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Affinity tools for the immunoprecipitation of GFP-fusion proteins

Preamble

This document compares affinity tools for the immunoprecipitation of GFP-fusion proteins:

- conventional anti-GFP antibodies, and
- GFP-Trap®, a ready-to-use pulldown reagent that comprises an anti-GFP-Nanobody.

What is the GFP-Trap? What are the differences between the ChromoTek GFP-Trap and conventional anti-GFP antibodies? How does the GFP-Trap have a superior performance for the immunoprecipitation of GFP-fusion proteins?

Introduction of GFP as a protein-tag

Green Fluorescent Protein (GFP) and variants are extensively used to study protein localization, interactions, and dynamics in cell biology.

In many cases, data generated from microscopy studies requires complementary assays to obtain a more comprehensive set of information. Additional aspects including posttranslational modifications, DNA binding, enzymatic activity, and protein-protein interactions are investigated: Immunoprecipitation (IP), Co-IP, Co-IP for mass spectrometry (MS) analysis, and chromatin IP (ChIP) are used to complement microscopy measurements.

Researchers now can apply GFP, GFP derivatives, and other fluorescent proteins using the constructs from microscopy analysis as epitope tags for reliable and sensitive biochemistry assays rather than re-cloning the genes coding for their protein of interest into vectors with traditional epitope tags. Furthermore, the performance of GFP-Trap makes GFP a first choice for biochemistry application.

What is the GFP-Trap?

The GFP-Trap is an anti-GFP-Nanobody covalently bound to agarose, magnetic agarose, or Dynabeads™ (Figure 1). It is used to immunoprecipitate GFP-fusion proteins from cell extracts of various organisms like mammals, plants, bacteria, fungi, insects, etc.

What is a Nanobody?

The antigen binding domain of heavy chain only IgG antibodies from alpaca and other camelids is called Nanobody, V_HH or single domain antibody (Figure 1, left: blue domain; green circle). ChromoTek's anti-GFP-Nanobody is a single peptide chain protein of 13.9 kDa size that binds to GFP with high affinity and low background.

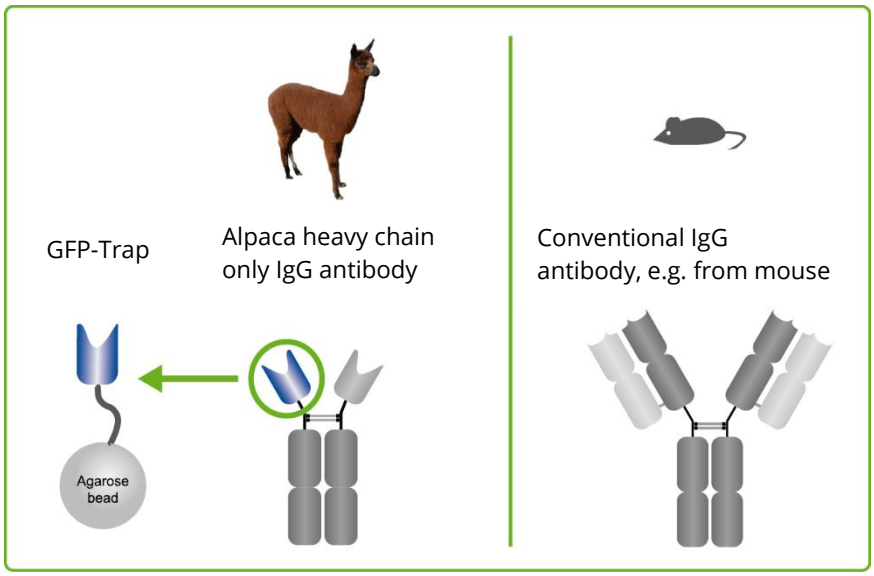

Figure 1: What is a Nanobody? Next to conventional IgG antibodies, which are composed of 2 heavy chains and 2 light chains (right), the immune repertoire of alpacas contains so-called heavy chain only IgGs (left). These antibodies lack the C_H1 domain of the heavy chain and are devoid of any light chain. The antigen binding domain of a heavy chain only IgG is called V_HH, Nanobody or single domain antibody (blue; green circle). Conjugated to either agarose, magnetic agarose or Dynabeads™, the resulting affinity reagent is called ChromoTek GFP-Trap.

Conventional IgG antibodies (Figure 1, right) are also powerful affinity tools. However, the large and complex structure of two heavy and two light chains can be troublesome in certain applications as discussed in this whitepaper.

What are the experimental steps for pulldown of GFP-fusion proteins?

The IP protocols using either the GFP-Trap or conventional anti-GFP-antibodies are shown in Figure 2. In general, both workflows have (1) cell lysis, (2) binding of GFP-fusion proteins to the affinity resin, and (3) washing of non-bound and unspecific proteins in common.

Note, that the conventional anti-GFP-antibody has to be coupled to Protein A/G beads for IP, which requires additional experimental steps, processing and incubation times. In contrast, the GFP-Trap is a ready-to-use reagent and requires less handling time.

For all affinity tools, **cell lysis** buffer and conditions need to be optimized with regards to cell type, cell compartment in which the GFP-fusion protein is expressed, protein type, i.e. soluble or membrane protein, and protein stability.

The **immunoprecipitation** step, which is the the binding of the GFP-tagged protein to the GFP-affinity beads, shall be conducted at 4 °C in the presence of protein inhibitors and end-over-end rotation. Longer incubation times like overnight are generally not recommended because the protein sample may degrade, fragile protein complexes may dissociate, and proteins of the cell lysate can bind unspecifically to the bead matrix.

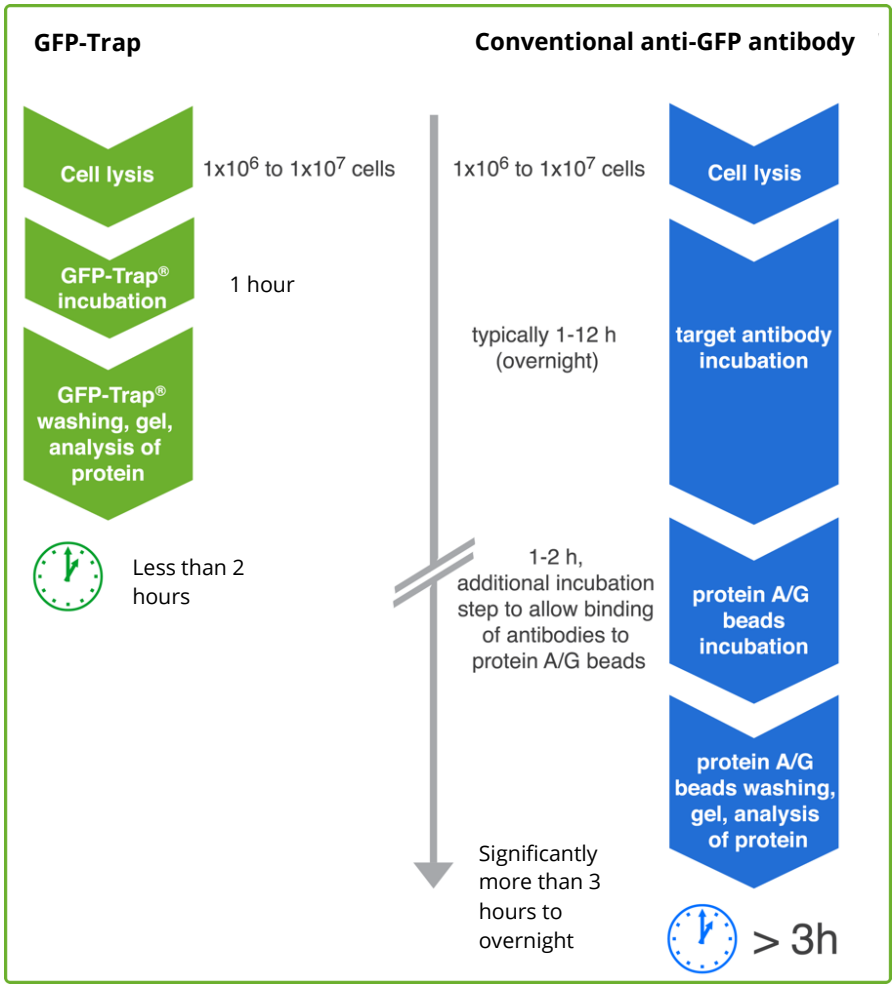

Figure 2: Immunoprecipitation workflows of GFP-fusion protein using GFP-Trap (left) and conventional anti-GFP IgG antibodies (right).
The high binding rate of the ChromoTek GFP-Trap enables a considerable shorter processing time than conventional anti-GFP-antibodies. After washing, the immunocprecipitated protein fraction may be subject to Western blotting, mass spectrometry analysis, ChiP, iCLIP, RIP or enzyme activity assays.

Because the GFP-Trap has a fast binding rate, the IP is typically accomplished after a one-hour incubation time. Our experiments indicate that the pulldown of the GFP-fusion protein is nearly completed after 10 minutes. As the GFP-Trap has an impressively high affinity of 1 pM (picomolar), very low abundant proteins like GFP-fusion proteins expressed at endogenous levels, which have been introduced using CRISPR/Cas or similar methods, can also be effectively immunoprecipitated.

Alternatively, with conventional antibody-Protein A/G tools, it is common to first incubate the conventional anti-GFP-antibody in the cell lysis buffer for several hours up to overnight to form the complex of GFP-fusion protein and anti-GFP-antibody. Next, for binding of the protein-antibody complex to Protein A/G beads, additional incubation and handling are required, which prolongs the total process time further.

Washing steps shall effectively remove unbound protein and minimize background by adjusting the stringency of wash buffer composition. Because of the extraordinary stability of the GFP-Trap itself and GFP-fusion protein:GFP-Trap complex, harsh washing buffers can be applied that may include detergents, high salt, glycerin, denaturating and reducing reagents. Conventional anti-GFP-antibodies may be sensitive to these conditions as the IgG may disassemble into heavy and light chains,so less stringent conditions must be applied, which may increase unspecific binding.

In summary, the GFP-Trap protocol for the IP of GFP-fusion proteins requires less time and experimental steps, treats the immunoprecipitated protein of interest in a more gently manner, and is more versatile than the protocol using a conventional anti-GFP-antibody.

Performance comparison of immunoprecipitation

The pulldown results of GFP-Trap and conventional anti-GFP-antibodies have been compared using HeLa cells that express GFP (Figure 3). For analysis, Input (I), non-bound (FT) and bound, i.e. immunoprecipitated (B) fractions were separated by SDS-PAGE followed by Coomassie staining and Western blotting. GFP has been pulled down using both affinity resins, but there are considerable differences.

Whereas the bound fraction (B) of the GFP-Trap shows only a GFP band at about 29 kDa: "single band purification", the bound fraction of the conventional anti-GFP-antibody contains two prominent bands in addition to the GFP band. These extra bands of approximately 50 kDa and 25 kDa are heavy and light chains from the conventional anti-GFP-antibody. The contaminating bands may be a serious problem if your GFP-fusion protein of interest has a similar size. When polyclonal anti-GFP-antibodies are used, instead of distinct bands broad smears may be detected.

Furthermore, there is less background in the bound fraction of the GFP-Trap than in the fraction of the anti-GFP-antibody, where multiple bands can be detected in the SDS-PAGE. This indicates the superior binding specificity of the GFP-Trap and its advanced coupling. In addition, the background of GFP-Trap can be minimized by using more stringent wash buffer conditions when required by the experiment.

The IP effectiveness has been determined by Western blots: The non-bound fraction (FT) of the GFP-Trap does not show any GFP indicating that all protein (to the detection level) has been effectively immunoprecipitated, because of the high affinity and binding capacity of the GFP-Trap.

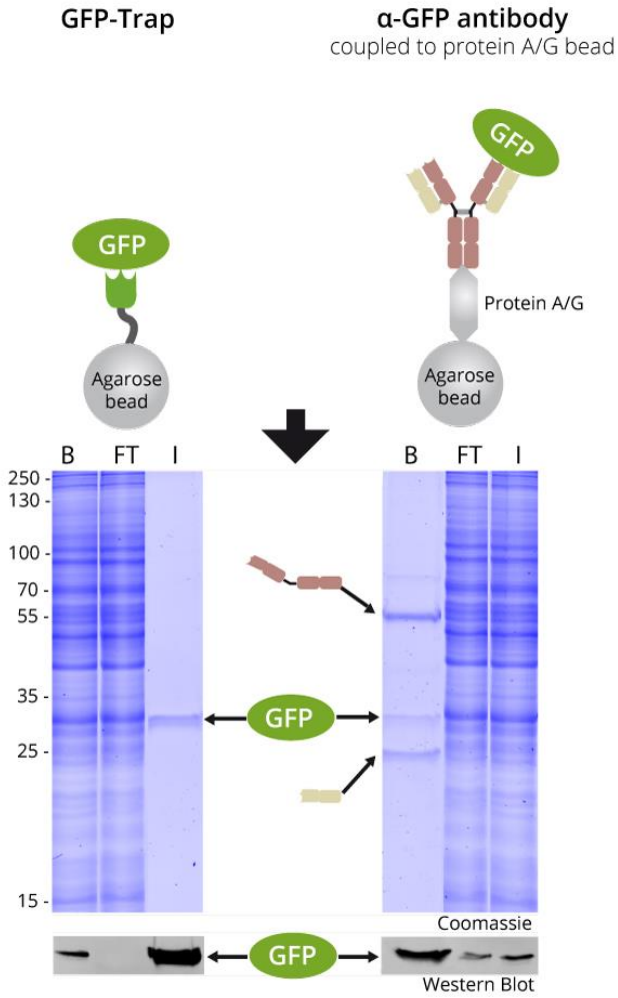

Figure 3: SDS-PAGE and Western blotting analysis of immunoprecipitation of GFP from cell extracts. Protein extracts of GFP-expressing HEK 293T cells were subjected to IP with GFP-Trap or monoclonal anti-GFP antibody. Input (I), flow-through (FT), and bound fractions (B) were separated by SDS-PAGE and stained with Coomassie Blue staining (top) or by immunoblotting (bottom). The bands of precipitated GFP (green), denatured heavy chain (brown) and light chain (beige) of the IgG are marked by arrows. The bound fraction of the GFP-Trap shows a GFP band only whereas the anti-GFP-antibody beads show heavy and light antibody chains plus additional background bands. Note effective binding of GFP-fusion proteins: No GFP-fusions protein bands are left in non-bound (FT) lane in Western Blot.

Very high binding affinity of GFP-Trap

Binding of a target protein to an antibody/Nanobody is a steady state reaction, i.e. there is simultaneous binding and dissociation. The dissociation rate divided by the binding rate is called dissociation constant K_D or affinity (= reciprocal K_D).

The GFP-Trap has a very low dissociation rate; only very small amounts of GFP dissociates even during lengthy washing. The binding rate of GFP-Trap however is very high. The combination of low disscociation and high binding rates results to a very low K_D respectively high affinity. GFP-Trap’s affinity has been measured as 1 pM (Figure 4).

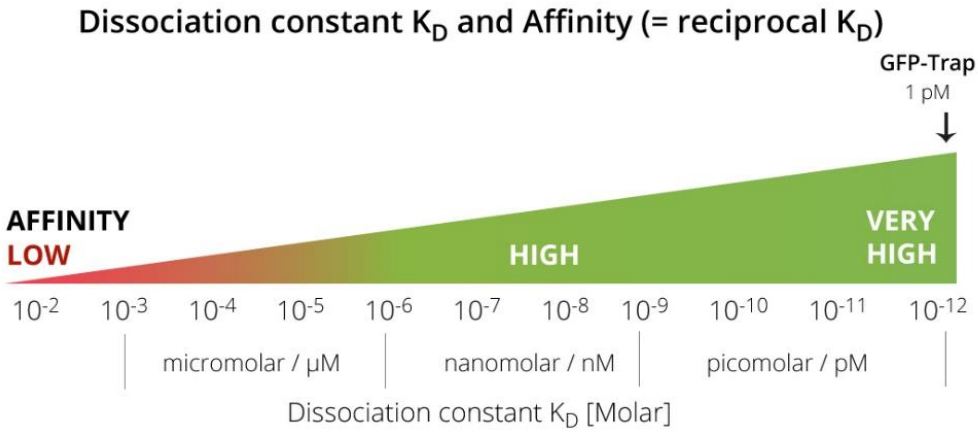

Figure 4: Affinities of antibodies/Nanobodies

The practical implications of a 1 pM very high affinity for the IP of GFP-fusion proteins are: (1) short incubation time for pulldown, (2) GFP-Trap effectively binds very low GFP-fusion protein concentrations in your sample, and (3) GFP-Trap will effectively bind all GFP-fusion protein (to the detection level).

Other GFP pulldown reagents may have lower affinities if these have been measured and characterized to that level at all.

Validation of GFP affinity tools for immunoprecipitation

The validation and reliable production of antibodies are intensively discussed (Bradbury and Plückthun 2015; Baker 2015). This debate complains about insufficient antibody quality and, consequently, wasted research time and funds and seeks options to resolve these issues by standardizing antibodies and their production.

Based on the discussion in the literature, ChromoTek has utilized genetic strategies and comparison with independent anti-GFP-antibodies for the validation of the monoclonal GFP-Nanobody that is used in the GFP-Trap. The GFP-Nanobody is sequenced and its crystal structure has been determined (Figure 5).

Furthermore, the GFP-Trap has been thoroughly characterized: affinity, melting point, aggregation, GFP-binding specificity and chemical & thermal stability have been determined.

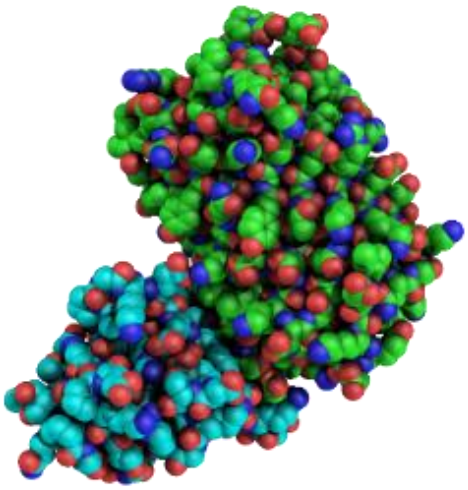

Figure 5: Crystal structure of the ChromoTek GFP-Nanobody (blue) binding to GFP (green): GFP-Nanobody-GFP complex. Note the large surface area of interaction that causes the extraordinary strong binding.

The GFP-Nanobody is recombinantly produced. In combination with ChromoTek’s high QC standards the GFP-Trap is a reliable affinity tool virtually without lot-to-lot variations.

High characterization and validation levels are very important for ChromoTek. These thorough characterization and validation levels may not be applied for other GFP affinity beads.

References of GFP immunoprecipitation tools

The GFP-Trap has been referenced in more than 1,600 scientific publications so far. Therefore GFP-Trap is the gold-standard for the immunoprecipitation of GFP-fusion proteins.

You may want to search our literature database at https://www.chromotek.com/references/.

Discussion and summary

One challenge of the postgenomic era is the seamless integration of genetic, biochemical, and cell biological data. Here, we have discussed how GFP can be efficiently used as an affinity tag for biochemical assays:

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Whitepaper "Affinity tools for the immunoprecipitation of GFP-fusion proteins"

GFP-Trap	Anti-GFP-antibody
⊕ Ready-to-use	⊖ Additional steps for binding to Protein A/G beads needed
⊕ Selective IP and Co-IP with low background and without heavy & light antibody chain contaminations	⊖ Contaminating heavy & light antibody chains and additional background may compromise downstream applications
⊕ Very high binding affinity of 1 pM	⊖ Low to moderate binding affinities
⊕ Harsh wash conditions may be applied when required by protocol	⊖ Limited buffer/reagent compatibility of IgG
⊕ Short incubation time of 1 hour	⊖ Long incubation times may compromise Co-IPs of fragile protein complexes and increase unspecific background
⊖ GFP-Nanobody can't be used for Western blotting	⊕ May be used for Western blotting
⊕ Thoroughly validated and characterized	⊖ Typically, lower validation and characterization levels
⊕ High reproducibility due to recombinant expression with very low batch-to-batch variation	⊕ Reproducibility of monoclonal anti-GFP antibodies varies and may be high but not to the level of recombinantly produced antibodies.
	⊖ Reproducibility of polyclonal anti-GFP-antibodies may vary

Request your free GFP-Trap test sample here:

Free sample

Further reading:

How to plan an Immunoprecipitation using the GFP-Trap
Unique Stability of the ChromoTek GFP-Trap:GFP Complex