"Mag"nificent Protein Pull-Down

In Vitro His-Tag Pull-Down Assay Using MagZ™ Particles

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Abstract

Recently we developed a novel product, the MagZ™ Protein Purification System, for the purification of expressed his-tagged protein from rabbit reticulocyte lysate. This protein purification system has a unique advantage over nickel-based immobilized metal ion affenity chromatography (IMAC) systems in that it specifically purifies His-tagged proteins from rabbit reticulocyte lysate without co-purifying hemoglobin. This article demonstrates that MagZ™ Binding Particles can be used to develop in vitro His-tag pull-down assays. By using a His-tagged Id protein attached to MagZ™ Particles, we show that we can detect the interaction with MyoD.

The MagZ™ Particles can be used for many applications including confirming two-hybrid assays, proteome-wide pull-down assays and DNA:protein

Introduction

With recent advances in human genome and proteome mapping, elucidating the protein:protein interaction map ("interactome") of the whole proteome is one major focus of functional proteomics (1,2). Various methods have been used for studying protein:protein interactions. These include yeast, bacterial and mammalian two-/three-hybrid systems, immunoaffinity purifications, affinity tag-based methods and mass spectrometry (reviewed in 1–3).

Though the yeast two-hybrid system is the most popular technique for analyzing protein:protein interactions, it has some limitations, including high rates of false positives and false negatives. Thus, experimental results from yeast two-hybrid assays are often further confirmed by in vitro tag-based pull-down assays. Moreover, tag-based affinity pull-down approaches enable analysis of protein:protein interactions by gel analysis or by techniques such as mass spectrometry, which is also useful in identifying known and unknown interacting partners. Like two-hybrid systems, in vitro pull-down assays can also be used for genome- or proteome-wide identification of protein:protein interactions (2–6). In vitro protein pull-down assays can be performed using cell lysates, in vitro-expressed lysates, tissue samples, etc. These options are not possible with two-hybrid approaches. Moreover, in vitro pull-down-based techniques such as tandem affinity purification (TAP) are being widely used for isolating protein complexes (7).

There are several reports describing the use of in vitro pull-down assays for analyzing protein:protein interactions using proteins translated in vitro using cell-free expression systems such as rabbit reticulocyte lysate-based expression systems (8–10). Cell-free expression is a powerful method for expressing cDNA libraries. This technique is also amenable for high-throughput protein expression and identification. Cell-free expression systems, especially rabbit reticulocyte lysate-based methods, have been extensively used for in vitro pull-down assays because of the ease of performing these experiments (8–10). There are also reports describing high-throughput identification of protein:protein interactions using TNT® Reticulocyte Lysates[a–d] (10). However, reports using His-tagged-based pull-down assays in rabbit reticulocyte lysate are very limited compared to other fusion tags (e.g., GST). One major reason for this is the co-purification of hemoglobin with His-tagged proteins. As a result, a second purification using ion-exchange chromatography is sometimes required after the nickel purification to remove the contaminating hemoglobin.

The MagZ™ Protein Purification System allows the purification of His-tagged proteins expressed in rabbit reticulocyte lysate without hemoglobin contamination (Figure 1; 11). The MagZ™ Particles have a high binding capacity that can be used for immobilizing His-tagged bait proteins. By using a His-tagged ubiquitin protein (MW ~7.5KDa) we found that the binding capacity of MagZ™ Particles is approximately 1mg/20µl of particles (data not shown). Here we test MagZ™ Particles in an in vitro pull-down assay. We used the Id-MyoD protein interaction system to demonstrate that we could use the MagZ™ Particles for analyzing protein:protein interactions using proteins expressed in rabbit recticulocyte lysate-based, cell-free expression systems.

Results and Discussion

MyoD is expressed in skeletal muscle and is a myogenic regulatory protein. The Id protein acts as a negative regulator of myogenic differentiation. MyoD and Id are members of the helix-loop-helix family of nuclear proteins. MyoD-Id interactions have been reported earlier using various methods (12,13). Figure 2 shows a His-tag pull-down assay using the MagZ™ Particles to detect the His-Id-MyoD interaction. Among the various conditions we tested, we found that the addition of imidazole to the washing conditions at a concentration of ≥40mM was optimal for assaying the Id-MyoD interactions. These results show that the pull-down of [^{35}S]met-labeled MyoD is Id protein-dependent.

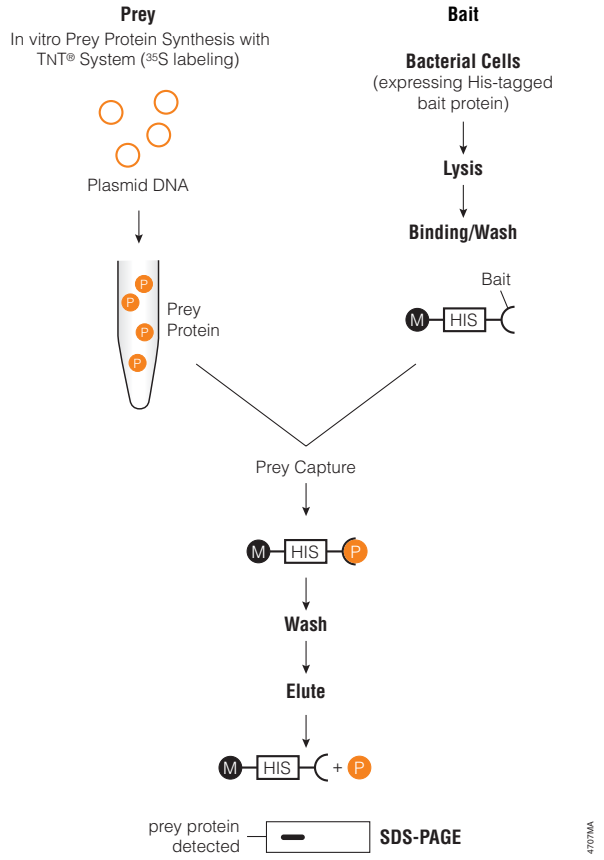

Figure 1. A schematic representation of His-tag pull-down using the MagZ™ Particles. P = prey protein.

Even though we saw some nonspecific binding of MyoD to the MagZ™ particles, the His-Id-MyoD interaction was 2–4 times greater than that of the controls, depending on the washing conditions. We further confirmed these results by Western blot analysis (Figure 3). Interestingly, the Id-MyoD interaction is stable at a higher concentration of imidazole, 500mM. These conditions may not be ideal for all proteins.

Table 1 shows examples of some of the binding and wash buffers described in the literature. Imidazole concentrations from 5–50mM have been used in interaction and wash buffers. Salts at concentrations from 100–300mM have been reported. It is evident from this table that different interaction experiments require different conditions. Thus, the observed interaction of His-Id with MyoD at higher concentrations of imidazole is not surprising. However, it is important to note that His-Id protein did not elute off the MagZ™ Particles at 500mM imidazole. This indicates that conditions should be optimized for each protein.

As we reported in our accompanying article in this issue, the amount of imidazole required for protein elution is protein-dependent (11). Some His-proteins require 1M imidazole for efficient elution, whereas other His-proteins elute off at 100mM imidazole, so researchers should be

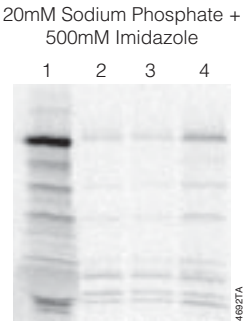

Figure 2. MagZ™ Protein Purification System used for pull-down assay. His-tagged Id was used as bait. His-Id is expressed and induced in *E. coli* according to standard protocols. The cultures were pelleted, resuspended at a 10X concentration, and sonicated. Untagged MyoD prey protein was expressed in TNT® T7 Coupled Transcription/Translation System[a–e] (Cat.# L1170) using 1µg of MyoD DNA and 2µl of [35S]met. The TNT® reaction was performed as recommended in the Technical Manual (#TM045). One hundred microliters of the bacterial lysate containing the His-Id bait protein was added to 30µl MagZ™ Binding Particles and incubated for 15 minutes on a shaker. The MagZ™ Particles were washed three times with 200µl of 20mM sodium phosphate (pH 7.4) and resuspended with 30µl of MagZ™ Binding/Wash Buffer. Five microliters of particles was transferred to new tubes. Particles were resuspended in 175µl of the MagZ™ Binding/Wash Buffer. Twenty microliters of the TNT® reaction was added to the prepared bait His-Id/MagZ™ Particles and incubated for 60 minutes on a shaker. The particles were washed three times in the same final wash buffer used in the immobilization followed by an additional wash of 500mM imidazole. Twenty microliters SDS buffer was added to the particles and incubated for 5 minutes with shaking, and the samples were collected. The eluted sample was diluted 1:10 in SDS buffer, heated at 95°C and loaded onto a 4–20% Tris-glycine gel. The gel was transferred to a PVDF membrane, exposed to a PhosphorImager® plate overnight and read on a Storm® fluorescent imager. Lane 1, MyoD expressed in TNT® Lysate ([35S]met-labeled); lane 2, MagZ™ Particles plus MyoD; lane 3, His-RNase H bound to MagZ™ Particles plus MyoD; lane 4, His-Id bound to MagZ™ Particles plus MyoD.

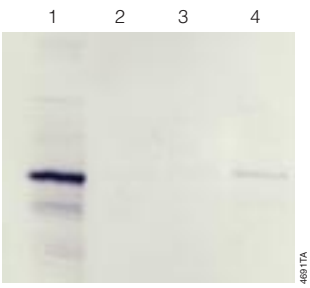

Figure 3. Western blot analysis. The membrane from Figure 2 was blocked with TBST/1% BSA for 1 hour at room temperature. Purified mouse anti-MyoD monoclonal antibody (BD Biosciences Cat.# 554130) was added at 1µg/ml in TBST and incubated for 1 hour. The membrane was washed 3 times in TBST. Anti-Mouse IgG (H+L), AP Conjugate (Cat.# S3721), diluted 1:7,500 in TBST, was added and incubated for 1 hour. The membrane was washed three times with TBST, twice with with TBS, and bands were visualized using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841). Lane 1, MyoD expressed in TNT® Lysate ([35S]met-labeled); lane 2, MagZ™ Particles plus MyoD; lane 3, His-RNase H bound to MagZ™ Particles plus MyoD; lane 4, His-Id bound to MagZ™ Particles plus MyoD.

careful when performing experiments at higher concentrations of imidazole. The conditions for binding and wash buffers will vary from protein to protein and will need to be optimized for individual experiments.

Conclusions

In this report, we demonstrated the use of MagZ™ Particles for analyzing protein:protein interactions by His-tag pull-down assays. In our experiments, we used His-Id as the bait protein and untagged MyoD as the prey protein. Thus, MagZ™ particles could be used for many applications including confirming two-hybrid assays, proteome wide pull-down assays and DNA:protein interaction analysis.

Acknowledgments

We would like to thank Marjeta Urh and Jacqui Sankbeil for providing the His-tagged Id and untagged MyoD clones.

Table 1. Binding/Wash Buffers Used for His-Tag Pull-Down Assays.

Binding/Wash Buffer	Pull-Down Expression System	Reference
50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.3% Nonidet® P-40, 10mM $ZnCl_2$, 1mM DTT, 0.35% BSA	TNT® Rabbit Reticulocyte Lysate	8
20mM Tris-HCl (pH 7.5), 100mM NaCl, 1mM EDTA, 0.5% Nonidet® P-40, 1mM DTT	TNT® Rabbit Reticulocyte Lysate	9
50mM Tris-HCl (pH 7.5), 300mM KCl, 20–30mM imidazole	TNT® Rabbit Reticulocyte Lysate	10
20mM Tris-HCl (pH 7.6), 100–125mM NaCl, 15mM imidazole, 1% Nonidet® P-40	Gold TNT® Rabbit Reticulocyte Lysate[a,c,d,e]	13
20mM Tris-HCl (pH 8.0), 100mM NaCl, 15mM imidazole, 10% glycerol	Crude insect cell extracts	14
20mM Tris-HCl (pH 8.0), 150mM NaCl, 10–15mM imidazole, 1% Triton® X-100	Crude cell extracts	15
20mM MOPS (pH 7.0), 4.5mM $MgCl_2$, 150mM NaCl, 0.5% (v/v) Triton® X-100, 50mM imidazole	Cell lysate	16
10mM Tris-HCl, 100mM NaH_2PO_4, 25mM imidazole, 1% Nonidet® P-40	Cell extract	17
20mM Tris-HCl (pH 8.0), 10% glycerol, 100mM NaCl, 15mM imidazole	Cell extract	18
50mM phosphate buffer (pH 8.0), 300mM NaCl, 2mM BME, 10–20mM imidazole	Cell extract	19
20mM Tris-HCl (pH 8.0), 10% glycerol, 90mM KCl, 10mM BME, 50µg/ml ethidium bromide, 0.1% Triton® X-100, 20mM imidazole, 20µg/ml BSA	Cell extract	20
PBS, Tween®-20	Purified protein	21
10mM HEPES (pH 7.4), 200mM KCl, 10% glycerol, 0.25% Triton® X-100, 30mM imidazole, 0.2mM EDTA, 10mM BME	Cell lysate	22
50mM NaH_2PO_4 (pH 8), 300mM NaCl, 20mM imidazole	Purified protein	23
10mM Tris buffer (pH 7.9), 50mM NaCl, 50mM imidazole	Purified protein	24

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Protocol

- *MagZ™ Protein Purification System Technical Bulletin* #TB336, Promega Corporation. (www.promega.com/tbs/tb336/tb336.html)
- *TNT® T7 Coupled Transcription/Translation System Technical Manual* #TM045, Promega Corporation. (www.promega.com/tbs/tm045/tm045.html)

Ordering Information

Product	Size	Cat.#
MagZ™ Protein Purification System	30 purifications	V8830

[a] U.S. Pat. Nos. 5,324,637 and 5,492,817, Australian Pat. No. 660329 and other patents.

[b] The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

[c] U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

[d] Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.

[e] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.