How to plan an immunoprecipitation of your Myc-tagged protein using the ChromoTek Myc-Trap

This document offers tips for planning an immunoprecipitation experiment of a Myc-tagged protein of interest using the ChromoTek Myc-Trap®.

Content

ChromoTek GmbH pioneers a new class of immunologic research tools derived from single domain camel antibodies for cell biology and proteomics. ChromoTek is the inventor of GFP-Trap®, the gold standard for immunoprecipitation of GFP-fusion proteins.

How to plan an IP of your Myc-tagged protein when using the ChromoTek Myc-Trap®

Introduction

ChromoTek Myc-Trap is an affinity resin that binds the Myc-tag EQKLISEEDL derived from c-Myc. Myc-Trap consists of a Myc-binding protein derived from an Alpaca single domain antibody, also called V_HH or nanobody (figure 1). The V_HH is coupled to a matrix like agarose beads. The anti-Myc-tag V_HH has particular properties and provides some advantages over conventional IgG antibodies when applied in immunoprecipitation (IP) experiments:
Conventional antibodies comprise two heavy and light chains each, which can contaminate the pulled down protein of interest. This may be a serious problem if you want to detect proteins of similar size. The anti-Myc V_HH used in Myc-Trap is the binding domain antibody of a single domain and therefore results in single band purification.

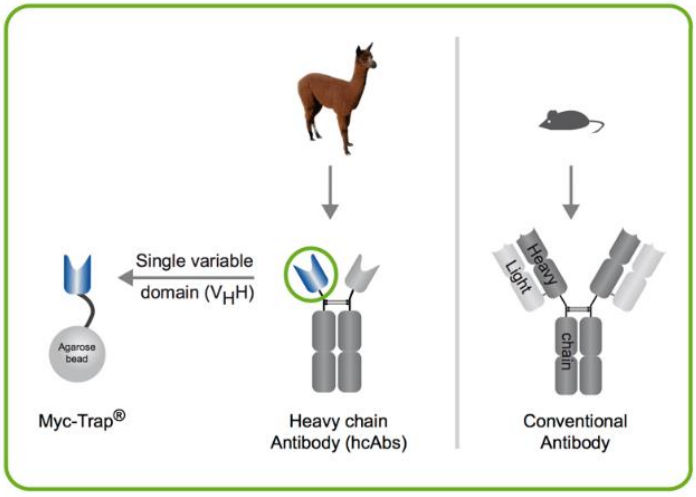

Figure 1: The ChromoTek Myc-Trap consists of an anti-Myc V_HH coupled to agarose or magnetic agarose beads. Unlike traditional antibodies the Myc-Trap's V_HH does not contain uncoupled heavy and light chains that may interfere with downstream application

Planning of the experiment

There are some experimental aspects that you should consider when planning immuno-precipitation of your Myc-fusion protein using the ChromoTek Myc-Trap. Below, we guide you step by step through the experimental workflow.

Tag selection

The Myc-tag is a very popular peptide tag. However, there is a difference whether you use the Myc-tag 1x (EQKLISEEDL) or 2x (EQKLISEEDL*EQKLISEEDL*) fused to your protein of interest:

1xMyc-tagged protein:

- Suitable for all kind of IPs and Co-IPs
- Affinity purification
- Ideal if effective and/or gentle elution of native protein required

2xMyc-tagged protein:

- Suitable for all kind of IPs and Co-IPs
- Affinity purification
- Ideal for low expressed/abundant proteins
- Extraordinary binding affinity
- Favors downstream on-bead processing

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Matrix-Selection

To select the appropriate matrix for protein immunoprecipitation, just consider your experimental convenience needs. You will need to make this decision based on your individual preferences.

There are two matrices for Myc-Trap available:

Feature	Agarose beads	Magnetic agarose beads
Matrix	Agarose, 4% highly cross-linked	Agarose, 6% cross-linked
Particle size	45-165µm	20-40µm
Binding capacity	7µg per 10µl bead slurry	7µg per 10µl bead slurry
Magnetic	no	yes

Table 1: Properties of Myc-Trap matrices: Binding capacity is determined in µg Myc-MBP. We have made the experience that for some samples the Myc-Trap coupled to agarose beads seems to have lower background from unspecific protein binding than the magnetic agarose beads of Myc-Trap. We do not know why as both agarose and magnetic agarose should have the same surface composition.

Background resulting from nonspecifically bound protein

Pre-clearing:
Pre-clearing is an optional step to remove proteins or DNA, which bind non-specifically to the solid-phase (= matrix). This is achieved by incubation of the cell extract with plain beads (e.g. binding control beads, see table of products and complementary products at end of document) before performing the actual immunoprecipitation experiment. After successful removal of these, the immunoprecipitation of the Myc-fusion protein of interest is conducted.

CRAPome:
By nature, every matrix and binding molecule may non-specifically bind some proteins resulting in protein background. 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.

Endogenous c-Myc protein:
The Myc-tag is derived from the c-Myc transcription factor. This protein is engaged in cell cycle progression, apoptosis, and cellular transformation. Thus, one could expect binding of endogenous c-Myc protein. However, we could not detect endogenous c-Myc in neither SDS-PAGE nor Western Blot applications.

Some epitope residues that have shown to be crucial for binding (see below) are actually buried in the three-dimensional structure of the c-Myc protein. Hence, under native conditions, c-Myc protein is not a suitable binding partner for the ChromoTek Myc-trap.

Specificity

There are indications that the Myc-peptide and 2xMyc-peptide do have a 3D structure. This is in line with the general fact discussed in the literature that nanobodies tend to bind three-dimensional epitopes, i.e. 1xMyc-tag or 2x-Myc-tag. This leads to two experimental features:

- Generally, the nanobodies' elongated CDR3 (Complementarity Determining Region 3) allows to reach into clefts of the epitopes, which are not accessible to conventional

antibodies (IgGs). This results in very strong binding and very low dissociation constants (= high binding affinities) of ChromoTek Nano-Traps. The anti-Myc nanobody has a very low dissociation constant K_D of 0.5 nanoMolar, when 2xMyc-tagged protein is bound.
- The anti-Myc nanobody is not suitable for detection of Myc-fusion protein in Western Blots. For Western Blot detection of Myc-tagged proteins, ChromoTek recommends the conventional anti-Myc antibody 9E1 (rat monoclonal antibody, see list of products and complementary products at end of document).

The Myc-Trap recognizes the Myc-tag sequence at the N-terminus, C-terminus, or internal site of the fusion protein.

Controls – What controls should I conduct to validate the experimental data?

Below find some suggestions by application:

For Immunoprecipitation (IP):
- Myc-Trap for IP of Myc-fusions and a non-relevant Nano-Trap as negative control, e.g. GFP-Trap, GST-Trap or MBP-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

Cell Lysis – What to consider when preparing a cell lysate?

Lysis buffers:
- A non-denaturing lysis buffer is recommended for Co-IP, because proteins will remain in their native conformation
- The RIPA (Radio Immunoprecipitation Assay) buffer might denature proteins or disrupt protein complexes

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 samples on ice!

Immunoprecipitation – Binding of Myc- and 2xMyc-fusion proteins

Since the Myc-binding protein is covalently coupled to the beads, the Myc-Trap beads are ready-to-use and can be directly added to the prepared lysate. Because of the fast binding of 1xMyc- and 2xMyc-tagged proteins to the Myc-nanobody (high binding rates k_{on}), the binding of Myc-fusions is completed within 30 minutes (table 2). However, the low dissociation rate (k_{off}) of bound 2xMyc-fusion proteins allows for prolonged incubation and washing, whereas the relative high dissociation rate (k_{off}) of 1xMyc-fusion protein requires quick (short) washing steps.

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2xMyc-tagged proteins are even more tightly bound to Myc-Trap, which manifests in the dissociation constant K_D (table 2). Myc-Trap binds 1xMyc-tagged MBP with a low K_D of 400nM. Although this K_D value already matches general requirements of immunoprecipitation, our additional experiments have shown that 2xMyc-tagged MBP is even considerably stronger bound with a K_D of just 0.5nM. This explains the high pulldown effectiveness of 2xMyc-tagged protein and the gentle and effective elution capability of 1xMyc-tagged protein when using Myc-Trap (see below).

Kinetic parameter	1xMyc-MBP	2xMyc-MBP
Dissociation constant K_D	400nM*	0.5nM**
Binding rates k_{on}	5.3×10^5 M^{-1} s^{-1}	2.9×10^5 M^{-1} s^{-1}
Dissociation rate k_{off}	2.1×10^{-1} s^{-1}	1.4×10^{-4} s^{-1}

Table 2: Kinetic parameters of 1xMyc and 2xMyc-fusion proteins bound to Myc-Trap *Experiments were conducted using Maltose Binding Protein (MBP). *The K_D value for binding 1xMyc-MBP indicates strong binding and is in range what is generally required for IP. It does allow gentle and effective elution. **The K_D value for 2xMyc-MBP is about 1,000 fold smaller, indicating a considerable increase of affinity and binding strength and demonstrating the outstanding IP performance of Myc-Trap. It does enable binding of 2xMyc-tagged proteins at low concentrations and under harsh binding conditions.*

Buffer compatibility of the Myc-Trap for binding and washing

The Myc-Trap is compatible with most wash buffers and stable under relative harsh conditions, particularly when bound to 2xMyc-tagged protein of interest.

Myc-Trap Epitope and Binding Mechanism

The nanobody making up the Myc-Trap was designed to recognise the c-Myc peptide EQKLISEEDL. We have determined the resulting epitope of the Myc-tag-binder using a peptide micro-array and an alanine scan of the c-Myc peptide. The minimal epitope of the ChromoTek Myc-tag-binder is the sequence LISEEDL. Within this motif, the amino acid residues isoleucine (I), aspartic acid (D) and the last leucine (L) are essential for binding.

EQKL**I**SEE**DL**

Highest binding, however, is achieved when using a double-Myc peptide (EQKLISEEDLEQKLISEEDL). It is likely that the ChromoTek Myc-tag binder interacts strongly with the second LISEEDL motif and then additionally with the first EQKLI motif:

EQKLI**SEEDL**EQK**L**I**SEEDL**

The sum of these two binding events yields a high-affinity interaction with the 2xMyc peptide.

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Elution strategies

Do I need to elute my Myc-tagged protein of interest from Myc-Trap at all?

IMPORTANT: Please consider whether you ***really*** need to elute the bound protein of interest from the beads rather than conduct the downstream analysis "on-bead". An "on-bead" processing has the advantage of less experimental steps and a potential higher yield:

- Proteins can be digested when still coupled to the beads for subsequent mass spectrometry analysis. Click here for "on-bead digest protocol for mass spectrometry"
- Enzymatic activity assays can also be performed when still coupled to the beads if the active center is not blocked.

When your experiment requires the elution of Myc-tagged protein of interest from Myc-Trap, you have various elution options dependent on your experimental strategy, i.e. for gentle elution or effective pull-down. Four elution options and their performances are presented in table 3. As you can see, the effectiveness of these options actually depends on whether you immunoprecipitate/purify 1xMyc- or 2xMyc-tagged protein.

Keeping the Myc-Trap binding mechanism in mind, you may want to consider at the beginning of the IP & Co-IP experiment what Myc-construct you want to use:

(i) If you like to effectively or gently elute your protein of interest in native conditions use 1xMyc-tagged protein in your experiment

(ii) If you like to effectively pull-down/bind low expressed/abundant proteins, or low concentration proteins use 2xMyc-tagged protein' in your experiment

Elution with	Elution Effectiveness	
	1xMyc-MBP	2xMyc-MBP
Myc-peptide (40 µM)	++	o
2xMyc-peptide (40 µM)	+++	+
Urea (8 M)	+++	+
Glycine (0.2 M) pH 2.5	+	+

Table 3: Effectiveness of Myc-Trap elution options. Experiments were conducted using 1x and 2xMyc-tagged Maltose Binding Protein (MBP). Instead of 2x Myc-tag also 3xMyc-tagged protein can be used. Abbreviations: o: <50%, +: ca. 50%, ++: ca.70%, +++ ca. 90% of bound protein eluted.

Bound Myc-fusion proteins can be eluted competitively with Myc-peptide and 2xMyc-peptide at different performances. Alternatively, bound Myc-tagged proteins are eluted by means of the chaotropic effect of 8M urea, or by means of pH shift. For SDS-PAGE and western blotting the bound MYC-Protein may be boiled directly in SDS sample buffer.

- Elution by Myc or 2xMyc-peptide:
  50µg peptide added to 25µl bead slurry in 50 to 100µl dilution buffer
  15 min incubation

- Elution by pH shift:
  50 µl of 200mM Glycine pH 2.5 added to 25µl bead slurry
  2 pipetting cycles (permanent up & down pipetting) of 30 sec each
  The repetition will improve the elution efficiency.
  Very important: Don't forget to neutralize proteins *immediately* after elution!

- Chaotropic Elution:
  50 µl of 8M urea added to 25µl of bead slurry
  2 times 15 min at 37°C
- For SDS-PAGE and Western Blotting:
  2x SDS sample buffer
  10 min at 95°C
  The elution results in denatured MYC-fusions.

Downstream applications

ChromoTek collects publications, which describe the use of the Myc-Trap, on its website at www.chromotek.com/references. Please check this database for your planned application.

Reproducibility

The Myc-Trap's nanobody is a small, soluble and stable single polypeptide chain that is recombinantly expressed in bacteria virtually without lot-to-lot variations. This, in combination with stringent quality control procedures makes its production robust and reproducible for reliable results.

Summary of experimental design considerations

1xMyc-fusion protein	2xMyc-fusion protein
Overview: • Suitable for all kind of IPs & Co-IPs plus affinity purification • For efficient and gentle elution of native protein	Overview: • Suitable for all kind of IPs & Co-IPs plus affinity purification • Extraordinary binding affinity for low expressed/abundant proteins
Consideration aspects: • Effective one step elution: both 2xMyc-peptide and urea result in fairly complete elution of bound Myc-tagged protein • Small elution volume to concentrate Myc-fusion protein of interest • Both Myc and 2xMyc-peptides enable gentle elution for recovery of native proteins • Fast binding for short incubation time • Fast dissociation requires quick wash processes	Consideration aspects: • Effective binding, optimal for low or endogenous expressed 2xMyc-fusion protein • Stringent wash condition can be applied • Complete pulldown/binding • Fast binding enables short incubation of 2xMyc fusion protein • Slow dissociation of 2xMyc-fusion protein enables long wash steps • Incomplete elution favors on-bead downstream assays & digestion of 2xMyc fusion protein

Further reading

Although recently launched, many researchers have already successfully used the Myc-Trap for a number of high impact publications that ChromoTek collects in a web-based database. We are frequently updating our database. You can search for organism and type of experiment at our website www.chromotek.com/references, to find publications relevant for you. Also, you will find a section on frequently asked questions at www.chromotek.com. Below there is a selection of publications introducing nanobodies and their applications.

Request a free Myc-Trap sample and see for yourself (www.chromotek.com/order/test-samples)

Free sample

Selected references (www.chromotek.com/references):

Mass spectrometry, on bead-digestion
Affinity Purification of Protein Complexes from Drosophila Embryos in Cell Cycle Studies.
Zoltan Lipinszki, Peng Wang, Rhys Grant, Catherine Lindon, Nikola S. Dzhindzhev, Pier Paolo D'Avino, Marcin R. Przewloka, David M. Glover, Vincent Archambault, in Methods Mol Biol. 2014;1170:571-88. doi: 10.1007/978-1-4939-0888-2_33.

ChromoTek Protocol "on bead-digestion"
On-bead digest protocol for mass spectrometry following immunoprecipitation with Nano-Traps

ChromoTek Protocol Mass spectrometry, sample preparation
How to prepare protein interaction partners for MS analysis

General introduction of Nanobodies
Nanobodies as probes for protein dynamics in vitro and in cells
Dmitriev, O. Y., Lutsenko, S. and Muyldermans, S. in: Journal of Biological Chemistry, 2015 – jbc-R115.

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Products and complimentary products (for research only)

Product name	Size	Code
Myc-Trap A ▸ coupled to agarose beads	10 rxns	yta-10
	20 rxns	yta-20
	100 rxns	yta-100
	200 rxns	yta-200
	400 rxns	yta-400
Myc-Trap A Kit ▸ Myc-Trap A ▸ incl. lysis, wash and elution buffers	20 rxns	ytak-20
Myc-Trap MA ▸ coupled to magnetic agarose beads	10 rxns	ytma-10
	20 rxns	ytma-20
	100 rxns	ytma-100
	200 rxns	ytma-200
	400 rxns	ytma-400
Myc-Trap MA Kit ▸ Myc-Trap MA ▸ incl. lysis, wash and elution buffers	20 rxns	ytmak-20
Myc Peptide ▸ EQKLISEEDL	1mg	yp-1
	10mg	yp-10
Multiple-Myc Peptide ▸ EQKLISEEDLEQKLISEEDL	1mg	2yp-1
	10mg	2yp-10
Spin columns	10 units	sct-10
	20 units	sct-20
	50 units	sct-50
Binding control ▸ agarose beads	20 rxns	bab-20
Binding control ▸ magnetic agarose beads	20 rxns	bmab-20
Myc-tag antibody [9E1] ▸ rat monoclonal	20µl	9e1-20
	100µl	9e1-100

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