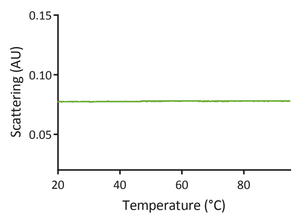
- **Single peptide of 131 amino acids**
- **Small size: 14.7 kDa**
- **Binds Spot-Tag with high affinity**
- **Thorough biochemical and structural characterisation**
- **Recombinant production and thus high batch-to-batch consistency**
- **Binds Spot-Tag fused to N- or C-terminus of a protein**
- **Easily derivatised using fluorophores or agarose matrices**



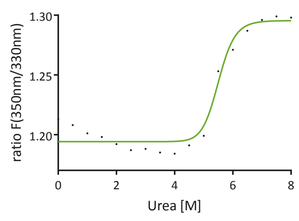
Crystal structure of the anti-Spot nanobody.[2]

High stability

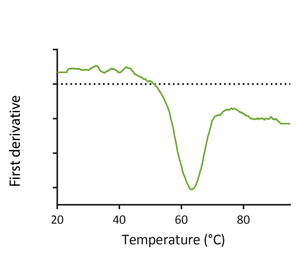




No aggregation observed for anti-Spot nanobody (1 g/l) over a temperature gradient.



Chemical stability of anti-Spot nanobody in a chaotrope (urea).



Differential scanning fluorimetry analysis of anti-Spot nanobody.

The anti-Spot nanobody

→ has a melting temperature T_m of 63 °C.

→ shows unusually high colloidal stability.

→ is highly resistant to chaotropes.

Applications of the Spot-Tag system

Purification and immunoprecipitation using Spot-Trap®

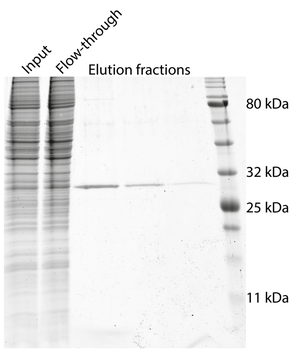
Anti-Spot nanobody covalently coupled to agarose beads or magnetic agarose beads (called Spot-Trap) enables the immunoprecipitation and purification of Spot-Tag fusion proteins.

→ High affinity allows the purification of low-abundance proteins.

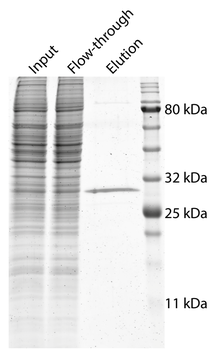
→ Bound protein can be eluted using free Spot peptide.

→ High chemical stability leads to extraordinary chemical compatibility of Spot-Trap

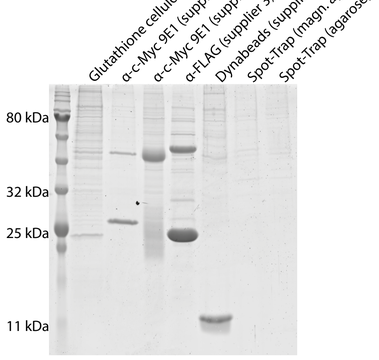
→ Chemical stability also permits repeated use of Spot-Trap



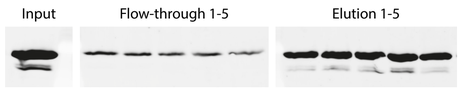
Spot-Trap affinity purification of GFP-Spot from HEK 293T lysate followed by step-wise Spot peptide (100 µM) elution.



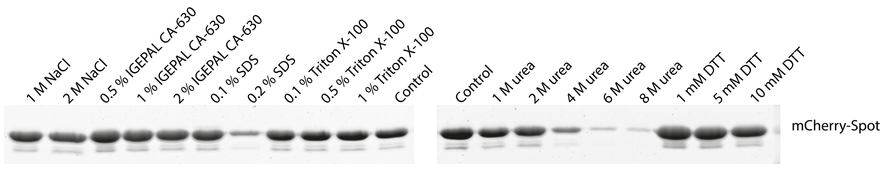
Spot-Trap immunoprecipitation of GFP-Spot from HEK 293T lysate.



Background of different affinity media in immunoprecipitation from HEK 293T lysate without cognate antigen present.



Test of long-term stability of Spot-Trap. Spot-Trap was subjected to five full cycles of affinity purification (loading, washing, peptide elution, regeneration) of mCherry-Spot from *E. coli*. Analysis using Western blotting.



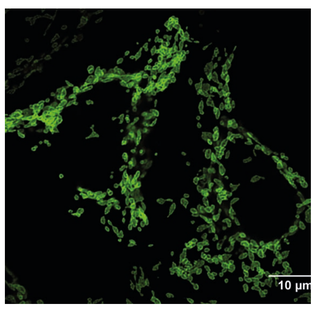
Buffer compatibility test for the Spot-Trap. After precipitation of mCherry-Spot from *E. coli*, Spot-Trap was washed using control buffer or buffer supplemented with indicated additives.

Immunofluorescence microscopy using Spot-Label

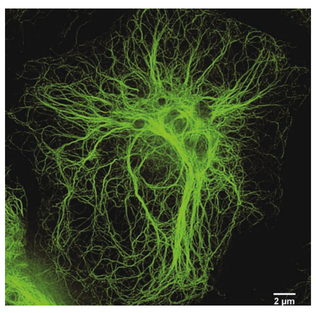
A bivalent format of anti-Spot nanobody conjugated to fluorophores (called Spot-Label), allows the imaging of cellular proteins and structures using fluorescence microscopy.

→ Small size of Spot-Label leads to better tissue penetration.

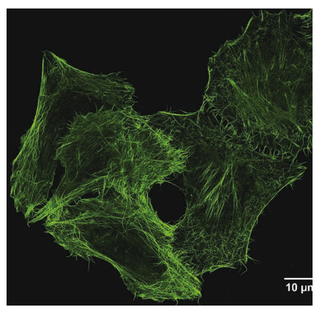
→ Spot-Label is the first detection tool directed against a small peptide tag that is applicable to super resolution microscopy studies (STED and also STORM[3]) owing to minimal label displacement.



Spot-Label ATTO594 confocal microscopy imaging of Tom70-GFP-Spot in HeLa cells.

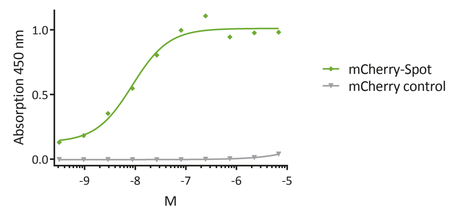


Spot-Label ATTO594 STED super resolution imaging of Vimentin-Spot in HeLa cells.



Spot-Label ATTO594 STED super resolution imaging of Actin-Chromobody-Spot in HeLa cells.

ELISA



Coating of mCherry-Spot followed by ELISA analysis using varyiing concentrations of anti-Spot nanobody and anti-llama-HRP.

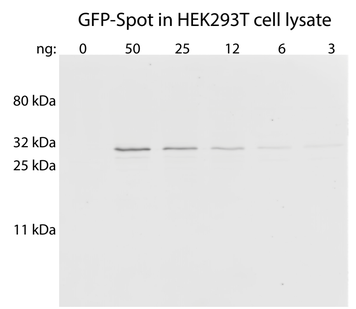
→ Anti-Spot nanobody allows detection and capture of Spot-Tag proteins in ELISA.

Western blot

For Western blot detection of Spot-Tag proteins, monovalent Spot-Label conjugated to a fluorophore or in conjuction with a secondary antibody can be used.

→ Spot-Label ATTO488 allows sensitive one-step Western blot detection with minimal background.

→ Alternatively, anti-Spot nanobody can be detected using anti-llama HPR antibody.



Western blot detection using monovalent Spot-Label ATTO488.

Conclusion

The Spot-Tag system combines the high affinity and specificity of an antibody-epitope tag system with the stability and small size of an alpaca nanobody. This results in a universal tag-system that will simplify the purification and concurrent analysis of target proteins.

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References:
[1] Tränkle, B. et al.: Monitoring interactions and dynamics of endogenous β-catenin with intracellular nanobodies in living cells. Molecular & Cellular Proteomics 14(3), pp. 707-723 (2015).
[2] Braun, M.B. et al.: Peptides in headlock - a novel high-affinity and versatile peptide-binding nanobody for proteomics and microscopy. Scientific reports 6, 19211 (2016).
[3] Virant, D. et al.: A peptide tag-specific nanobody enables high-quality labeling for dSTORM imaging. Nature Communications 9(1) 930 (2018).