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Whitepaper

GFP-Trap

How to plan the (co-) immunoprecipitation of your GFP-fusion protein with the ChromoTek GFP-Trap

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Preamble

The use of ChromoTek GFP-Trap for immunoprecipitation of GFP-fusion proteins is simple and straight forward. In order to obtain the very best pulldown results, when using very challenging experimental conditions like the pulldown of very low expressed GFP-fusion protein or when working at harsh buffer conditions, you may consider the recommendations of this document to fine tune your IP or co-IP experiments.

Content summary

1. Introduction

With more than 1,600 literature references the ChromoTek GFP-Trap is the gold standard for the (co-) immunoprecipitation of GFP-fusion proteins. GFP-Trap is used in hundreds of laboratories worldwide because of its excellent performance.

The affinity ligand of the GFP-Trap is an anti-GFP Nanobody that is derived from a heavy chain antibody from alpaca. The antigen binding domain of a heavy chain antibody is called VHH or Nanobody. The GFP VHH has several outstanding advantages: high affinity of 1 pM, extreme stability even in high concentrations of chaotropic reagents and detergents, and binding to correctly folded GFP (fusion protein) only. Furthermore, the GFP-VHH is thoroughly characterized and highly validated.

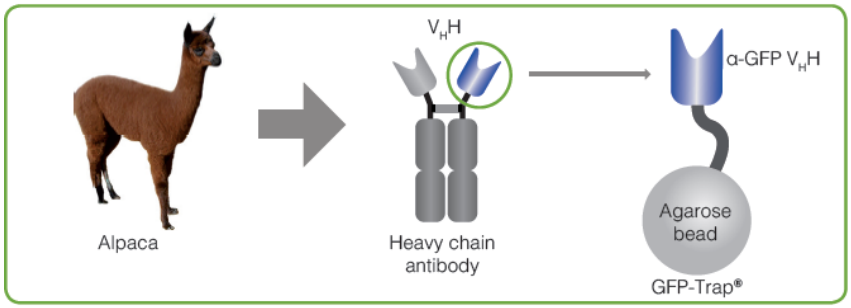

Figure 1: GFP-Trap: anti-GFP-VHH coupled to an agarose, magnetic agarose, bead or Dynabead

2. Planning of the experiment

Here, we discuss certain aspects you may want to consider when planning the (co-) immunoprecipitation of your GFP-fusion protein of interest.

2.1. What type of GFP-Trap beads shall I use?

GFP-Trap is available in 3 kinds of beads: agarose, magnetic agarose, and magnetic particles M-270. What GFP-Trap shall I use?

- GFP-Trap Agarose, when lowest background and high binding capacity IP is needed.
- GFP-Trap Magnetic Agarose, when magnetic separation and high binding capacity IP is needed.
- GFP-Trap Magnetic Particles M-270, when very large proteins/complexes are investigated, and magnetic separation is needed for IP.

Specifications of GFP-Trap Agarose, Magnetic Agarose, and Magnetic Particles M-270:

	GFP-Trap		
	Agarose	Magnetic Agarose	Magnetic Particles M-270
Matrix	Agarose (4% cross-linked)	Magnetic agarose (6% cross linked)	Magnetic Particles M-270
Bead form	Porous	Porous; sold iron core	Solid
Ligand	GFP-VHH	GFP-VHH	GFP-VHH
GFP-tagged protein size*	Small to large size	Small to large size	Small to very large size; no size limitation
Color	White	Black	Brown
Medium particle size	90 µm	40 µm	2.8 µm
Binding capacity	12 µg/ 10 µL	8 µg/ 10 µL	1 µg/ 10 µL
Background	Very low	Low	Low
Magnetic separation & automation	No	Yes	Yes
May be centrifuged up to	2,500 x g	800 x g	8,000 x g

*Does depend on protein size and shape, protein multimers, complexes and interaction partners

2.2. Specificity - What fluorescent proteins are captured by the GFP-Trap

The GFP-Trap specifically binds to most of the common GFP derivatives. Visit www.chromotek.com for a complete list. The ChromoTek GFP-Trap only binds properly folded, i.e. active, GFP variants. Crystal structure data show that this is because the anti-GFP-Nanobody binds to a three-dimensional epitope of GFP. Therefore, the anti-GFP-Nanobody is not suitable for detection of GFP-fusion protein in Western Blots. For Western Blot detection of GFP (-fusion proteins) ChromoTek recommends the traditional monoclonal anti-GFP antibody [3H9] or the polyclonal anti-GFP antibody [PABG1] (see list of products and complementary products at end of document).

2.3. Very high affinity of GFP-Trap – Binding of lowest amounts of GFP

The capture of proteins by Nanobodies and antibodies is a steady state reaction. The amount of bound protein depends on the affinity/dissociation constant of the Nanobody and the protein

concentration. When the protein concentration equals the dissociation constant of the Nanobody, 50% of the cell lysate's protein is captured. At a protein concentration of 10 times the KD 90% and at a protein concentration of 100 times the KD 99% of the protein from the cell lysate is bound.
The 1 pM very high-affinity (low KD) GFP-Trap effectively immunoprecipitates even lowest concentrations of GFP-fusion proteins, possibly at or even below the detection limit of SDS-PAGE and Western blotting.

2.4. What controls should I conduct to validate the experimental data?

Below find some suggested controls for IP and Co-IP applications:

For Immunoprecipitation (IP):

- GFP-Trap for IP of GFP-fusions and a non-relevant Nano-Trap as negative control, e.g. GST-Trap

For Co-Immunoprecipitation (Co-IP) of protein complex AB:

- lysate obtained from untransformed cells
- lysate from cells expressing protein A
- lysate from cells expressing protein B
- lysate from cells expressing both A and B

2.5. What to consider when preparing a cell lysate?

Lysis buffers:

- A non-denaturing lysis buffer is recommended because proteins will remain in their native conformation. This is also important when doing a Co-IP.
- The RIPA (Radio Immunoprecipitation Assay) buffer might denature proteins or disrupt protein complexes.
- Even harsher buffers comprising high concentrations of chaotropic reagents like 8 M urea are recommended to completely inhibit protease activity for the MS analysis of phosphorylation sites.

Inhibitors:

- Add protease inhibitors to prevent proteolysis!
- Preserve posttranslational modifications of your protein and add e.g. phosphatase inhibitors!
- Prevent degradation of your protein by keeping your cell lysates on ice!

2.6. Short incubation time

Since the GFP-Nanobody is covalently coupled to the beads, the GFP-Trap beads are ready-to-use and can be directly added to the prepared lysate. No pre-incubation with an antibody with protein A/G is necessary.
The GFP-fusion proteins bind quickly to GFP-Trap because the GFP-Nanobody has a high binding rate (kon): the binding of GFP-fusions is completed within 5-30 minutes. However, the GFP-VHH's low dissociation rate (koff) allows for prolonged incubation times and if the protocol requires extensive washing steps. Please note that for co-immunoprecipitation also the binding and dissociation rates of the protein complex' partners may have to be considered (if known). Hence, ChromoTek typically recommends a 1-hour incubation time for the immunoprecipitation. An overnight incubation is not recommended, because the risk of protein degradation and non-specific protein binding increase over time.

2.7. Buffer compatibility of the GFP-Trap for binding and washing

The GFP-Trap is compatible with most wash buffers and stable under harsh conditions:

Salt	**Chaotropic reagents**	**Reducing agents**	**Detergents**	**Other**
2 M NaCl	8 M Urea 4 M Guanidinium•HCl	1 mM DTT* 0.2 mM TCEP	1% SDS* 1% Triton™ X-100 2% Nonidet™ P40 Substitute 3% Deoxycholate	30% Glycerol

*GFP-Trap Magnetic Particles M-270: 10 mM DTT; 0.2% SDS

2.8. Elution strategies

Bound GFP fusion proteins cannot be eluted from GFP-Trap by means of competitive elution or chaotropic reagents like 8 M urea, because of the formation of an extraordinary stable complex of the GFP-fusion protein with the GFP-Trap (for further discussion see whitepaper Unique Thermal & Chemical Stability of the ChromoTek GFP-Binding Protein:GFP Complex).

Therefore, harsher elution conditions are needed to disrupt the GFP-Nanobody:GFP complex or alternatively to release the fusion protein by proteolytic cleavage:

- Elution by pH shift (200 mM glycine pH 2.5)
- Elution with SDS-sample buffer (Laemmli) for SDS/PAGE and/or Western blotting
- Elution by enzymatic cleavage using TEV protease

See the Application Note How to elute bound GFP-fusion protein from GFP-Trap for step-by-step description of the above elution options.

NB: Please consider whether you really need to elute the bound protein of interest from the beads rather than conduct the downstream analysis "on-bead":

- Proteins can be digested when still coupled to the beads for subsequent mass spectrometry analysis.
- Enzymatic activity assays can be performed when still coupled to the beads if the active center is not blocked.

Click here for "on-bead digest protocol for mass spectrometry"

2.9. Background resulting from non-specifically bound protein

By nature, every type of bead and every binding molecule may non-specifically bind some proteins. This results in unwanted protein background during the IP:

- Non-specific binding to the beads: Proteins and DNA of the cell lysate can bind non-specifically to the beads. Besides enhanced washing, preclearing the cell extract with plain beads (e.g. binding control beads, see table of products and complementary products at end of document) prior to the actual immunoprecipitation experiment can reduce the background.
  The Magnetic Particles M-270 are very inert and hence show only minor non-specifically binding. Therefore, unconjugated Magnetic Particles M-270 shouldn't be used for preclearing. (To investigate unspecific binding to GFP-Trap Magnetic Particles M-270, we recommend conducting the IP with mock cell lysate without GFP-fusion or with GFP only.)
- Non-specific binding to the GFP Nanobody: The affinity ligand can also non-specifically bind to proteins of the cell lysate. Frequently this is the case if the buffer composition partly denatures cell lysate proteins, which may bind to the GFP Nanobody through exposed hydrophobic patches. Harsher washing conditions (e.g. addition of detergents) and increased washing steps can help to reduce non-specific binding. Alternatively, you may use a Nano-Trap with a different specificity (i.e. Myc-Trap, Spot-Trap, GST-Trap etc.) for preclearing.
- Non-specific binding to the fusion protein: Similar to non-specific binding to the GFP Nanobody, (partly) unfolded proteins can also bind to the GFP-fusion protein. In this case, harsher washing conditions (e.g. addition of detergents) and increased washing steps can help to reduce non-specific binding.

CRAPome:

Background levels by non-specific binding to proteins may not be an issue when evaluating your IP reaction by SDS-PAGE and/or WB. However, for the high sensitivity mass spectrometry

analysis, it is recommended to perform the IP with mock cell lysate without GFP-fusion or with GFP only as appropriate negative control. Furthermore, to better deal with that issue, scientists have established the internet-based database CRAPome at www.crapome.org. This database stores and annotates negative controls generated by the proteomics research community. CRAPome helps to

determine the background contaminants--for example, proteins that interact with the solid-phase support, affinity reagent or epitope tag.

2.10. Reproducibility

The GFP-Trap's GFP-Nanobody is a very stable single polypeptide chain binding protein that is recombinantly expressed virtually without lot-to-lot variations. This recombinantly produced GFP-Nanobody in combination with stringent quality control procedures makes GFP-Trap a robust and reproducible tool for reliable GFP-fusion protein immunoprecipitation results.

3. Further reading

Many researchers have used the GFP-Trap for multiple applications in several high impact publications that ChromoTek collects in a literature database. We are frequently updating our database, currently there are about 1,600 publications for the GFP-Trap (as January 2020). You can search by organism, product and application at www.chromotek.com/references/, to find publications relevant for you.

Also, you will find a section on frequently asked questions at www.chromotek.com/faq/ and a trouble shooting guide here https://www.chromotek.com/fileadmin/content/PDFs/Troubleshooting_guide/Troubleshooting_guide_Nano-Traps.pdf .

4. List of GFP-Trap and complimentary products (for research only)

Product	Product code
GFP-Trap® Agarose (10, 20, and 100 reactions)	gta-10; -20; -100
GFP-Trap® Agarose Kit (20 reactions)	gtak-20
GFP-Trap® Magnetic Agarose (10, 20, and 100 reactions)	gtma-10; -20; -100
GFP-Trap® Magnetic Agarose Kit (20 reactions)	gtmak-20
GFP-Trap® Magnetic Particles M-270 (10, 20, and 100 reactions)	gtd-10; -20; -100
GFP-Trap® Magnetic Particles M-270 Kit (20 reactions)	gtdk-20
iST GFP-Trap Kit for IP/MS (8 reactions)	gtak-iST-8
Binding Control Agarose (20 reactions)	bab-20

Binding Control Magnetic Agarose (20 reactions)	bmab-20
Spin columns (10, 20, and 50 each)	sct-10; sct-20; sct-50
GFP VHH, recombinant binding protein (250 µg)	gt-250
GFP VHH, biotinylated recombinant binding protein (250 µg)	gtb-250
EGFP, recombinant purified protein (250 µg)	EGFP-250
GFP antibody [3H9] (rat monoclonal) (20 and 100 µl)	3h9-20; -100
GFP antibody [PABG1] (rabbit polyclonal) (20 and 100 µl)	PABG1-20; -100
Alpaca anti-rabbit IgG, recombinant VHH, Alexa Fluor® 488 (10 and 100 µl) Alpaca anti-rabbit IgG, recombinant VHH, Alexa Fluor® 568 (10 and 100 µl) Alpaca anti-rabbit IgG, recombinant VHH, Alexa Fluor® 647 (10 and 100 µl)	srbAF488-1-10; -100 srbAF568-1-10; -100 srbAF647-1-10; -100
GFP-Booster Alexa Fluor® 488 (10 and 50 µl) GFP-Booster Alexa Fluor® 568 (10 and 50 µl) GFP-Booster Alexa Fluor® 647 (10 and 50 µl) GFP-Booster ATTO488 (10 and 100 µl) GFP-Booster ATTO594 (10 and 100 µl) GFP-Booster ATTO647N (10 and 100 µl)	gba2AF488-10; -50 gba2AF568-10; -50 gba2AF647-10; -50 gba488-10; -100 gba594-10; -100 gba647n-10; -100

GFP-Trap is for research use only.