Immunoprecipitation of proteins at very low expression levels using GST-Trap and MBP-Trap

Abstract

The use of GST- and MBP-fusion proteins in combination with corresponding affinity matrices is a well-established method to analyze protein interactions using co-precipitation assays. However, if the GST- or MBP-fusion protein of interest is expressed at low levels, conventional affinity matrices may fail to precipitate the target protein.
Here we describe two innovative tools, the ChromoTek GST-Trap and the ChromoTek MBP-Trap. These two affinity matrices exploit the exceptional high affinity of specific camelid single-domain antibodies (also called "nanobodies" or V_HHs) to GST and MBP. Both allow the efficient immunoprecipitation of GST- and MBP-fusion proteins even at very low expression levels (Table 1).

Protein tag	Affinity matrix	K_D	Fraction of bound protein at given protein concentration		
			1 nM	10 nM	50 nM
MBP	ChromoTek MBP-Trap	4 nM	20 %	71 %	93 %
	Amylose resin	150 nM	<1 %	6 %	25 %
GST	ChromoTek GST-Trap	1 nM	50 %	91 %	98 %
	Glutathione cellulose	50 nM	2 %	17 %	50 %

Table 1: The fraction of MBP- or GST-fusion protein captured by an affinity matrix is dependent on protein concentration and on the dissociation constant K_D of the matrix-protein interaction.
Fraction of bound protein is defined as the fraction of the total protein added to the affinity matrix, which is actually bound by the affinity matrix. For more details, see chapter "The impact of affinity on pull-down experiments and immunoprecipitation: a biophysical background".

We present a theoretical framework explaining the superior performance of the GST- and MBP-Trap. Particularly, we demonstrate why the low dissociation constants (K_D) of the GST- and MBP-Trap translate to the efficient immunoprecipitation of proteins expressed at low levels or from dilute solutions. **Finally, we will consider practical implications of the low dissociation constant of GST- and MBP-Trap and derive recommendations for the planning of a pull-down experiment:**

1. **Concentration matters:**
   If the GST- or MBP-fusion protein of interest is difficult to express or expressed at natural levels, you should consider using a high-affinity matrix such as the ChromoTek MBP-Trap or GST-Trap high affinity means low KD value (table 1).
2. **Volume matters:**
   Use a sample volume as small as practically possible. If the protocol requires the use of a substantial lysis volume, apply a high-affinity (low K_D) matrix such as ChromoTek MBP-Trap or GST-Trap.
3. **Time matters:**
   Avoid lengthy washing. However, if required by the protocol use an affinity matrix that has a slow dissociation rate (low k_{off}) such as the ChromoTek MBP-Trap or GST-Trap.

Introduction

Pull-down assays and protein purification in general often make use of the fusion of protein tags to the protein of interest. Two prominent protein tags are glutathione S-transferase (GST) from *Schistosoma japonicum* and maltose-binding protein (MBP) from *Escherichia coli*. Commonly, GST- and MBP-fusion proteins are pulled-down using small molecules, glutathione for GST and amylose for MBP, immobilized on a matrix such as agarose. These conventional affinity matrices will easily enrich any fusion protein that is expressed at high levels. However, owing to the suboptimal affinity of GST and MBP to glutathione and amylose matrices, this binding efficiency is rapidly lost with decreasing concentrations of fusion protein.
For fusion proteins expressed at low levels, we suggest to apply the high-affinity matrices ChromoTek GST-Trap and ChromoTek MBP-Trap. Both GST-Trap and MBP-Trap are based on immobilized highly affine and specific camelid single-domain antibodies (V_HHs, also called "nanobodies").
The difference in affinity between ChromoTek GST-Trap or MBP-Trap and conventional affinity matrices, as expressed through the dissociation constant K_D, is in the range of orders of magnitude. This diverging affinity directly impacts on an experiment's set-up and outcome, as will be shown in the following exemplary experiments and a theoretical discussion thereof.

A titration pull-down experiment of GST and MBP fusion proteins highlights the importance of affinity for immunoprecipitation

To illustrate the effect of affinity on how ChromoTek GST-Trap and MBP-Trap perform in comparison with conventional affinity matrices, we set up pull-down experiments with varying concentrations of fusion proteins. Our aim was to mimic real-life experimental conditions, but also to control the concentration of the fusion protein of interest. To this end, we added defined amounts of purified fusion-protein (MBP-GFP or GST-β-catenin) to lysate of HEK cells and performed pull-down experiments followed by Western blot according to standard protocols (Figure 1).
Both ChromoTek GST-Trap and MBP-Trap efficiently precipitated the target fusion proteins even at the lowest test concentration of 1 nM (Figure 1). They also comprehensively depleted the lysate of fusion protein at concentrations of 10 and 50 nM. In contrast, both conventional affinity resins, glutathione cellulose and amylose resin, depleted only a fraction of the fusion proteins at concentrations of 10 and 50 nM. The conventional affinity resins also failed completely to precipitate detectable amounts of the target proteins at a concentration of 1 nM (Figure 1). Thus, ChromoTek GST-Trap and MBP-Trap were significantly more efficient at the precipitation of low-concentration fusion proteins than glutathione cellulose and amylose resin.

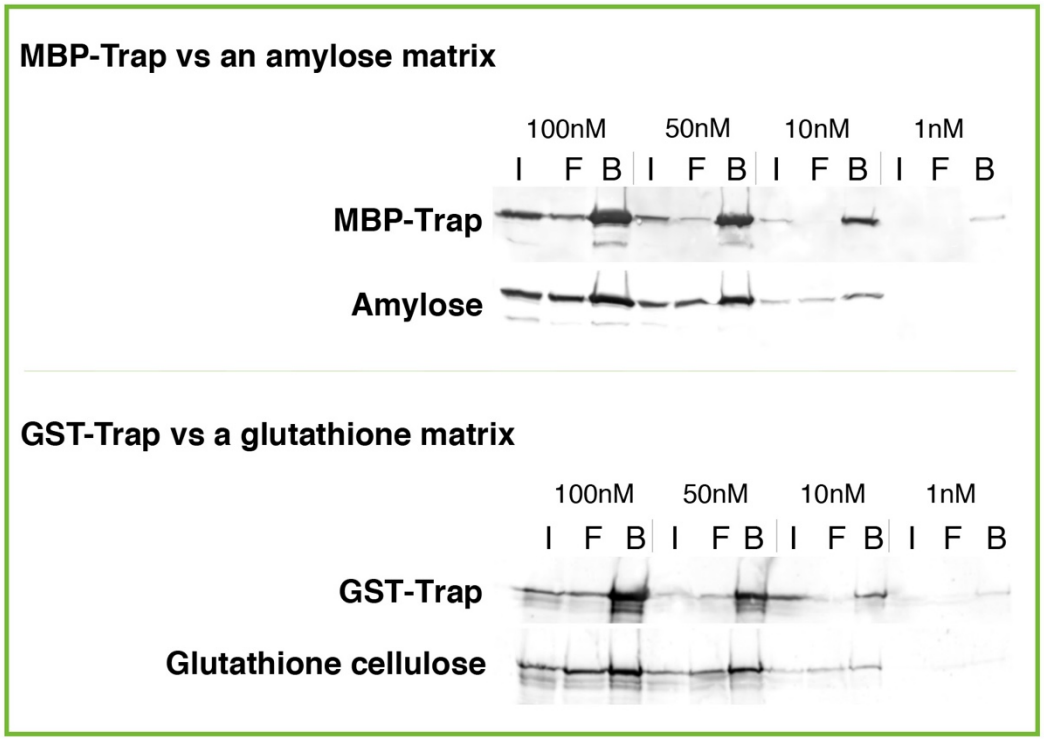

Figure 1. Comparison of the Nano-Traps vs conventional affinity matrices: MBP-Trap vs an amylose matrix (top) and GST-Trap vs glutathione matrix (bottom): *HEK cell lysate was spiked with either purified His-MBP-TEV-Xa-GFP (top) or GST-β-catenin (bottom) at given concentrations and incubated with Nano-Traps or conventional affinity matrices (20 µl slurry each). Protein was detected using Western blot with anti-GFP antibody 3H9 (ChromoTek) respectively anti-GST antibody 6G9 (ChromoTek). The stated concentrations of the input correspond to 70 ng MBP-GFP/ 114 ng GST-β-catenin (100 nM), 35 ng/ 57 ng (50 nM), 7 ng/ 12 ng (10 nM), and 0.5 ng/ 1 ng (1 nM) MBP-GFP respectively GST-β-catenin fusion protein in a volume of 500µl each. HEK cell lysate was used to mimic "real" experimental conditions. I: Input; F: Flow-Through; B: Bound (beads or resin)*

The selected concentration range of 1-100 nM is well within the range of many proteins in a cell. For example, the concentration of β-catenin in Xenopus eggs was determined to be 35 nM (REF: Lee, PLoS Biology 2003). In our experiments, the lower limit of the test range of 1 nM corresponds to 1 ng/ml GST-β-catenin or 0.5 ng/ml MBP-GFP. Using a reaction volume of 500 µl, this results in a total amount of fusion protein of 0.5 ng GST-β-catenin or 0.25 ng MBP-GFP (Figure 1). Thus, even complete precipitation of the tested fusion proteins would lead to a signal that is well below the detection limit of Coomassie Blue staining. Moreover, although the tested protein concentration range mirrors natural and thus relevant experimental conditions, conventional affinity matrices lack efficiency in this range, and the ChromoTek GST-Trap and MBP-Trap are better suited tools.

The impact of affinity on pull-down experiments and immunoprecipitation: a biophysical background

Distinct biophysical properties, namely binding kinetics, explain the difference in performance between the ChromoTek GST-Trap and MBP-Trap and conventional affinity matrices such as glutathione cellulose or amylose resin. In order to precipitation of a fusion protein to occur, it

has to bind the respective affinity matrix. On a molecular level, this binding means the formation of a complex. In the case of ChromoTek GST-Trap and MBP-Trap, the complex consists of the protein tag (GST, MBP) and an immobilized single-domain antibody; in the case of conventional affinity matrices, GST or MBP form a complex with glutathione or amylose, respectively. The formation of a complex P-L between a fusion protein P and an affinity matrix ligand L (antibody or small molecule) can be formally described as follows:

$$P + L \rightleftarrows P\text{-}L$$

The formation of a non-covalent complex is a reversible process (hence the two arrows going in both directions). In a solution with constant concentrations, both association and dissociation of the complex can be observed. The rate of association and the rate of dissociation are called k_{on} and k_{off}, respectively, and are specific to the respective complex:

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} P\text{-}L$$

Importantly, this process of association and dissociation of the complex is a product of both the rates k_{on} and k_{off}, but also of the concentrations of P and L. At equilibrium, the concentrations [P-L], [P] and [L] remain constant (but association and dissociation still occur!), and are in the following relationship with the rates k_{on} and k_{off}:

$$[P] \cdot [L] \cdot k_{on} = [P\text{-}L] \cdot k_{off}$$

This equation may be rearranged thus:

$$k_{off} / k_{on} = [P] \cdot [L] / [P\text{-}L]$$

The ratio of the rates k_{off} and k_{on} defines the equilibrium dissociation constant K_D:

$$K_D = k_{off} / k_{on} = [P] \cdot [L] / [P\text{-}L]$$

The dissociation constant K_D is a commonly used measure for the affinity of a binder such as a single-domain antibody. Conveniently, the unit of K_D is the same as for concentrations, M (molar).

Please note, however, that the dissociation constant K_D is a ratio. Thus, two binders may have the same K_D, but one may both have high association and dissocation rates k_{on} and k_{off}, whereas the other may have both a low k_{on} and k_{off} rate.

As implicated in the equation $K_D = k_{off} / k_{on} = [P] \cdot [L] / [P\text{-}L]$, the dissociation constant K_D is also defined by the ratio of the concentrations of free P and L and of the complex P-L. This relationship emphasizes the importance of protein/ligand concentrations for complex formation, and thus also for pull-down experiments.

The equations above may be further developed into a good measure of the efficiency of a pull-down experiment: the fraction of fusion protein captured by the affinity matrix. The fraction of bound ligand or protein is the ratio of the concentration of protein-ligand complex and the sum of the concentrations of free and complexed protein or ligand. As this is a ratio of concentrations of complexed and free protein or ligand, a substitution of [P-L] with above equations will eventually lead to following simple equation for the fraction of captured protein *Y*:

$$Y = [P]/([P] + K_D)$$

It is no mere coincidence that this equation corresponds to the Langmuir adsorption isotherm, which describes the adsorption of molecules to a surface. It should be noted that protein adsorption to an affinity matrix does not fulfill all the assumptions that are prerequisite to the full applicability of the Langmuir isotherm model, but it is still a useful approximation. Importantly, this equation shows how binding efficiency depends only on the concentration of the fusion protein and the dissociation constant K_D of the binder, e.g. a single-domain antibody.

In practical terms, the Langmuir isotherm approximation may be used to predict the fraction of captured fusion protein. Figure 2 plots such predictions for three different dissociation constants K_D (1 pM, 1 nM, and 1 μM). Please note that the fraction of bound protein is 50 % (Y = 0.5), when the concentration of the fusion protein equals K_D of the affinity matrix. If the fusion protein concentration is a tenth of the K_D, about 9 % will be bound the affinity matrix. And at ten times K_D, 90 % will be bound. 99 % binding will be reached at 100 times K_D.

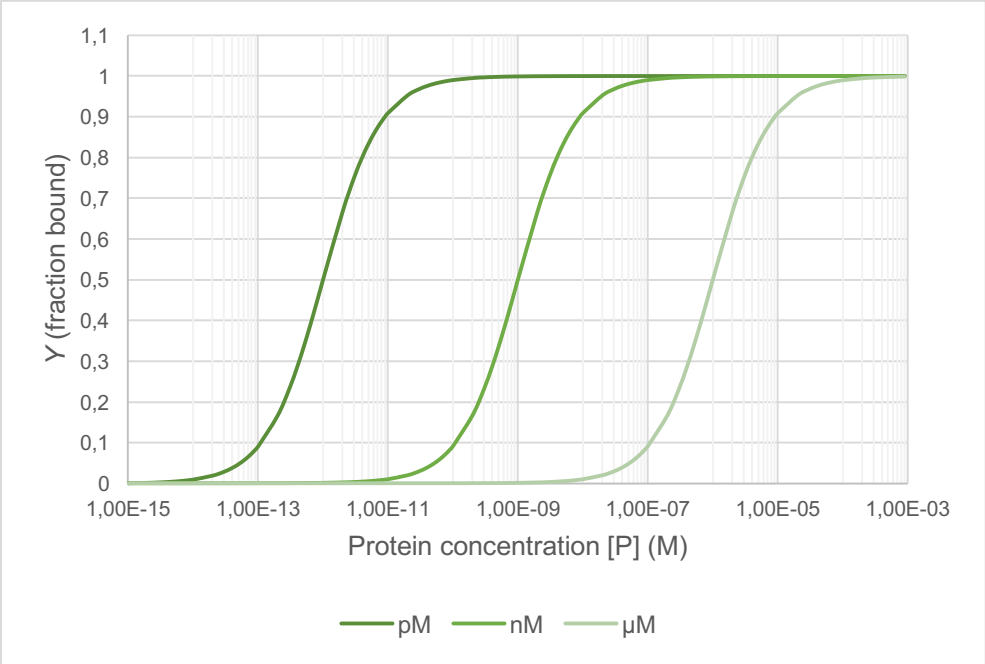

Figure 2. Theoretical binding curves of antigen-antibody complexes: *Calculated binding curves for antibodies with dissociation constants of 1pM (dark green curve), 1nM (light green curve), and 1µM (grey curve) are shown.*

These binding predictions explain our results for the comparison of the ChromoTek GST-Trap and MBP-Trap with conventional affinity matrices. The ChromoTek MBP-Trap used in the experiments above has a K_D of 4 nM. Thus, if the MBP fusion protein concentration is 50 nM,

93 % will be captured by the MBP-Trap. At the concentrations of 10 nM and 1 nM, the capture rate is still 71 % and 20 %, respectively. In contrast, the K_D of the conventional affinity resin amylose resin is 150 nM. Thus, amylose resin will bind 25 % of MBP fusion protein at 50 nM, and only 6 % and less than 1 % at 10 nM and 1 nM, respectively. These calculated values correlate well with the experimental data in Figure 1, taking into account the semi-linear nature of Western blot signals[1]. Thus, the superior performance of the ChromoTek MBP-Trap is a function of its low K_D, i.e. its high affinity to MBP.

The dissociation constant K_D of the ChromoTek GST-Trap is 1 nM. Thus, at fusion protein concentrations of 50, 10, and 1 nM, the fraction of captured protein is predicted to be 98, 91, and 50 %, which correlates again with our experimental results shown in Figure 1 and Table 2. The literature seems to offer widely diverging values for the K_D of the GST-glutathione complex ranging from 50 nM to 2 mM. The lowest value, 50 nM would agree with our pull-down experiment that suggests a K_D at least one order of magnitude higher than that of the ChromoTek GST-Trap, as illustrated by the low binding efficiency of glutathione cellulose at 1-50 nM GST fusion protein. In contrast, the low K_D of the ChromoTek GST-Trap leads to high efficiency at low concentrations.

Compared with GST-cellulose, a 10 times faster k_{on} rate and a 10 times smaller k_{off} rate have been observed for the ChromoTek GST-Trap. This indicates a faster association, although relatively slow, and a slower dissociation of GST. Hence, the resulting low K_D of the ChromoTek GST-Trap results in a high efficiency at low concentrations.

The ChromoTek MBP-Trap and amylose resin differ not only in their K_D, but also strongly in their rates of association and dissociation (k_{on}/k_{off}). The ChromoTek MBP-Trap has a relatively slow association rate k_{on} of $3.2 \cdot 10^4$ M^{-1} s^{-1}, but also a slow dissociation rate k_{off} of $1.2 \cdot 10^{-4}$ s^{-1}. In contrast, the k_{on} of amylose is fast with $2.5 \cdot 10^7$ M^{-1} s^{-1}, but k_{off} is extremely fast at 8.4 s^{-1}.

Affinity matrix	K_D	k_{on}	k_{off}
ChromoTek MBP-Trap	4 nM	$3.2 \cdot 10^4$ M^{-1} s^{-1}	$1.2 \cdot 10^{-4}$ s^{-1}
Amylose resin	150 nM	$2.5 \cdot 10^7$ M^{-1} s^{-1}	8.4 s^{-1}
ChromoTek GST-Trap	1 nM	$2.1 \cdot 10^4$ M^{-1} s^{-1}	$2.9 \cdot 10^{-5}$ s^{-1}
Glutathione cellulose	50 nM	$2.6 \cdot 10^3$ M^{-1} s^{-1}	$1.3 \cdot 10^{-4}$ s^{-1}

Table 2: Kinetic binding parameters of MBP- and GST-tagged fusion proteins to various affinity media. *Generally, higher* K_D *values have been reported for the GST-glutathione interaction. Kinetic parameters of the MBP- and GST-Trap have been measured using Dynamic Biosensors' switchSENSE® technology[2]. For details see text.*

This discrepancy in k_{off} is highly relevant for two steps during pull-down experiments, washing and elution. After an affinity matrix has been incubated with a solution of a fusion protein (commonly a cell lysate), binding is at an equilibrium. Once the solution of fusion protein has been removed, i.e. its concentration is 0, the complex between the affinity matrix and the fusion protein will dissociate. In the case of the ChromoTek MBP-Trap, the low k_{off} translates to very

[1] NB: The binding of an antibody to an antigen in a Western blot experiment is of course also governed by K_D and concentrations!
[2] SwitchSENSE® is a proprietary technology from Dynamic Biosensors to analyze molecular interactions using electro-switchable nanolevers. See www.dynamic-biosensors.com for details.

slow dissociation; 50 % of protein will remain in complex with the MBP-Trap single-domain antibody even after 2 h of washing. In strong contrast, the MBP-amylose complex will dissociate almost immediately – it will take less than a second to remove 50 % of bound MBP from amylose resin. The only reason that amylose resin is suitable for pull-downs and purification at all is the fast association rate, which means that dissociated MBP will bind back to amylose very fast. Nonetheless, captured MBP is leached from amylose resin during washing, whereas the ChromoTek MBP-Trap will retain most of the bound protein. Conversely, the high k_{off} of amylose resin is an advantage for elution, where high concentrations of free maltose can readily compete with amylose and thus elute MBP-fusion proteins. Elution from ChromoTek MBP-Trap requires either denaturing conditions or the use of a specific protease such as thrombin or TEV protease, which remove the protein of interest from MBP.

Practical implications of binding characteristics of affinity matrices

Knowledge of the biophysical characteristics of an affinity matrix, i.e. its dissociation constant K_D or its association/dissociation rates k_{on}/k_{off}, may support the design of an experiment. In the following, we would like to distill some of the theoretical arguments discussed above into practical advice for a pull-down or immunoprecipitation experiment.

Concentration matters. If your fusion protein of interest is highly over-expressed, a conventional affinity matrix will suffice. If, however, you are interested in a protein that is difficult to express or expressed at natural levels, you should consider using a high-affinity, low K_D affinity matrix such as ChromoTek MBP-Trap or GST-Trap.

Volume matters. Concentration is of course a function of volume. Using a sample volume as small as practically possible may increase the efficiency of your pull-down by keeping protein concentrations high (above K_D). ChromoTek recommends to perform immunoprecipitation experiments in a volume of 0.5-1 ml for 10^6-10^7 cells in the case of HeLa or HEK cells. If, owing to the nature of the sample to analyze, a protocol requires the use of a substantial lysis volume, leading to high sample dilution and thus low protein concentration, a high-affinity (low K_D) matrix such as ChromoTek MBP-Trap or GST-Trap may be the affinity matrix of choice.

Time matters. An experiment may demand extensive and lengthy washing or otherwise require a fusion protein to remain bound to the affinity matrix for an extended duration of time. In such a case, conventional affinity matrices often suffer from substantial leaching of bound protein owing to their high dissociation rate k_{off}. Consequently, you will benefit from using an affinity matrix that has a slow (low) k_{off} such as the ChromoTek MBP-Trap or GST-Trap.

Nano-Trap Technology: GST- and MBP-Trap

Conventional antibodies are powerful tools in life science research. However, their large and complex structure -two heavy and two light chains- can be troublesome in certain applications. *Camelidae* (alpacas, llamas, camels and dromedaries) possess a second type of antibody called heavy chain antibodies (hcAbs). HcAbs are devoid of light chains and bind their antigen via a single variable domain (V_HH), also known as a “nanobody”, see figure 4. These V_HH domains have excellent binding properties and can be produced at constant high quality without batch-to-batch variations. Coupled to an immobilizing matrix like agarose beads, V_HHs are superior tools for immunoprecipitations.

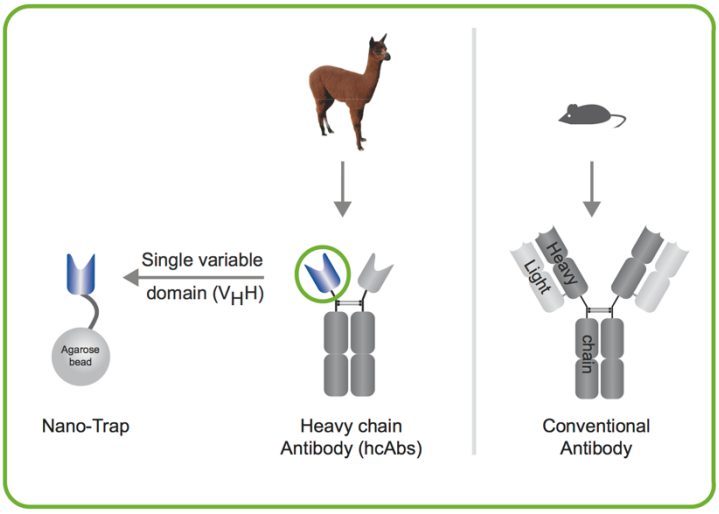

Figure 4: Nano-Traps GST- or MBP-Trap: anti-GST or anti-MBP VHH coupled to agarose & magnetic agarose beads *The ChromoTek Nano-Traps GST- and MBP-Traps consist of an anti-GST or anti-MBP V_HH coupled to agarose beads. Unlike traditional antibodies the Nano-Traps GST- and MBP-Traps' V_HH don't contain heavy and light chains that may interfere with downstream applications.*

Further reading:

We collect publications that our customers have published successfully using the ChromoTek Nano-Traps in a web-based database. We are frequently updating this database. You can filter your selection by organism, and type of experiment at our website www.chromotek.com/references, to find relevant publications relevant for your studies. You can also find a section on frequently asked questions at www.chromotek.com.