Dynabeads magnetic beads—the key to successful immunoprecipitation

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Introduction

Immunoprecipitation (IP) is an extensively used method in many different research fields, aimed at isolation of the target antigen or its binding partners for downstream analysis. In its basic form, the method is very simple—just conjugate an antibody to a solid surface, add it to the sample, and pull out the target of interest for use in any downstream analysis.

As protein–protein binding often involves transient and weak interactions, it is critical to use a method that offers rapid binding kinetics and low nonspecific binding. Invitrogen™ Dynabeads™ magnetic beads are nonporous, uniform, superparamagnetic, monodispersed polystyrene beads that are widely used for IP. The coating provides a defined surface area for the adsorption or conjugation of various molecules, in particular antibodies. Bead uniformity and shape provide consistent physical and chemical properties and are instrumental in minimizing nonspecific binding (Figure 1). In addition, optimal binding kinetics and high reproducibility allow for rapid and efficient binding of the target; sometimes binding is completed in less than 10 min. Dynabeads magnetic beads help ensure high antibody-binding capacity and accessibility, low nonspecific binding, and high yield. The chemical properties of the beads also eliminate the pre-clearing step and reduce the consumption of antibodies, making these magnetic beads ideal for IP.

Here we describe automated IP using Dynabeads magnetic beads on the Thermo Scientific™ KingFisher™ Flex instrument. This procedure uses a simple 7-step, 40 min protocol that enables high reproducibility and scalability, with minimal hands-on time (Figure 2). Results of this study show that the parameters of the automated protocol most critical to success include the antibody subtype, washing conditions, and elution volume. The data shown were generated on the KingFisher Flex instrument, but the optimized protocol has also been adapted to the Thermo Scientific™ KingFisher™ Duo Prime and Apex instruments.

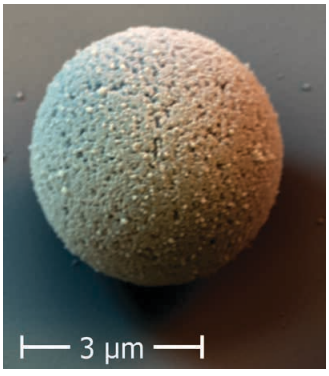

Figure 1. Scanning electron micrograph of Dynabeads magnetic beads. The nonporous, uniform nature of the beads enable low nonspecific binding, high reproducibility, low antibody consumption, and fast binding kinetics.

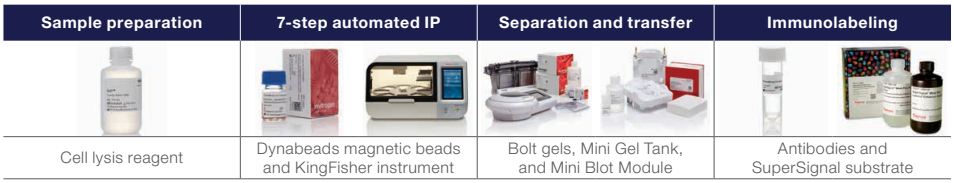

Figure 2. Immunoprecipitation workflow from cell lysis to protein analysis. In the 7-step automated IP protocol, the KingFisher instrument uses magnetic rods with single-use tip combs to collect Dynabeads magnetic beads and move them from plate to plate. This process allows the instrument to carry out the same steps as a manual IP protocol but with greater consistency and minimal hands-on time.

Materials and methods

The KingFisher Flex instrument with Thermo Scientific™ BindIt™ Software was used for automated processing of Invitrogen™ Dynabeads™ Protein G (Cat. No. 10004D). Various parameters of the automated protocol (e.g., incubation time, mixing conditions) were tested by measuring binding efficiency of acridinium-labeled mouse IgG1 to Dynabeads Protein G. Binding efficiency of acridinium-labeled mouse IgG1 was assessed by measuring chemiluminescence on the Centro LB 960 Luminometer (Berthold Technologies).

The complete automated protocol for IP was performed using Invitrogen™ CD9 monoclonal antibody (Cat. No. 10626D) to capture CD9 protein from HeLa cell lysate. A similar procedure for co-IP was performed using cofilin polyclonal antibody (Abcam, Cat. No. ab42824). Invitrogen™ beta-actin polyclonal antibody (Cat. No. PA1-183) was used to detect the binding partner of cofilin. Invitrogen™ Bolt™ gels, Mini Gel Tank, and Mini Blot Module were used for standard electrophoresis and western blotting onto PVDF membranes.

Results

Incubation time for antibody conjugation

Antibodies can be noncovalently coupled to beads using Dynabeads Protein G or Invitrogen™ Dynabeads™ Protein A. The binding efficiency will depend on the antibody subclass and whether Protein A or Protein G are utilized. Here, IgG1 was used to estimate the antibody binding efficiency to Dynabeads Protein G on the KingFisher Flex instrument. The incubation time for binding primary antibodies to magnetic beads varies substantially between protocols. Long incubation times are often used. By measuring chemiluminescence as a result of antibody binding to the beads, the binding efficiency was measured at different incubation times. The data demonstrated that due to rapid binding kinetics, 94% of the antibodies were bound to the beads after only 10 min, and extending the incubation time to overnight did not result in further gains (Figure 3).

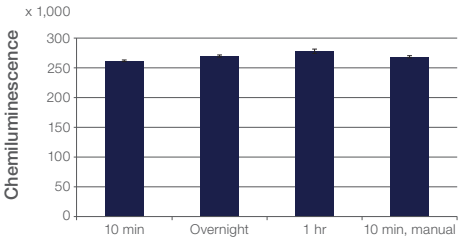

Figure 3. Incubation time for binding of antibody to Dynabeads Protein G. Acridinium-labeled mouse IgG1 was incubated with Dynabeads Protein G for 10 min, 1 hr, or overnight using the KingFisher Flex instrument. Chemiluminescence was then measured to determine binding efficiency. The binding efficiency was compared with the manual protocol using 10 min incubation time.

Mixing conditions during washing of conjugated antibody

To avoid loss of antibodies and ensure the highest yield, it is critical to select the optimal mixing conditions during the antibody conjugation washing step. A variety of mixing conditions and devices are used when conjugating antibodies and targets to solid surfaces such as magnetic beads. Three different mixing conditions were tested on the KingFisher Flex instrument during the washing step, following antibody binding. The mixing conditions selected were slow (baseline), medium, or fast. Chemiluminescence was then used to measure binding efficiency (Figure 4). By performing medium mixing during the washing steps, 4% of the conjugated antibodies were lost from the surface of the magnetic beads compared to slow mixing. By increasing the mixing intensity even further by using the fast condition, 32% of the antibodies were lost. Therefore, to avoid loss of antibodies from the bead surface (leading to reduced target yield), mixing of the antibody-coated beads should be performed in a gentle manner.

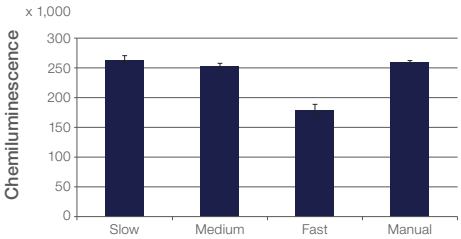

Figure 4. Comparison of mixing speeds during washing of conjugated antibody. After conjugation to acridinium-labeled mouse IgG1, Dynabeads Protein G was exposed to different mixing conditions during washing to measure the potential reduction in binding efficiency. The binding efficiency was compared with manual mixing.

Number of washing steps after antibody conjugation

The number of washing steps used after antibody conjugation varies significantly depending on the target, sample type, and objectives. Here, the effect of the number of washing steps was examined to ensure minimal loss of primary antibody (which impacts target yield). The effect of 1, 2, or 3 washing steps was examined using the KingFisher Flex instrument. The loss of acridinium-labeled mouse IgG1 was measured by chemiluminescence (Figure 5). Increasing the number of washing steps from 1 to 2 resulted in 8% loss of antibody from the beads. Adding a third washing step resulted in 12% of the antibody being lost. The results indicate that using excessive washing steps after antibody binding may result in partial loss of antibody from the surface of Dynabeads Protein G.

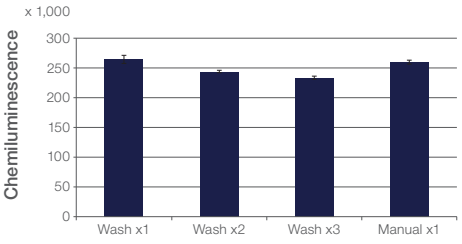

Figure 5. Number of washing steps to preserve antibody binding. Acridinium-labeled mouse IgG1 bound to Dynabeads Protein G was exposed to different numbers of washing steps (1–3) to measure the potential drop in binding efficiency. The binding efficiency was compared with manual washing (x1).

Washing volume after antibody conjugation

Washing volume is another parameter that varies significantly in different workflows and laboratories. The effect of the volume of washing buffer used after antibody binding was studied in the range of 100–500 µL per sample (Figure 6). A volume of 200 µL of washing buffer was used as the baseline (100%). Reduction of volume to 100 µL resulted in 3% loss of antibody. Increase of volume to 500 µL resulted in 5% loss of antibody. These findings indicate that the volume of the washing buffer does not significantly impact the loss of primary antibody from the Dynabeads Protein G surface.

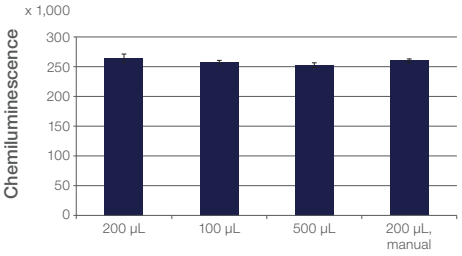

Figure 6. Effect of washing buffer volume on binding efficiency. Acridinium-labeled mouse IgG1 bound to Dynabeads Protein G was exposed to different volumes of washing buffer (100–500 µL) to measure the potential reduction in binding efficiency. The binding efficiency was compared with manual washing using 200 µL.

Binding temperature during antibody conjugation

Antibody binding and target capture at lower temperatures (4°C) are very common when performing IP. Antibody binding was analyzed at 4°C and room temperature, with incubation time set to 10 min. Only a 9% difference in signal was observed for incubation at room temperature (91%) compared to 4°C (100%), indicating minor temperature sensitivity of the antibody capture (Figure 7).

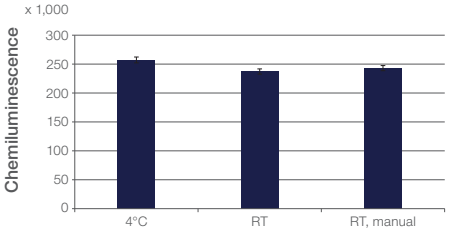

Figure 7. Effect of temperature on binding efficiency. Binding of acridinium-labeled mouse IgG1 to Dynabeads Protein G at room temperature (RT) was compared to low temperature (4°C). Binding efficiency was measured by chemiluminescence.

Effect of antibody concentration and isolation volume

To illustrate the effect of antibody concentration and isolation volume, the amount of antibody and the amount of magnetic beads was kept constant (5 µg antibody and 50 µL Dynabeads Protein G) while the isolation volume varied from 100 µL to 1 mL (200 µL was used as baseline) (Figure 8). Decreasing the antibody isolation volume from 200 µL to 100 µL (increasing the antibody concentration by 2x) increased the amount of antibody bound to the beads by 3%. Increasing the volume from 200 µL to 500 µL (reducing the antibody concentration by 0.4x) reduced the amount of antibody bound to the beads by 8%. When increasing the volume to 1,000 µL, the binding was reduced by 16%. These data illustrate the importance of selecting the optimal antibody isolation volume and concentration in order to obtain the best possible signal after target capture. However, the difference in results is not dramatic due to the robustness of the Dynabeads magnetic beads.

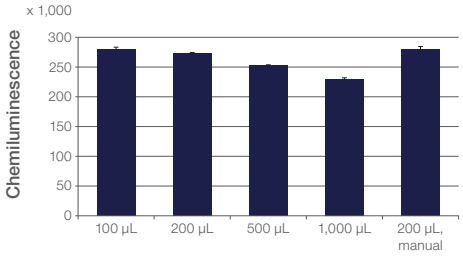

Figure 8. Effect of capture volume on binding efficiency. Binding of acridinium-labeled mouse IgG1 to 50 µL of Dynabeads Protein G was measured using 5 µg of antibody in various buffer volumes. Binding efficiency was measured by chemiluminescence. For comparison, the same quantities of antibodies and beads were incubated in 200 µL buffer using the manual approach.

Effect of different antibody subclasses on antibody conjugation

In IP, many different antibody subclasses are utilized. The ability of different subclasses to bind to Protein G on the magnetic beads varies significantly, which is important for optimization of the IP workflow. Figure 9 shows binding of different antibody subclasses to Dynabeads Protein G. The amount of antibody added was the same for each subclass (5 µg; general recommendations are 1–10 µg per 1.5 mg (50 µL) of Dynabeads Protein G). IgG1 was used as the reference point, and binding efficiency was presented as a percentage of IgG1 (Figure 9). When subclass IgG2a was tested, the binding efficiency was reduced to 55% compared to IgG1. For IgG2b, the binding was somewhat higher (79%). Finally, polyclonal rabbit IgG was tested and resulted in 62% binding. These observations highlight the importance in selecting the right antibody subclass to obtain maximal binding efficiency.

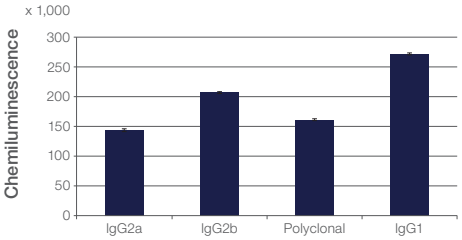

Figure 9. Binding efficiencies of 4 different antibody subclasses to Dynabeads Protein G. Binding efficiencies of IgG2a, IgG2b, polyclonal rabbit IgG, and IgG1 were compared by measuring chemiluminescence. Antibodies of different subclasses were bound to Dynabeads Protein G using the fully automated protocol on the KingFisher Flex instrument.

Background signal

To obtain the best possible signal-to-noise ratio, it is important to have very low nonspecific binding, especially if the target of interest is rare. In this experiment, nonspecific binding was estimated by binding irrelevant primary antibody to Dynabeads Protein G followed by exposure of the beads to cell lysate. The washing volumes tested were 100 µL, 200 µL, or 500 µL (Figure 10). After gel electrophoresis of the eluted total protein, nonspecific binding was revealed by silver staining of the gel. Cell lysate and antibody solution were included for comparison (Figure 10). The data demonstrate very low background, almost exclusively related to the antibodies.

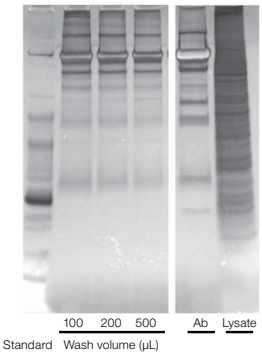

Figure 10. Assessment of nonspecific binding. Dynabeads Protein G incubated with irrelevant antibody were incubated with cell lysate and washed with different volumes of buffer. Total protein was eluted and subjected to electrophoresis. Silver staining of the gel was used to detect nonspecific binding. For comparison, irrelevant antibody and cell lysate were included.

Immunoprecipitation

Protein target was captured using the optimized and automated protocol on the KingFisher Flex instrument, followed by gel electrophoresis and western blot analysis. Dynabeads Protein G were first coated with primary antibody targeting tetraspanin CD9. The antibody-coated magnetic beads were then used to isolate CD9 from HeLa cell lysate. The volumes of the wells were a limiting factor (20–30 µL), so the target was eluted in a volume compatible with the well volume. For applications not limited by well size, increased elution buffer volume may increase the yield (Figure 11). The sample was then subjected to electrophoresis and western blotting. The blot was labeled with CD9 antibody to detect the isolated protein (Figure 12). Similar results were obtained for co-IP where cofilin was pulled out of the lysate and the cofilin-binding protein actin was detected by western blotting (Figure 13). The blot demonstrated high yield of the target and low nonspecific binding.

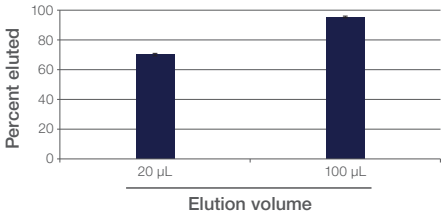

Figure 11. Effect of elution volume on release efficiency. The amount of antibody released from Dynabeads Protein G was measured using 20 µL or 100 µL elution buffer (low pH, glycine-based buffer). The data are presented as percent eluted vs. total bound antibody prior to elution.

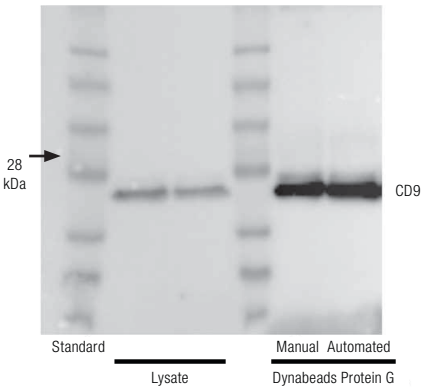

Figure 12. IP of CD9 protein. HeLa cell lysate was incubated with Dynabeads Protein G coated with CD9 antibody using a manual or automated workflow. The samples were analyzed by electrophoresis and western blot using CD9 antibody.

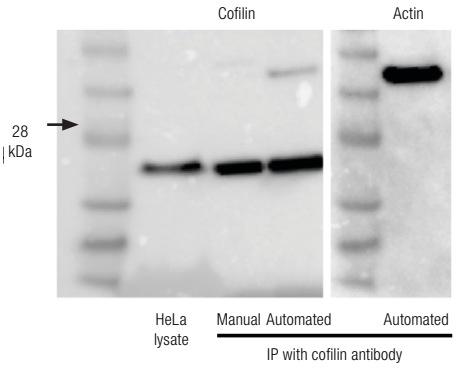

Figure 13. Co-IP of cofilin and actin. HeLa cell lysate was incubated with Dynabeads Protein G coated with cofilin antibody using a manual or automated workflow. The samples were analyzed by electrophoresis and western blot using cofilin antibody or actin antibody.

Discussion

Immunoprecipitation is a multistep process that includes manual handling of the samples, which is prone to user error and user-to-user variation. The automated KingFisher platform helps ensure high reproducibility (CV of 5%) and offers the ability to handle multiple samples at a time. An additional benefit is significantly reduced hands-on time. In our experience, hands-on time for manual handling of 20 samples is about 35 min, while hands-on time for preparing plates with reagents for the automated protocol takes about 13 min.

Establishment of a 7-step automated IP protocol calls for an in-depth understanding of every step of the protocol in order to develop a robust and reproducible method. Detailed examination of the process using the KingFisher Flex instrument revealed several critical parameters. Antibodies suitable for IP come in different concentrations and quality. Care should be taken to test and select the best possible capture antibody (clones and subclasses). The volume of antibodies is not critical for efficient conjugation of the antibodies to the magnetic beads. The binding of the antibody to the magnetic beads is a rapid process due to fast binding kinetics. Usually, most of the antibody is bound within 10 min; extending the incubation time will not result in coupling more antibodies to the bead surface. The critical factor is the amount of antibodies (µg) added per mg of magnetic beads. Extending the incubation time with the sample should be considered if target yield is low. This potential increase in yield may be at the expense of increased nonspecific binding; more stringent washing conditions may counteract this effect. Tween™ 20 or Triton™ X-100 detergent can be added to the washing buffer in concentrations between 0.01 and 0.1%. Alternatively, the number of washing steps may be increased, and the duration of each washing step may be extended. The elution efficiency depends on the elution volume, with increased volume resulting in higher efficiency.

Conclusion

A rapid, 7-step automated protocol for IP has been established using Dynabeads magnetic beads on the KingFisher platform. This 40 min protocol enables high reproducibility and scalability, with minimal hands-on time for 10 or 96 samples in one run. Scripts are available for the KingFisher Flex, Duo Prime, and Apex instruments. The automated protocol allows flexibility in each step if further fine-tuning is required.

Ordering information

Product	Quantity	Cat. No.
Dynabeads Protein G for Immunoprecipitation	5 mL	10004D
DynaMag-2 Magnet	1 each	12321D
CD9 Monoclonal Antibody	200 µL	10626D
Mini Gel Tank	1 each	A25977
Mini Blot Module	1 each	B1000
Bolt 4-12% Bis-Tris Plus Gels, 10-well	10 gels	NW04120BOX
4X Bolt LDS Sample Buffer	10 mL	B0007
Bolt Western Pack B (PVDF)	1 kit	B1000B
SeeBlue Plus2 Pre-stained Protein Standard	500 µL	LC5925
SuperSignal West Dura Extended Duration Substrate	20 mL	37071
KingFisher Duo Prime Purification System	1 each	thermofisher.com/kingfisher
KingFisher Flex Purification System*	1 each	
KingFisher Apex Purification System*	1 each	

* For Laboratory Use.

Find out more at **thermofisher.com/immunoprecipitation**

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