Application Note

Automated Immunoprecipitation of PP2A using the PureProteome™ Magnetic Beads on KingFisher® Particle Processor

Introduction

Immunoprecipitation (IP) is a powerful technique for proteomic screening, biomarker discovery, and signaling network elucidation. It is frequently used to enrich target proteins from complex samples such as cell lysates or extracts. Traditional IP protocols use Protein A, Protein G or a mixture of Protein A and G coupled to a solid support resin, such as agarose beads, to capture an antigen/antibody complex in solution.

As the number of samples increase, the traditional, manual IP method can be time-consuming. Processing of multiple IP reactions in parallel can introduce complexity, variability and pipetting errors, which may affect reproducibility.

To meet requirements for throughput and reproducibility, many laboratories have turned to automated systems for immunoprecipitation using Protein A or Protein G magnetic beads. Liquid handling and bead handling robots are the two types of systems commonly used for automating magnetic bead separation protocols. In liquid handling robots, magnetic separation is achieved by integrating an external 96-well magnetic rack to separate the beads from the liquid. Alternatively, bead handling robots such as KingFisher® particle processors use magnetic rods covered with disposable plastic tips to move beads from one solution to the next.

PureProteome™ Protein A and G Magnetic beads are well suited for immunoprecipitation (IP) reactions. There are two main types of IP reactions, direct and indirect. In direct IP, the capture antibody is first immobilized onto the PureProteome™ magnetic bead, creating an immunoaffinity magnetic bead. These antibody-coupled beads are then added to the sample so that the bead-bound antibody can capture the antigen or protein complex of interest. In contrast, in indirect IP, the capture antibody is first incubated with the sample to allow formation of the antibody-antigen complex in solution. The PureProteome™ beads are then incubated with the pre-formed antibody-antigen complex for capture onto the beads. Here, we use the indirect IP method to evaluate the effectiveness of PureProteome™ Protein G Magnetic Beads for immunoprecipitation of protein phosphatase 2A (PP2A) in a fully automated purification process using the KingFisher® Duo particle processor. To obtain the best results, parameters such as mixing speed, bead collection time and duration of binding and washing steps were optimized for use with PureProteome™ Protein G Magnetic Beads.

Materials and Methods

Immunoprecipitation of PP2A – Standard Manual Protocol

For each reaction, 25 µg of Jurkat cell lysate was combined with 5 µg of anti-PP2A monoclonal antibody (Isotype IgG2bκ, Cat. No. 05-421) in a total volume of 150 µL with binding buffer. Phophate-buffered saline (PBS) containing 0.01% Tween® 20 detergent was used as binding/wash buffer. The reaction was incubated at room temperature for 2 hours with end-over-end mixing. 25 µL of suspended PureProteome™ Magnetic Bead slurry (Cat.No. LSKMAGG10) was added to a 1.5 mL microcentrifuge tube (6 replicates). Using the PureProteome™ Magnetic Stand (Cat. No. LSKMAGS08), the beads were washed with 500 µL of binding buffer by vigorously vortexing for 10 seconds, collecting the beads on the magnet and removing the buffer with a pipette. 150 µL of the antibody-antigen sample was added to the beads, and the mixture was incubated at room temperature for 30 minutes with continuous end-over-end mixing. The unbound fraction was discarded and, as described above, the beads were washed three times with 500 µL of wash buffer using the PureProteome™ Magnetic Stand to capture the beads. Denaturing elution was performed by adding 100 µL of reducing 1X SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample loading buffer to the beads and incubating at 70 °C for 10 min. An additional elution was performed in a similar manner with 50 µL to achieve maximum yield. Both elutions were collected using the PureProteome™ Magnetic Stand into the same microcentrifuge tube and saved for further analysis.

Immunoprecipitation of PP2A – KingFisher® Duo Protocol

For each reaction, 25 µg of Jurkat cell lysate was combined with 5 µg of anti-PP2A and was brought up to a total volume of 150 µL with binding buffer. PBS containing 0.01% Tween® 20 detergent was used as binding/wash buffer. The reaction was incubated at room temperature for 2 hours with end–over-end mixing. All the reagents and samples were pipetted into the KingFisher® Duo plates (Microtiter Deepwell 96 plate and elution strips) and a protocol was set up on KingFisher® Duo System (Thermo Fisher) according to the plate layout and optimized conditions (mixing and collecting parameters for PureProteome™ magnetic beads) outlined in Table 1. 25 µL of suspended bead slurry was combined with 175 µL of binding buffer to obtain sufficient volume for use with the KingFisher® Duo System. 150 µL of the antibody-antigen sample was pipetted into row D of the Microtiter Deepwell 96 plate and the binding step was performed for 30 minutes using medium mixing speed. Denaturing elution was carried out with 100 µL of reducing 1X SDS-PAGE sample loading buffer at 75 °C for 15 min in elution strips for better recovery of the sample. The protocol was executed and the plates were loaded into the KingFisher® Duo System. After the run was completed, the plates were removed and eluted samples were collected for further analysis.

Plate/Row Elution Strip	Row Name	Content	Volume	Mixing Time/Speed	Collecting Count/Time
1-A	Beads	Protein G Beads + Bind Buffer	200 µL	1 min/Medium	4/10 sec
1-B	Tip	12-Tip Comb	-	-	-
1-C	Equilibration	Binding Buffer	500 µL	1 min/Medium	4/10 sec
1-D	Bind	Antibody + Antigen Sample	150 µL	30 min/Medium	4/10 sec
1-E	Wash 1	Wash Buffer	500 µL	1 min/Medium	4/10 sec
1-F	Wash 2	Wash Buffer	500 µL	1 min/Medium	4/10 sec
1-G	Wash 3	Wash Buffer	500 µL	1 min/Medium	4/10 sec
Elution Strip 1	Elution 1	Denaturing Elution Buffer	100 µL	15 min/Medium	4/10 sec
Elution Strip 2	Elution 2	Denaturing Elution Buffer	50 µL	15 min/Medium	4/10 sec

Table 1. **Pipetting, mixing and collecting instructions for automated immunoprecipitation using the KingFisher® Duo System**

SDS-PAGE and Western Blotting

Immunoprecipitated samples (10 µL of the eluted fractions) and 2 µg of control lysate (reduced and denatured at 70 °C for 10 min) were loaded onto 1 mm thick 4-12% gradient NuPAGE® Bis-Tris gels (Life Technologies). Proteins were separated at 200 V for 35 min. Gels were removed from the cassette and equilibrated for 10 min in transfer buffer (25 mM Tris, 192 mM glycine), supplemented with 10% methanol. After equilibration, the proteins were transferred to Immobilon®-P PVDF membrane using a semidry transfer system for 35 min at 10 V. Blots were briefly rinsed in Milli-Q® Water and assembled directly into the SNAP i.d.® 2.0 blot holder for immunodetection.

Immunodetection using SNAP i.d.® 2.0 system

Each blot processed in the SNAP i.d.® 2.0 system was assembled according to the user guide. Once blot holders were placed in the SNAP i.d.® system, blocking buffer was added and the vacuum immediately activated. Primary antibody (anti-PP2A monoclonal antibody) diluted in blocking buffer was added to the blot holder and incubated for 10 min at room temperature. The vacuum was initiated and blots were washed four times with Tris-buffered saline with Tween® 20 (TBST). After the vacuum was turned off, the blots were incubated with anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody diluted in blocking buffer for an additional 10 min. The vacuum was activated and blots were washed four times with TBST. Probed blots were visualized with 5 mL Luminata™ Forte HRP substrate for 5 minutes. Blots were exposed to x-ray film for 1 min followed by development.

Results

We compared the overall performance and reproducibility of the PureProteome™ Protein G Magnetic Beads in an automated immunoprecipitation process using the KingFisher® Duo particle processor to the performance of a standard manual protocol. Eluted fractions were analyzed by western blotting followed by immunodetection.

Visual inspection of the Western blot (Figure 1) indicated that high-throughput immunoprecipitation using the KingFisher® particle processor and the manual method resulted in an intense, anti-PP2A-reactive band at the expected molecular weight of PP2A (36 kDa). Since the same antibody was used for both immunoprecipitation and detection, immunoglobulin heavy (55 kDa) and light (25 kDa) chains are present in the immunoprecipitated fractions due to the cross-reactivity of the secondary antibody.

All fractions (both using the KingFisher® system as well as the manual method) generated similar results, indicating that PureProteome™ Protein G Magnetic Beads can be used to reproducibly immunoprecipitate PP2A in KingFisher® particle processor.

Conclusions

The results confirm that PureProteome™ magnetic beads are very well suited for an automated immunoprecipitation process using the KingFisher® particle processor. Furthermore, the process is reproducible and is comparable to that of a standard manual protocol using PureProteome™ Magnetic Stand. With any IP experiment, the results will depend on the antibody affinity toward the target protein. Therefore, the time required for the immune complex formation will vary and needs to be empirically determined.

The paramagnetic properties of PureProteome™ magnetic beads make them ideal for automation. Because bead performance is critically dependent on using the right particle processing parameters, such as mixing speed, bead collection time and binding/washing duration, we have carefully optimized these parameters on the KingFisher® system specifically for PureProteome™ beads. Protein G was used in this study; however, the protocol can be implemented for PureProteome™ Protein A and PureProteome™ Protein A/G Mix Magnetic Beads as well, depending on the subtype of the primary antibody.

Because PureProteome™ magnetic beads can be used manually or on any automated system for low, medium or high-throughput sample preparation, they save time and costs for researchers who wish to process multiple samples in parallel.

Ordering Information

Description	Qty/Pk	Catalogue No.
PureProteome™ Protein A Magnetic Beads	2 x 1 mL	LSKMAGA02
	1 x 10 mL	LSKMAGA10
PureProteome™ Protein G Magnetic Beads	2 x 1 mL	LSKMAGG02
	1 x 10 mL	LSKMAGG10
PureProteome™ Protein A/G Mix Magnetic Beads	2 x 1 mL	LSKMAGAG02
	1 x 10 mL	LSKMAGAG10
PureProteome™ Kappa Ig-Binder Magnetic Beads	2 x 1 mL	LSKMAGKP02
PureProteome™ Lambda Ig-Binder Magnetic Beads	2 x 1 mL	LSKMAGLM02
PureProteome™ Magnetic Stand, 8-well	1	LSKMAGS08

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