Pulling Down the Protein You Want

Detection of Protein:Protein Interactions Using the MagneGST™ Pull-Down System

By Marjeta Urh, Ph.D., Jacqui Sankbeil, M.S., Don Creswell, B.S., and Dan Simpson, Ph.D., Promega Corporation

Abstract

The MagneGST™ Pull-Down System was designed to detect protein interactions between GST-fusion proteins expressed in E. coli *lysates and prey proteins expressed in vitro in the TNT® T7 Quick Coupled Transcription/Translation System. The MagneGST™ System provides glutathione (GSH)-linked magnetic particles that allow immobilization of GST-fusion proteins (bait) from bacterial lysates. The magnetic nature of the particles allows easy and quick processing of multiple samples and provides enhanced recovery. Prey protein synthesized in the TNT® T7 Quick Coupled Transcription/Translation reaction is captured using bait protein (GST-fusion protein) immobilized on MagneGST™ (GSH) Particles. Nonspecifically bound proteins are washed away, and the prey protein is analyzed. We tested the method described with two different protein:protein interaction systems and detected the results by either radioactive labeling or Western blotting.*

The MagneGST™ Pull-Down System offers a unique format for the convenient analysis of protein:protein interactions.

Introduction

Many proteins function with partners or as components of a large multiprotein complex. Understanding these interactions is critical to our understanding of biological pathways and cellular function. Glutathione-S-Transferase (GST) pull-down (1) is becoming an increasingly important tool for validation of suspected protein:protein interactions and also for identification of new interacting partners (2–5). GST pull-down uses a GST-fusion protein (bait) bound to glutathione (GSH)-coupled particles to affinity purify any proteins (prey) that interact with the bait from a pool of proteins in solution. Bait and prey proteins can be obtained from multiple sources including cell lysates, purified proteins and in vitro transcription/translation systems.

The MagneGST™ Pull-Down System[a–d] is optimized for the detection of protein:protein interactions where the bait protein is prepared from *E. coli* lysate and mixed with the prey protein synthesized in the TNT® T7 Quick Coupled Transcription/Translation System[a,b,d,e,f] (Cat.# L1170). The magnetic nature of the MagneGST™ GSH-linked particles in this system offers significant advantages over traditional resins, which require lengthy preparation and equilibration and are hard to dispense accurately in small amounts. The MagneGST™ Particles are easy to dispense in volumes less than 5µl, equilibration is quick and easy and does not require any centrifugation steps. Another advantage of this system is that the pull-down reaction is performed in one tube. The particles are easily and efficiently separated from supernatants using a magnetic stand and don't require centrifugation, which increases reproducibility and should reduce sample loss. The flexible format of the MagneGST™ Pull-Down System allows optimization of experimental conditions including modification of particle volume to fit specific requirements of each unique protein:protein interaction. Additionally, the system allows easy processing of multiple samples at once.

MagneGST™ Pull-Down Method

The MagneGST™ Pull-Down System provides GSH-linked magnetic particles that allow simple immobilization of bait proteins from bacterial lysates and an in vitro transcription/translation system for expressing prey proteins. The MagneGST™ Pull-Down protocol can be divided into three phases: 1) the prey protein is expressed in the TNT® T7 Quick Coupled System; 2) bait protein present in crude *E. coli* lysate is immobilized on the MagneGST™ Particles; 3) the prey protein is mixed with MagneGST™ Particles carrying the bait protein and captured through bait-prey interaction. Nonspecifically bound proteins are washed away, and the prey and bait proteins are eluted from the resin with SDS loading buffer. Prey proteins can be analyzed by SDS-PAGE gel followed by detection with specific antibodies or by autoradiography if the prey protein was radioactively labeled during the TNT® synthesis. Phases 1 and 2 are performed simultaneously. An overview of the MagneGST™ Pull-Down System is depicted in Figure 1.

Examples of Protein:Protein Interactions Detected by the MagneGST™ Pull-Down System

We developed the guidelines for the MagneGST™ Pull-Down System primarily using the well-known MyoD:Id interaction as a model. The MyoD and Id proteins are members of the helix-loop-helix family of nuclear proteins shown to regulate myogenic differentiation (6,7) and known to interact in vitro (8).

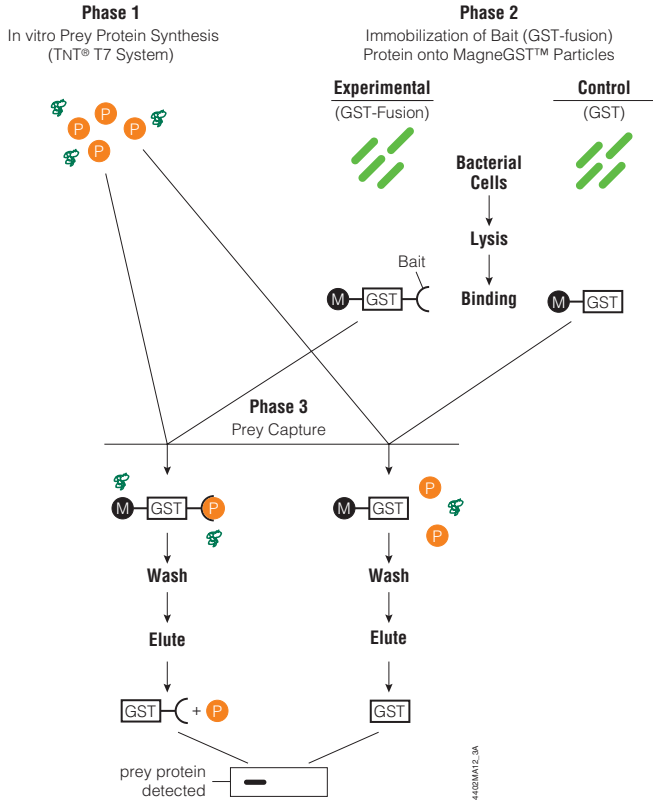

Figure 1. Overview of the MagneGST™ Pull-Down System protocol. P = Prey Protein, M = MagneGST™ Particles.

We cloned the MyoD sequence into the pCI-neo Mammalian Expression Vector[g,h] (Cat.# E1841) and expressed the protein as prey in the TNT® T7 Quick Coupled System. The prey protein was either left unlabeled (to be detected by Western) or labeled with ^{35}[S]methionine. The Id sequence was cloned as a GST-fusion into the pGEX-6P-1 vector (Amersham Biosciences), and the GST-Id protein was overexpressed in *E. coli*. We also harvested cells expressing GST alone (pGEX-6P-1 vector) to use as a negative control. The bacterial cells were lysed and the proteins immobilized as bait onto the MagneGST™ Particles. We used a portion of each of these precharged particles to capture the MyoD prey protein. Figure 2 shows the prey proteins we recovered. The much stronger signal in the GST-Id lanes compared to the GST control lanes indicates that the GST-Id bait protein specifically pulls down the MyoD prey.

We also tested the interaction between the simian virus 40 (SV40) T-antigen and the tumor suppressor p53 protein (9,10). We excised the p53 sequence from the pGBKT7-53 control vector (BD Biosciences Clontech) and cloned it into the pGEX-6P-1 vector to create a GST fusion protein. The SV40 T-antigen protein was expressed from the pGADT7-T plasmid (BD Biosciences Clontech) in the TNT® Quick Coupled System and

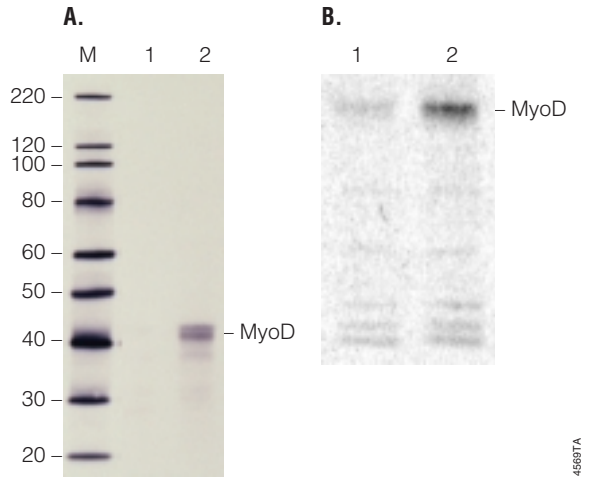

Figure 2. Non-radioactive and radioactive detection of MyoD:Id MagneGST™ Pull-Down reactions. Pull-down experiments were performed as described in the Technical Manual #TM249 and Figure 1 and included 1% BSA. Lanes: M, MagicMark™ XP Western protein standards (Invitrogen Cat.# LC5602); lanes 1, GST-only control; lanes 2, GST-Id pull-down. **Panel A.** Western blot analysis. Twenty-five percent of the total eluted samples were analyzed by SDS-PAGE followed by Western blotting with anti-MyoD antibody (BD Biosciences Cat.# 554130), an alkaline phosphatase-conjugated secondary antibody and Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841). **Panel B.** Radioactive detection. One percent of the total eluted samples were analyzed by SDS-PAGE and the image developed on a Storm® PhosphorImager® instrument.

labeled with ^{35}[S]methionine. We used a portion of each of the GST-p53 or GST control bait particles to capture the SV40 T-antigen prey. The results were analyzed on an SDS-PAGE gel and visualized using a PhosphorImager® instrument (Figure 3). The results demonstrate that the TNT® reaction was efficient and that the GST-p53 bait protein specifically pulls down the T-antigen prey.

As seen with these samples, a small amount of nonspecific interaction between the prey protein and the GST-only control may be observed in some cases. Nonspecific interactions of proteins with resins through charge or other noncovalent interactions are common and well-documented (11–13). In most cases the nonspecific interactions can be reduced or eliminated by high-stringency washes (high salt or detergents) and by addition of bovine serum albumin (BSA) to the pull-down reaction. We found that the addition of 1% BSA reduced nonspecific binding detected with the GST-Id:MyoD and GST-p53:T-antigen pairs. The addition of IGEPAL CA-630 (NP40 analog)(Sigma Cat.# I-8896) to a final concentration of 0.5% had a similar effect (data not shown). Furthermore, the nonspecific interactions could be affected by the amount of the resin in the pull-down reaction. Excess resin in the reaction may result in an increase in nonspecific interactions (14). Thus the amount of the resin in each experiment may need to be optimized; the flexibility of the MagneGST™ Pull-Down System allows for easy and accurate adjustments in resin volume.

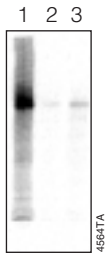

Figure 3. Radioactive detection of p53:SV40 T-antigen MagneGST™ Pull-Down reactions. Pull-down experiments were performed as described in the Method section and Figure 1 and included 1% BSA. SV40 T-antigen was expressed in the TNT® T7 Quick System and labeled with ^{35}[S]methionine. One percent of the TNT® reaction and 1% of the eluted samples were run on an SDS-PAGE gel and the results detected with a Storm® PhosphorImager® instrument. Lane 1, 1% of the TNT® reaction; lane 2, 1% of the GST-only control; lane 3, 1% of the GST-p53 pull-down.

Importance of Controls

The MagneGST™ Pull-Down System is designed to allow researchers to verify the success of each phase of the process (Figure 1). The most critical control for each pull-down experiment is a control sample where nontreated particles, or particles carrying GST alone, is processed in parallel with experimental samples (Figure 4). The proteins eluted from the GST-alone control and experimental samples are analyzed on SDS-PAGE gel. The bands that associate preferentially with bait protein and do not (or to a lesser extent) associate with the GST control indicate specific interacting protein partners. This control helps identify and eliminate false positives resulting from nonspecific interactions of proteins with the MagneGST™ Particles or with the GST moiety of the fusion protein.

We also recommend that the products from Phase 1 and 2 be tested to ensure that the bait and prey proteins are present (Figure 4). The efficiency of the first phase, the TNT® reaction, can be tested by running a small fraction of the reaction on an SDS-PAGE gel and verifying that the prey protein was successfully synthesized. For Phase 2, controls should be performed to show that the bait protein is bound to the MagneGST™ Particles. To check the efficiency of immobilization of the bait protein (or GST alone when used as a control in Phase 3) a fraction of the particles used in the immobilization step is removed and run on an SDS-PAGE gel. The amount of the bait protein and GST protein immobilized on the particles can be estimated by the band intensity when compared to the intensity of bands containing known amounts of protein. This control allows estimation of the efficiency of immobilization of the bait protein and GST-alone control.

Conclusion

GST pull-down is one of the most commonly referenced methods for the characterization of protein:protein interactions. We have demonstrated the effectiveness of the MagneGST™ Pull-Down System using two well-known interacting protein pairs: MyoD:Id and p53:SV40 T-antigen. The MagneGST™ Pull-Down System offers a unique format for the convenient analysis of protein:protein interactions. A bait protein is expressed and easily purified from *E. coli* by the use of magnetic particles. The complementary prey protein is expressed in the provided TNT® T7 Quick Coupled System. Since detection of all protein:protein interactions require optimization, the guidelines we describe offer a starting point that can be optimized for other protein pairs. In addition the system allows maximum flexibility and provides options for either radioactive or non-radioactive detection.

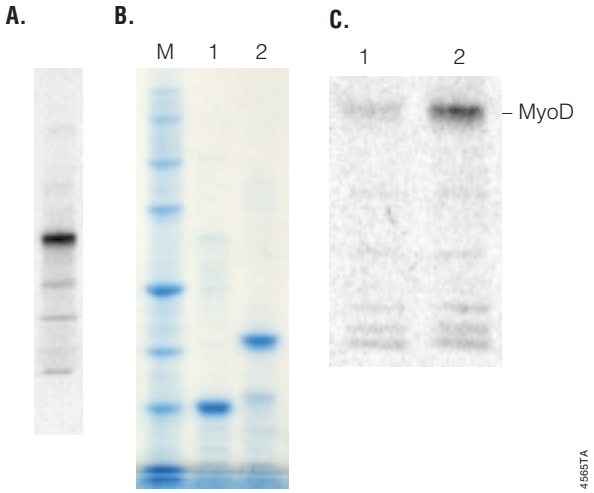

Figure 4. MagneGST™ Pull-Down controls. Panel A. Phase 1 control. One percent of a TNT® reaction with ^{35}[S]methionine-labeled MyoD protein was separated by SDS-PAGE and detected by autoradiography. **Panel B.** Phase 2 control. Five microliters of particles charged with either the GST-only control (lane 1) or the GST-Id (lane 2) were resuspended in 15µl of SDS loading buffer, separated by SDS-PAGE and visualized by Coomassie® blue staining. Lane M, Promega Protein Markers (Cat.# V8491). **Panel C.** Phase 3 control. One percent of the eluted protein from the GST-only control and GST-Id sample were separated by SDS-PAGE and visualized by autoradiography.

References

1. Kaelin, W.G. *et al.* (1991) *Cell* **64,** 521–32.
2. Benard, V. and Bokoch, G.M. (2002) *Methods Enzymol.* **345**, 349–59.
3. Wang, Y., Sugita, S. and Sudhof, T.C. (2000) *J. Biol. Chem.* **275**, 20033–44.
4. Wada, H. *et al.* (1998) *Biochem. Biophs. Res. Commun.* **251**, 688–92.
5. Malloy, P.J. *et al.* (2001) *Mol. Genet. Metabol.* **73**, 138–48.
6. Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) *Cell* **51**, 987–1000.
7. Weintraub, H. *et al.* (1991) *Science* **251**, 761–6.
8. Benezra, R. *et al.* (1990) *Cell* **61**, 49–59.
9. Bargonetti, J. *et al.* (1992) *Genes Dev.* **6,** 1886–98.
10. Sheppard, H.M. *et al.* (1999) *Mol. Cell. Bio.* **19**, 2746–53.
11. Draveling, C. *et al.* (2001) *Protein Expr. Purif.* **22**, 359–66.
12. Brymora, A. *et al.* (2001) *Anal. Biochem.* **295**, 119–22.
13. Haney, P. *et al.* (2003) *Protein Expr. Purif.* **28,** 270–9.
14. Ren, L. *et al.* (2003) *Anal. Biochem.* **322**, 164–9.

Protocols

- *MagneGST™ Pull-Down System Technical Manual* #TM249 Promega Corporation. (www.promega.com/tbs/tm249/tm249.html)

Ordering Information

Product	Size	Cat.#
MagneGST™ Pull-Down System	1 system	V8870

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

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

[c] This product or portions thereof is manufactured under license from Amrad Corporation Limited. For non-commercial research use only. All other uses require a license from Amrad Corporation Limited, Unit 6, 663 Victoria Street, Abbotsford, Victoria 3067, Australia, under U.S. Pat. No. 5,654,176, Australian Pat. No. 607511, Canadian Pat. No. 1338903 and other issued patents.

[d] 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.

[e] 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.

[f] U.S. 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.

[g] The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

[h] U.S. Pat. No. 4,766,072.

Marjeta Urh, Ph.D.
Research Scientist

Jacqui Sankbeil, M.S.
Research Scientist

Don Creswell, B.S.
Research Scientist

Dan Simpson
Research Scientist