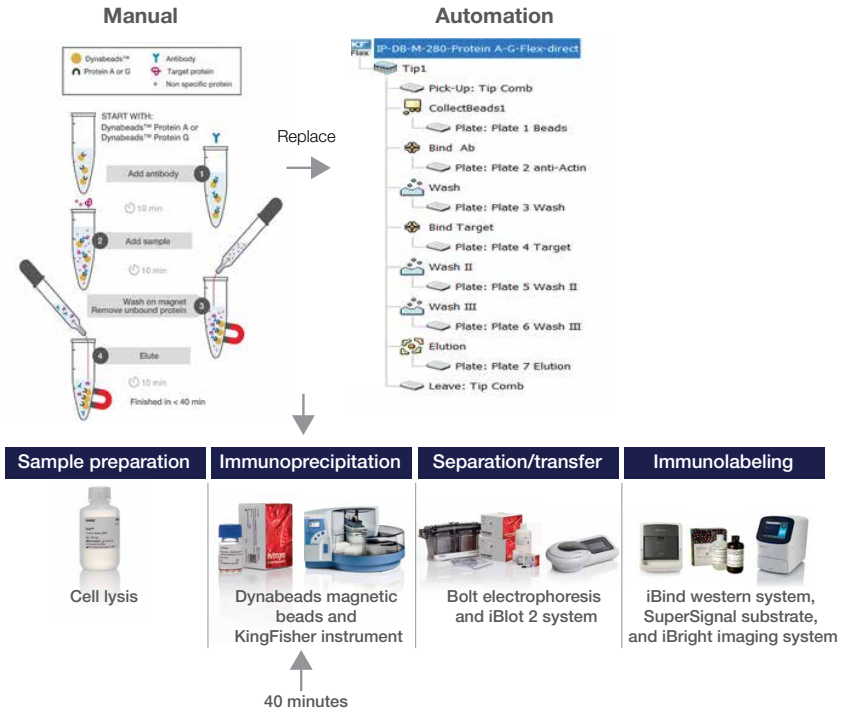
Automated immunoprecipitation with seamless integration to protein analysis

Introduction

Immunoprecipitation (IP) is an extensively used method in many different research fields, aimed at isolating a target antigen or its binding partners for downstream analysis. In its basic form, this complicated and lengthy procedure is performed manually. As protein–protein complexes often involve transient and weak interactions, it is critical to use a workflow that offers rapid binding kinetics and low nonspecific binding, in combination with minimized manual processes, to increase reproducibility and allow rapid simultaneous processing of several samples.

Here we present an automated IP method with seamless integration to protein analysis. IP was performed using Invitrogen™ Dynabeads™ magnetic beads on the automated Thermo Scientific™ KingFisher™ Duo Prime and Flex platforms. Protein transfer and labeling were automated using the Invitrogen™ iBlot™ 2 Gel Transfer Device for dry blotting and the iBind™ Western Device (Figure 1).

Automated IP with downstream integration to protein analysis ensures high yield and reproducibility, and low nonspecific binding with significant reductions in total and hands-on time compared to the manual protocol.



Benefits of Dynabeads products:

- Properties:
  - Monosized
  - Nonporous
  - Superparamagnetic
- Low nonspecific binding
- High reproducibility
- Low antibody consumption
- Fast binding kinetics
- IP critical factors
  - Washing steps
  - Mixing (washing)
  - Elution volume

Figure 1. Automated protein capture and detection workflow—from cell lysis to protein analysis.

Performing IP and downstream protein analysis is a time-consuming and labor-intensive process that can take up to 2–3 days before results can be evaluated. With this seamless IP and protein analysis workflow, it is now possible to evaluate the results in less than a day—all with minimal hands-on activities. The automated IP workflow provides a 20 minute reduction in time, compared to a manual workflow. The time is reduced by 1 hour when performing an automated blotting procedure, and finally the time can be reduced to 1 day when performing an automated immunolabeling procedure.

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Immunoprecipitation—reduced hands-on time

The manual IP protocol starts with addition of the primary antibody to the Dynabeads magnetic beads, followed by a 10 min incubation step. Unbound antibodies are washed off; the functionalized Dynabeads beads are then incubated with the sample containing the target of interest. Several washes are included to reduce the nonspecific binding, and finally the target is eluted. A considerable number of hands-on manipulations are required when performing IP manually. These procedures are tedious and error-prone, compared to quick up-front setup followed by automated IP on the KingFisher platform. To estimate the hands-on time saved by automated vs. manual IP, a stop-watch analysis was performed when handling 24 samples at a time (Table 1). Hands-on activities for the manual protocol included adding the beads to a tube, washing the beads after antibody binding, and three washes after target capture. Hands-on activities for the automated approach only included loading plates with the beads, reagents, and samples according to the protocol. The results demonstrated a significant reduction in hands-on time—for processing 24 samples, automated IP saved 22 minutes (63%) compared to manual IP. Importantly, in the automated approach, the hands-on time was solely limited to the initial phase of the workflow, while manual IP required the presence of the operator throughout the entire protocol.

Western blotting and immunolabeling—reduced protocol time

Proteins separated by electrophoresis are often transferred to a membrane (nitrocellulose, PVDF) by "wet transfer", which involves multiple manual procedures and requires preparation of several reagents and buffers. The blotting takes up to 60 min to complete. For the automated approach using the iBlot 2 Gel Transfer Device, where preparation of reagents, buffers, and stacking gel is not required, approximately 50 min were saved on the transfer process compared to wet transfer (Table 1). Even more time is saved when the labeling process is automated using the iBind Western Device. Immunolabeling of the blot usually includes several manual steps—blocking, washing and labeling, where extended labeling times (e.g., overnight) are frequently used. Limiting the hands-on activities to preparation of reagents required for all the steps in the labeling protocol, and using the iBind device for labeling, saves a significant amount of time (Table 1).

Automating conjugation of primary antibody to beads—increasing reproducibility

Reproducibility is critical when comparing data between different experiments. The KingFisher Flex instrument was utilized to assess the reproducibility of the antibody conjugation process. An acridinium-based assay was used where the amount of acridinium-labeled antibodies bound to Invitrogen™ Dynabeads™ Protein G was measured. A total of 24 samples were analyzed by two operators on two different days (Table 2). The data were presented as chemiluminescence

Table 1. Comparison of hands-on time using manual vs. automated approach for protein capture and detection, for 24 samples.

Hands-on time for 24 samples—manual vs. automated IP		
Protocol steps	**Manual**	**Automated**
Add antibody to Dynabeads beads	11 min	13 min (plate loading)
Incubate antibody–beads complex	10 min (incubation)	
Wash 1	6 min	
Capture target	10 min (incubation)	
Wash 2	18 min	
Wash 3		
Wash 4		
Elute target	10 min (incubation)	
Total hands-on time	**35 min**	**13 min**
Western blotting		
	Manual (1 blot)	Automated (1 blot)
Running time	60 min	7 min
Immunolabeling		
	Manual (1 blot)	Automated (1 blot)
Running time	2 days	<7 hr

and the coefficient of variation (CV) was calculated. The results showed a CV of only 5%, emphasizing that automation is very robust, and it minimizes the user variation that is typically significant for the manual protocol.

Automating IP—reducing background

To obtain a superior signal-to-background ratio, it is critical to have low nonspecific binding, especially if the target of interest is rare. In this experiment, nonspecific binding was estimated by binding irrelevant primary antibodies to Dynabeads Protein G beads followed by exposure to cell lysate and electrophoretic analysis with gel silver-staining. Cell lysate and antibody solution were included for comparison. IP was performed manually or automated on the KingFisher instrument, in triplicate (Figure 2). The results confirmed high reproducibility and low background for the automated approach.

Optimizing the automated IP protocol

An optimized, automated "walk-away" IP protocol was run on the KingFisher Duo Prime and Flex instruments and compared with the manual protocol (Figure 3). The target protein was captured, followed by gel electrophoresis and manual western blotting and immunolabeling analysis. Dynabeads Protein G were first coated with Invitrogen™ CD81 Monoclonal Antibody. The antibody-coated beads were then used to isolate CD81 from cell lysate. The results confirmed good performance on the two instruments, equivalent to those obtained with the manual protocol.

Table 2. Reproducibility estimated using an acridinium assay. The amount of acridinium-labeled antibodies bound to Dynabeads Protein G was measured by two different operators over two days. The data are presented in chemiluminescence units.

Day 1	Day 2	
Operator A	Operator A	Operator B
231,884	257,407	256,915
234,854	257,253	263,366
233,403	253,504	260,675
230,895	259,065	264,146
234,249	258,486	256,563
238,061	257,595	260,865
230,513	261,522	259,933
233,182	258,035	257,354
Mean ± SD: 250,405 ± 12,564 **CV:** 5%		

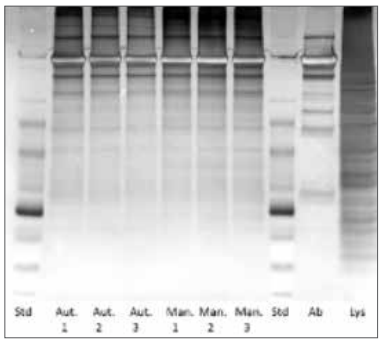

Figure 2. Estimation of background in results obtained using manual and automated procedures performed in triplicate and visualized by silver staining. The results confirmed high reproducibility and low background for the automated protocol.

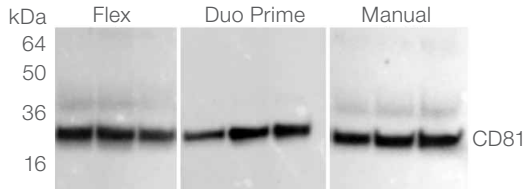

Figure 3. IP of CD81 using manual methods vs. KingFisher Flex and Duo Prime instruments. Jurkat cells expressing CD81 were lysed and incubated with Dynabeads Protein G coated with an anti-CD81 antibody using manual and automated methods in triplicate. The samples were prepared for electrophoresis and western blotting, and labeling was performed with the anti-CD81 antibody.

Automating IP, protein transfer, and immunolabeling

To avoid manual operations during protein transfer and immunolabeling, the automated IP protocol was used in combination with the iBlot 2 and iBind devices, and compared with manually performing wet transfer and labeling prior to analysis (Figure 4). The results clearly demonstrated that automated protein transfer in combination with automated immunolabeling was a highly efficient method and produced data equivalent to the manual protocol, thus dramatically reducing hands-on time.

Conclusions

IP and western blotting are time-consuming, multistep processes that include several manual steps of sample handling, which are prone to user errors and user-to-user variation. An automated workflow ensures a time-efficient and robust platform consistently generating high-quality data. The 7-step IP protocol on the KingFisher platform significantly reduced hands-on time and ensured consistent protein capture with high reproducibility (5% CV). The eluate generated at the final step of the IP protocol was adjusted in volume to be perfectly integrated with the Invitrogen™ Bolt electrophoresis system. Automating the transfer of proteins from gels to membranes helped reduce the total workflow time by 1 hour. Equally important, several manual handling steps were removed from the workflow, minimizing potential user errors.

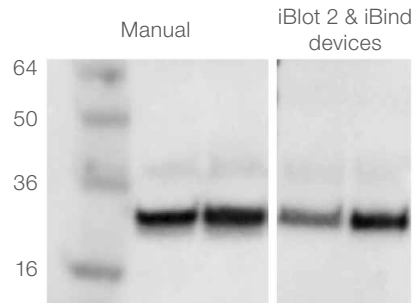

Figure 4. IP of CD81 using KingFisher instrument, and manual or automated blotting and labeling. Jurkat cells expressing CD81 were lysed and incubated with Dynabeads Protein G coated with anti-CD81 antibody using the automated approach in duplicate. The samples were prepared for electrophoresis and western blotting, and labeling was performed with the anti-CD81 antibody.

Ordering information

Product	Cat. No.
NP40 Cell Lysis Buffer	FNN0021
4X Bolt™ LDS Sample Buffer	B0007
Mini Gel Tank	A25977
Mini Blot Module	B1000
Bolt™ 4-12% Bis-Tris Plus Gels, 10-well	NW04120BOX
Bolt™ Western Pack B (PVDF)	B1000B
iBind Western Device	SLF1000
iBlot 2 Gel Transfer Device	IB21001
SeeBlue™ Plus2 Pre-stained Protein Standard	LC5925
SuperSignal™ West Dura Extended Duration Substrate	37071
DynaMag™-2 Magnet	12321D
Dynabeads Protein G for Immunoprecipitation	10004D
CD81 Monoclonal Antibody (M38)	10630D
KingFisher Duo Prime Purification System	5400110
KingFisher Flex Purification System	5400620

Dynabeads Protein G for Immunoprecipitation (Cat. No. 10004D) were used with Trueblot™ HRP-coupled anti-mouse IgG, Rockland (US), IgG sub-classes, Jurkat cells, NP40 Cell Lysis buffer, and anti-CD81 antibody. KingFisher platform with the Thermo Scientific™ BindIt™ Software (4.0) was used for IP automation. Downstream analysis included standard electrophoresis, and western blot with Bolt gels. The Berthold Centro LB 960 Luminometer was used for measuring chemiluminescence. For automated blotting and labeling, iBlot 2 and iBind devices were utilized.

Finally, by automating the immunolabeling of the blot, the hands-on time was limited to only the preparation of reagents used for the labeling, and the total protocol time was significantly reduced.

To summarize, a seamless integrated workflow has been established for IP and western blot with the following advantages over the manual workflow: (1) significantly shorter protocol time, (2) dramatically reduced “hands-on” time, and (3) consistent top results and robustness—due to elimination of “user-to-user” variation.

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

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