EXTRACTMAN® Delivers Expanded Analyte Isolation of Weakly- or Transiently-Bound Species through ESP™

APPLICATION NOTE AN1005

APPLICATION BENEFITS

Most proteins perform their function through interaction with other molecules, including other proteins. Co-immuoprecipitation (Co-IP) is a widely used technique for identifying physiologically relevant protein-protein interactions; however, harsh washing conditions can lead to loss of weakly- or transiently-bound interactions.

SOLUTIONS

EXTRACTMAN® offers a faster, gentler magnetic mixing approach to isolating target proteins and is ideal for isolating elusive, weakly-bound protein complexes critical to protein interaction research. Patented microplate technology enables highly efficient isolation of up to four samples in parallel and can easily be adapted to multiple washes, multiple targets, and even completely new magnetic bead applications.

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ABSTRACT

Co-immunoprecipitation (Co-IP) is a common technique for observing protein-protein interactions; however, weakly- or transiently-bound species often go undetected. Aggressive or repeated wash steps in the Co-IP process can result in a loss of these interactions. Exclusion-based sample preparation (ESP™) allows for rapid, yet gentle washing of protein solutions during Co-IP, thereby creating an environment that retains these elusive interactions. This application note describes a comparison of a conventional Co-IP protocol with an EXTRACTMAN® protocol using ESP technology. While the conventional Co-IP protocol did not pull down the protein-protein complex, EXTRACTMAN did, demonstrating the utility of ESP technology and EXTRACTMAN.

INTRODUCTION

Proteins are critical for cellular function, performing the majority of the 'work' within a cell, such as maintaining cellular structure (keratin), inter- and intracellular communication (growth hormones), enzymatic processes (DNA polymerases), and defense (antibodies). Most proteins perform their function through interaction with other molecules, including other proteins. These protein-protein complexes may occur constitutively or transiently with varying levels of affinity.

Figure 1
EXTRACTMAN® provides a rapid and reliable method for the isolation and purification of protein-protein interactions with paramagnetic particles (beads).

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Identifying the proteins that form these functional complexes may help researchers to understand a process or disease. Protein complexes and pathways[1] are attractive targets for drug discovery. There are several approaches for identifying protein-protein interactions, including X-ray crystallography, pull-down assays, crosslinking, label transfer, and far-western blotting; however, the primary method is co-immunoprecipitation (Co-IP).[2]

While Co-IP is a commonly used technique, limitations exist due to the mechanical and chemical stresses produced from repeated washing steps and incubations. Generally, only those protein-protein interactions that are constant or bound with a high affinity can be reliably purified using Co-IP, while transient and low-affinity interactions, such as those in secretory and signaling pathways (e.g., p53-MDM2-USP7 pathway) are lost.[2]

EXTRACTMAN

The use of exclusion-based sample preparation (ESP™) in the Co-IP process can dramatically reduce the mechanical stresses associated with rigorous washes while decreasing sample preparation time.[3,4] EXTRACTMAN® utilizes this technology to move paramagnetic beads coated with specific analyte binding surfaces through the wash steps[5] (Figure 1).

This application note describes how EXTRACTMAN was used to isolate a previously undetected, although hypothesized, protein-protein complex.

MATERIALS AND METHODS

Samples and Controls

Purified proteins, with and without tags (fusion protein), were used to evaluate the Co-IP protocols. Although the identity of the proteins used in this study is known to the researchers who carried out the work, due to pending publication in a peer-reviewed journal the proteins will be referred to as Unidentified Protein A and Unidentified Protein B. Unidentified protein A was tagged with Maltose Binding Protein (MPB-UPA). Unidentified Protein B was tagged with Glutathione-S-Transferase (GST-UPB). The tags without the target proteins (MBP and GST) were used as controls for the Co-IP protocol (Table 1).

Table 1

Samples used in the Co-IP experiments.

SAMPLE NAME	TAG/ FUSION PROTEIN	TARGET PROTEIN
GST	GST	-
MBP	MBP	-
GST-UPB	GST	UPB
MBP-UPA	MBP	UPA

Co-Immunoprecipitation Method

The primary pulldown for the Co-IP utilized an antibody specific for MBP bound to a magnetic bead (NEB: E8037S).

Four different Co-IP conditions were evaluated (Table 2).

1. The MBP tag was pulled down in the presence of the GST tag.
2. MBP-UPA was pulled down in the presence of GST.
3. The MBP tag was pulled down in the presence of GST-UPB.
4. MBP-UPA was pulled down in the presence of GST-UPB, which represented the primary focus of the experiment.

Table 2

Protein configurations used for co-Immunoprecipitation experiment.

CONDITION	BAIT PROTEIN	PREY PROTEIN
1	MBP	GST
2	MBP-UPA	GST
3	MBP	GST-UPB
4	MBP-UPA	GST-UPB

The anti-MBP magnetic beads were washed two times with ice-cold (0°C–4°C) phosphate buffered saline (PBS), and three times with Pulldown Buffer (25 mM Tris pH 7.5, 75 mM KCl, 0.04% NP40, 5 mM MgAcetate, 1 mM DTT, 10% Glycerol). Beads were then resuspended in Pulldown Buffer (1:1). The bait protein was added to 20 µL of beads, and then brought up to 500 µL total volume with Pull-down Buffer. The reaction was allowed to proceed for 2 h at 4°C on a rotating mixer.

The supernatant was aspirated off, and beads were washed two times with Pulldown Buffer. The prey protein (equimolar amount) was added to the beads and the reaction volume was brought up to 420 µL with Pulldown Buffer. The reaction was again allowed to incubate for 2 h at 4°C on a rotating mixer. The Co-IP was then performed with conventional method or with the EXTRACTMAN.

Conventional Co-IP Protocol

The supernatant was aspirated off, and beads were washed three times with 1 mL Pulldown Buffer. The reaction was incubated for 5 min at 4°C, before aspirating off the supernatant. The beads were retained for the SDS-PAGE procedure.

EXTRACTMAN Co-IP Protocol

The bead solution (420 µL) was added to the first sample well of EXTRACTMAN. Pull-down Buffer (100 µL) was added to wells 3, 4, and 5. The beads were moved via the EXTRACTMAN bead capture strip from well columns 1 to 3, 4, and finally to 5, allowing the beads to drop into each wash well. The supernatant was then removed from well 5, leaving the beads for the SDS-PAGE procedure.

> "...EXTRACTMAN can help to identify previously unidentified protein-protein interactions..."

SDS-PAGE and Western Blot

Laemmli sample buffer (20 µL of 2X) was added to the beads from both the conventional Co-IP and the EXTRACTMAN Co-IP procedures. Samples were boiled at 85°C for 5 min, then cooled down on ice, and centrifuged at ~15,500 rcf for 2 min. Supernatant was removed and loaded onto SDS-PAGE for protein separation.

Two gels were loaded (conventional vs. EXTRACTMAN) with supernatant from four different Co-IP conditions as described in Table 2. Two positive controls, which had not gone through the Co-IP process were also loaded onto each gel: GST tag alone and GST-UPB. The gels were then transferred to membranes for western analysis, using anti-GST antibody to look for the prey protein (UPB).

RESULTS AND DISCUSSION

The western blot analysis showed that the prey protein was pulled down with the bait protein when using EXTRACTMAN® but not with the conventional method (see Figure 2 on page 4).

Protein bands of the expected apparent molecular weight (~30 kDa) were observed in positive control lanes for samples processed with either conventional or EXTRACTMAN methodology. Additional, higher molecular weight bands were observed in the unprocessed GST-UPB sample, likely because of the presence of multimers. No bands were observed in either gel for the negative control conditions (1. MBP + GST, 2. MBP-UPA + GST, and 3. MBP + GST-UPB).

The conventional protocol did not produce a band for condition 4 (MBP-UPA + GST-UPB), while the EXTRACTMAN protocol did. This demonstrates that the rapid yet gentle washing performed on EXTRACTMAN can help to identify previously uncharacterized interactions. These transiently-acting or weakly-bound interactions would be lost with conventional protocols because of the increased time and more aggressive washing steps.

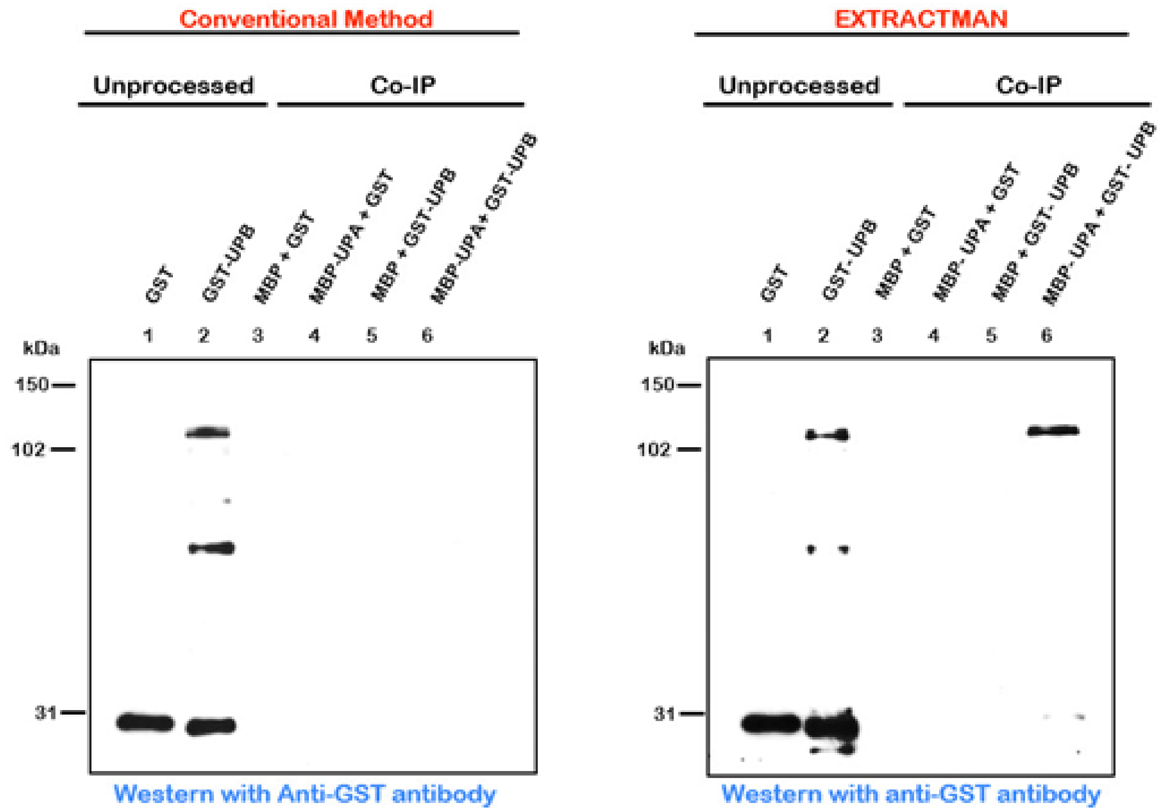

Figure 2

Western blot analysis of GST tagged proteins following Co-IP, by conventional protocol (left), and with the EXTRACTMAN® (right). Positive controls in lanes 1 and 2, GST and GST-UPB respectively, produced bands in both blots. Co-IP conditions 1–3 (1. MBP + GST, 2. MBP-UPA + GST, 3. MBP + GST-UPB) were loaded into lanes 3–5 respectively, and produced no bands. Co-IP condition 4 (MBP-UPA + GST-UPB) produced an observable band at the same size as the GST-UPB positive control (lane 2), demonstrating successful co-immunoprecipitation. The conventional protocol did not result in observa-ble band from the same condition.

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SUMMARY

- The data shown in this application note demonstrate that a previously undetected protein-protein interaction was observed when using the EXTRACTMAN®, but not with conventional Co-IP methodologies.
- EXTRACTMAN utilizes ESP™ technology, which creates a rapid and gentle technique for washing paramagnetic particles.
- EXTRACTMAN can be used for Co-IPs, to identify transient and weakly-bound protein-protein interactions.

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