Proteomics

Mass spectrometry–based proteomics workflow using Dynabeads Protein G and the KingFisher Flex Purification System for co-immunoprecipitation of YAP and its binding partners

Keywords
Dynabeads Protein G, non-small cell lung cancer research, NSCLC, mass spectrometry, western blot, yes-associated protein, YAP, TEAD, BAG3, Hsp70, KingFisher Flex Purification System, automation, proteomics

Highlights:

- Invitrogen™ Dynabeads™ Protein G efficiently captured YAP using an automated workflow with the Thermo Scientific™ KingFisher™ Flex Purification System.
- Dynabeads Protein G greatly reduces sample complexity by 90% compared to the full cell lysate and enables better resolution of low-abundance proteins.
- Captured YAP was able to isolate TEAD4 and the Hsp70-BAG3 chaperone complex.

Introduction

The yes-associated protein (YAP) is a tightly regulated nuclear transcription co-activator. In non-somatic cells, YAP is closely regulated by the Hippo signaling pathway. Activation of the Hippo signaling pathway promotes phosphorylation of S127 on the TEAD binding domain of YAP, which inactivates YAP and prevents its nuclear translocation. Instead, YAP is targeted for destruction by 14-3-3 proteins and the ubiquitin proteasome complex (Figure 1A). The binding of BAG3 with heat shock protein 70 (Hsp70) (protein product of the *HSPA9* gene) dephosphorylates YAP by releasing AMOT. This allows the binding of YAP to the BAG3-Hsp70 complex. This complex then chaperones YAP to the nucleus where it can bind to a range of nuclear transcription binding partners (Figure 1B) [1].

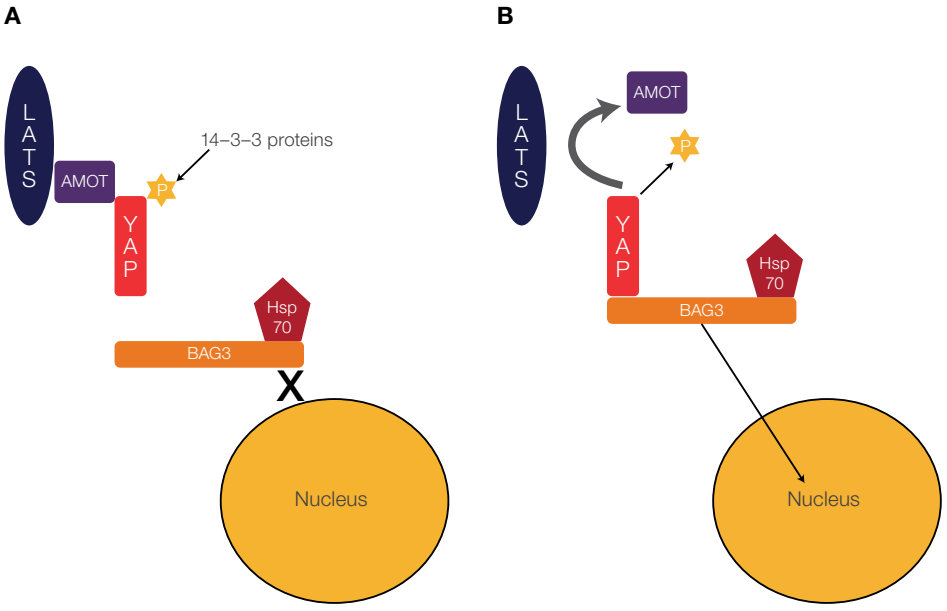

Figure 1. An illustration of the YAP-BAG3-Hsp70 chaperone complex. (A) Through the Hippo signaling pathway, YAP is phosphorylated on S127 from the LATS1/2-AMOT complex. The LATS1/2-AMOT complex then recruits 14-3-3 ubiquitin to the target and degrades YAP. **(B)** Through the YAP-BAG3-Hsp70 complex, AMOT is released and dephosphorylates YAP, which prohibits destruction of YAP and allows it to be translocated to the nucleus.

Some of the known nuclear transcription factors that bind to YAP include the P53 homolog P73 and TEAD1–4 [2]. These interactions promote genes responsible for cell survival and growth that render the cancer cell challenging to treat.

In recent years, YAP has been identified as an oncogenic driver in many cancers (lung, ovarian, pancreatic, breast, and prostate cancers) and is therefore at the forefront of cancer research [3]. It has also been found to drive drug resistance [4] that results in an aggressive phenotype, which promotes epithelial to mesenchymal transition following binding to its downstream transcription factors.

YAP is also of interest because it is characterized by nine isoform variants. Different splice variants have been found to be either oncogenic and cancer driving, or to be protective factors that lead to favorable outcomes. The key is for researchers to understand and find potential binding partners of YAP that may lead to new discoveries.

Proteomics research is a dynamically growing field. It enables direct discovery of biomarkers, which are protein targets for novel diagnostic, prognostic, or therapeutic purposes. Due to the high degree of complexity and dynamic range of the proteome, a key challenge in proteomics research is the detection of low-abundance proteins with optimal sensitivity as well as a robust and reproducible sample prep. Immunoprecipitation (IP) directly addresses this limitation by enriching specific target proteins or protein complexes and thereby reducing sample complexity.

Mass spectrometry (MS) is a powerful analytical technique widely used in proteomics research due to its sensitivity and specificity. It plays a crucial role in the identification and characterization of proteins within complex biological samples. Co-immunoprecipitation (co-IP) is a demanding, hands-on method that involves indirect capture of proteins that are bound to a specific target protein. A solution to the problem of high complexity is the use of automated workflows for accurate, reproducible, and standardized results that help researchers obtain the most information from every co-IP.

Here, we demonstrate the use of Dynabeads Protein G in a robust workflow for capturing YAP and its binding partners TEAD4, BAG3, and Hsp70.

Isolation of YAP–TEAD4/BAG3/Hsp70 using the KingFisher Flex Purification System

Western blot was used to demonstrate the ability of Dynabeads Protein G in a workflow (Figure 2) to successfully and rapidly capture YAP and several of its known binding partners. The Dynabeads Protein G conjugated with anti-YAP antibody successfully captured YAP. Dynabeads Protein G conjugated with an irrelevant antibody (anti-CD81) or beads without antibody failed to capture YAP (Figure 3A). Dynabeads Protein G conjugated with an anti-YAP antibody that captured YAP also successfully captured TEAD4, BAG3, and Hsp70 (Figures 3B, 3C, and 3D, respectively).

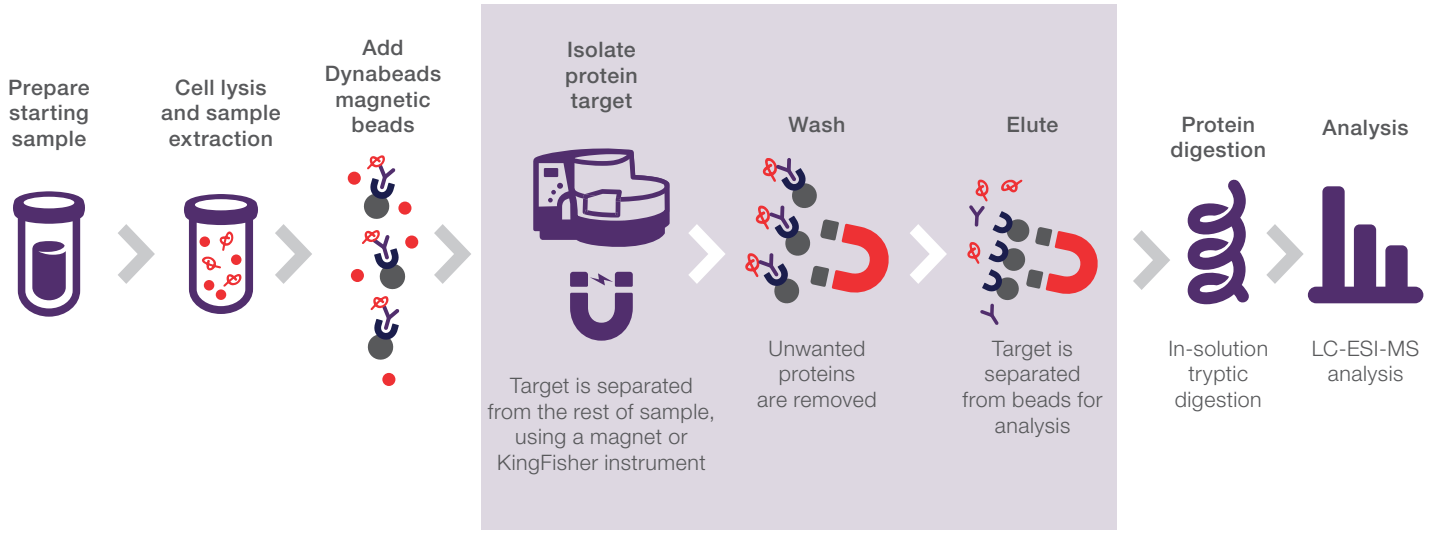

Figure 2. Overview of the workflow for co-IP.

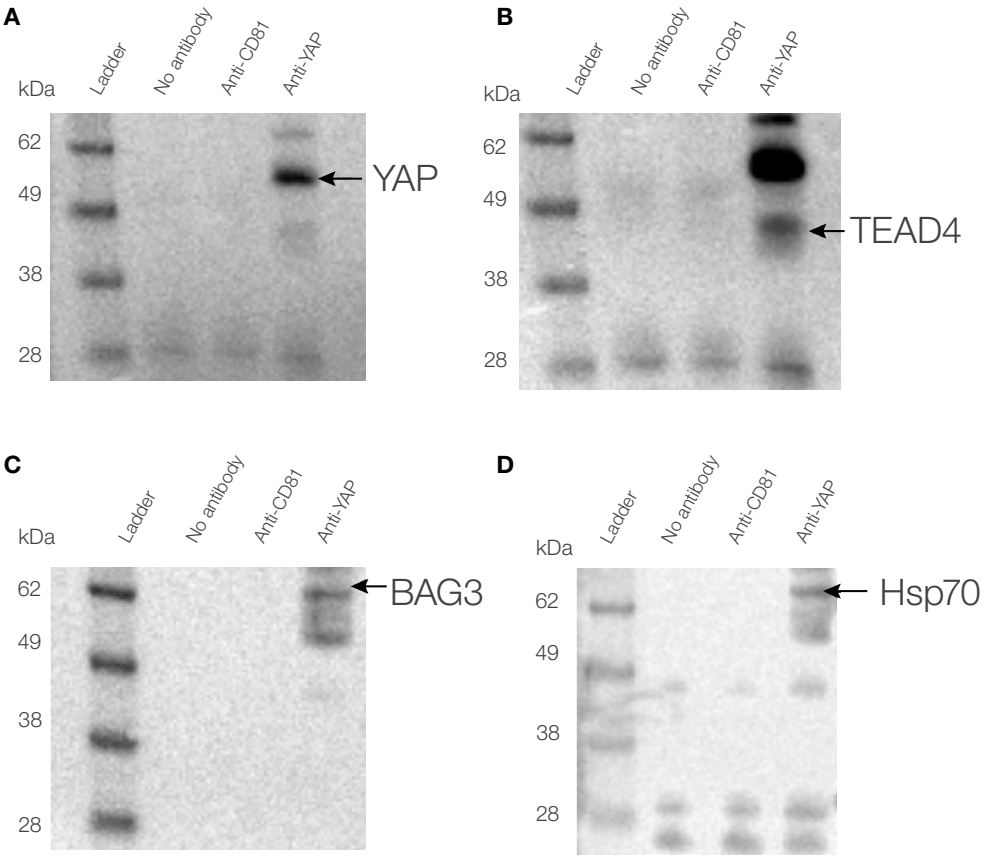

Figure 3. Co-IP of YAP and its binding partners TEAD4, BAG3, and Hsp70. (A) Successful capture of target protein YAP was only seen after the Dynabeads Protein G was conjugated with anti-YAP antibody. Dynabeads Protein G conjugated with anti-CD81 antibody or beads without antibody did not show any binding of the target protein YAP. **(B)** Successful co-IP of YAP also captured the TEAD4 transcription factor. **(C)** BAG3, part of the Hsp70–YAP chaperone complex, was captured after Dynabeads Protein G was conjugated with anti-YAP antibody. **(D)** Through capture of BAG3 by YAP, Hsp70 was also isolated following Dynabeads Protein G conjugation with anti-YAP antibody. Dynabeads Protein G conjugated with anti-CD81 antibody or beads without antibody failed to capture YAP and thus did not co-immunoprecipitate TEAD4, BAG3, or Hsp70.

Automated co-IP and MS analysis methods

A549 NSCLC adenocarcinoma cell lines were chosen for their endogenous expression of YAP. Thermo Scientific™ RIPA Lysis and Extraction Buffer (Cat. No. 89900) was used to lyse 2 x 10^6 cells for 15 min on ice before centrifugation at 14,000 x *g* for 15 min. The supernatant was carefully removed, leaving behind the cell debris and genetic material, and loaded into a Thermo Scientific™ KingFisher™ 96 Deep-Well Plate.

The Invitrogen™ YAP1 Polyclonal Antibody (Cat. No. PA578321) and the CD81 Monoclonal Antibody (M38) (Cat. No. 10630D) were diluted to 5 µg/IP in PBS with 0.05% Tween™ 20 detergent (PBST). Dynabeads Protein G (Cat. No. 10003D) was then added to another KingFisher 96-well plate at 1.5 mg/IP. The KingFisher plate setup is described as follows:

Plate 1. Elution mix: 1X Invitrogen™ Bolt™ LDS (Cat. No. B0007) in dH_2O, total volume 30 µL

Plate 2. Tip comb

Plate 3. Dynabeads Protein G

Plate 4. Antibodies in PBST, total volume 200 µL

Plate 5. Wash I: PBST

Plate 6. 200 µL cell lysate

Plate 7. Wash II: 200 µL PBS

Plate 8. Wash III: 200 µL PBS

The automation script for the KingFisher Flex Purification System ran for 40 min and the eluate was collected for further evaluation.

Dynabeads Protein G conjugated with anti-YAP antibody was analyzed in duplicate, while Dynabeads Protein G conjugated with an irrelevant antibody (anti-CD81) and nonconjugated beads were analyzed in triplicate. To evaluate the enrichment factor, the co-IP samples were compared to a full cell lysate in which the whole proteome of the cells was analyzed after lysis without any prefractionation (single analysis).

After the co-IP step, the beads were washed and proteins were digested off the beads into peptides in 50 mM ammonium bicarbonate buffer (pH 8) using 1 µg trypsin per sample. The cell lysate sample was digested using 5 µg trypsin in 50 mM ammonium bicarbonate buffer (pH 8). After overnight digestion, the supernatants containing the peptides were collected and desalted with solid-phase extraction tips (ZipTip™ Pipette Tips). Desalted samples were reconstituted in 10 µL of 2% acetonitrile and 0.1% formic acid and then analyzed with MS. Here, the samples were subsequently subjected to LC/MS analysis using a reverse- phase nano-HPLC system coupled to a high-resolution mass spectrometer that was operated in data-dependent mode. The LC/MS system was operated using 1 hr analysis time per sample.

The MS data were processed using the PEAKS™ Studio software (v11.6—PEAKS Studio Xpro) and searched against the canonical human SWISS-PROT™ database (20,435 entries). Results were analyzed in Microsoft™ Excel™ software, with sequence coverages and signal intensities, expressed as peak areas, compared to each other. Here, results of replicates were averaged and proteins were shortlisted if they appeared to be enriched with Dynabeads Protein G conjugated with anti-YAP antibody relative to the negative controls; in other words, a significantly increased sequence coverage or higher signal intensities were present in the Dynabeads Protein G anti-YAP samples. Shortlisted proteins were further evaluated by investigating the detailed replicate data.

Mass spectrometry confirmed successful co-IP of Hsp70

After performing a co-IP using the KingFisher Flex Purification System, LC/MS analysis was conducted on the co-IP samples and the full cell lysate identifying 5,115 proteins in total. The majority of proteins were identified in the full cell lysate (5,055). The sample complexity was drastically reduced in the bead samples, with 379 proteins identified in the Dynabeads Protein G conjugated with anti-YAP antibody sample, 698 proteins identified in the Dynabeads Protein G conjugated with anti-CD81 antibody sample, and 410 proteins identified in the nonconjugated Dynabeads Protein G sample (Figure 4).

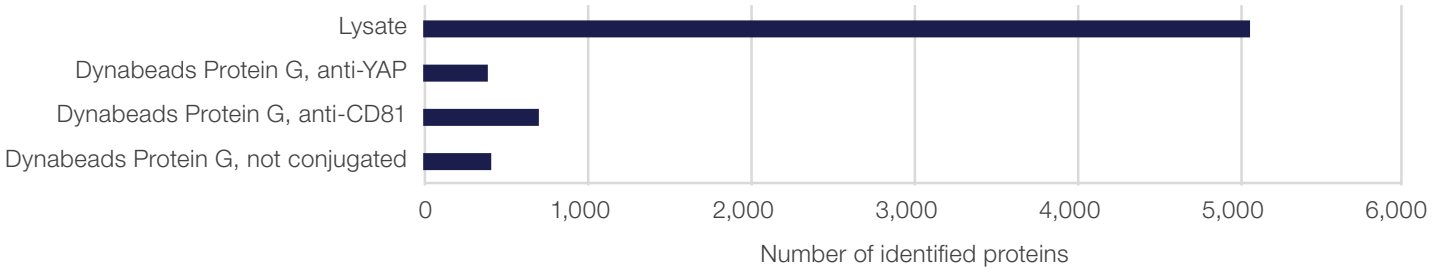

Figure 4. Dynabeads Protein G facilitates the enrichment for proteins of interest, greatly reduces sample complexity, and enables better resolution of low-abundance proteins. 90% of the sample complexity is reduced using Dynabeads Protein G compared to the full cell lysate.

Mass spectrometry is semiquantitative, and signal intensities are dependent on the physicochemical properties of the analyte and its surrounding matrix. Therefore, only signal intensities originating from the same molecule (same peptide) can be directly compared. For the data analysis, the sequence coverage of proteins and summed intensity of the mass spectrometer signals of corresponding peptides (expressed in the peak area) were compared to find potential candidates of co-immunoprecipitated proteins. The sequence coverage of a protein expresses the amount of covered sequence (by number of uniquely identified peptides) in relation to the full sequence by percentage. A higher sequence coverage of a protein (i.e., more identified peptides of a protein) may indicate a larger signal-to-noise ratio, a larger enrichment, and more abundance of that protein in the sample. The summed peak areas are a representation of the summed signal intensity of a protein, and a higher peak area may indicate a larger enrichment or higher presence of that protein in a sample. A combination of this information was used to shortlist candidates in the co-IP experiment. Care was taken to rule out false positive hits, and the nonspecific background was evaluated using beads conjugated to an irrelevant antibody and beads that were not conjugated.

The target protein YAP was successfully captured with Dynabeads Protein G conjugated with anti-YAP antibody and was not identified in the negative control samples (i.e., Dynabeads Protein G conjugated with an irrelevant anti-CD81 antibody or nonconjugated bead samples). The sequence coverage of YAP was increased to 42% and 47% (13 and 14 peptides), respectively, in the corresponding Dynabeads Protein G anti-YAP replicates compared to the full cell lysate (16%, or 6 peptides), demonstrating the successful enrichment of the target protein (Figure 5). A list of identified peptides and peak areas is given in Table 1.

Table 1. Peptides of YAP identified.

Rank	Peptide	Area Dynabeads Protein G, anti-YAP, rep 1	Area Dynabeads Protein G, anti-YAP, rep 2	Area lysate
1	R.DESTDSGLSMSSYSVPR.T	-	-	1440
2	R.YFLNHIDQTTTWQDPR.K	6570	5810	1070
3	R.TPDDFLNSVDEMDTGDTINQSTLPSQQNR.F	1120	1560	-
4	R.QASTDAGTAGALTPQHVR.A	1600	2220	-
5	R.GDSETDLEALFNAVMNPK.T	9100	8170	-
6	R.SQLPTLEQDGGTQNPVSSPGMSQELR.T	864	1320	-
7	K.TANVPQTVPMR.L	3820	2790	1060
8	R.KLPDSFFKPPEPK.S	744	1320	-
9	K.QPPPLAPQSPQGGVMGGSNSNQQQQMR.L		0	-
10	K.TANVPQTVPM(+15.99)R.L	148	0	-
11	R.GDS(+79.97)ETDLEALFNAVMNPK.T		1010	-
12	R.AHSSPASLQLGAVSPGTLTPTGVVSGPAATPTAQHLR.Q	2490	0	-
13	K.LDKESFLTWL	2200	2260	2580
14	R.NINPSTANSPK.C	528	378	-
15	R.QSSFEIPDDVPLPAGWEMAK.T	-	-	224
16	R.LQQLQMEK.E	-	-	20100

Hsp70 is a novel and known heat shock protein that aids in the translocation of YAP from the cytoplasm to the nucleus, where it becomes active and binds to its transcription factors.

The analysis of the co-IP data revealed an increased concentration of Hsp70 in the Dynabeads Protein G anti-YAP samples compared to the negative controls (i.e., Dynabeads Protein G conjugated with irrelevant anti-CD81 antibody or nonconjugated bead samples), which indicated a link of Hsp70 to the target protein YAP (Figure 6) that had been identified by previous research. Hsp70 is a relatively abundant protein that was identified in high quantity. In the full cell lysate, it was the 15th most abundant protein when ranked by intensity and some nonspecific binding to beads can be expected, especially for very abundant proteins. However, the high performance and low nonspecific binding of the Dynabeads Protein G allow for a distinction between nonspecific binding and actual biological co-IP, even though Hsp70 does not need precipitation to be detected on its own. Table 2 shows a heatmap reflecting the intensities of identified Hsp70 peptides.

Across all samples, 50 peptides were identified for Hsp70 (Table 2). The area under the peak corresponding to the intensity is indicated by color from red (low intensity) to yellow (medium intensity) to green (high intensity). A clear distinction can be made between Dynabeads Protein G conjugated with anti-YAP antibody and the negative controls (i.e., Dynabeads Protein G conjugated with irrelevant anti-CD81 antibody or nonconjugated bead samples), indicating a co-IP and therefore a biological relevance despite the large abundance in the full cell lysate.

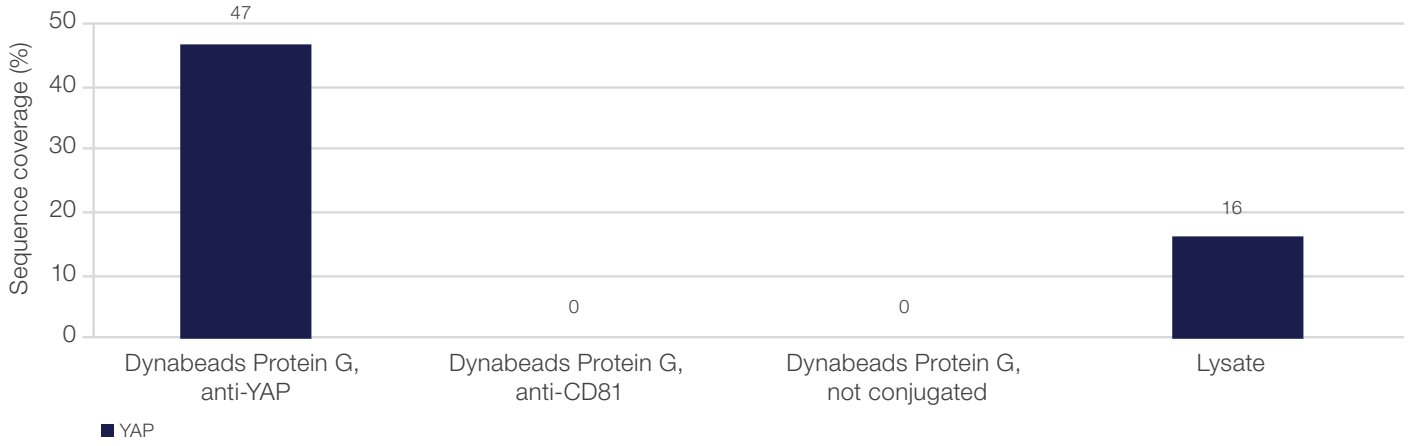

Figure 5. Using Dynabeads Protein G to enrich for the YAP target significantly improves its sequence coverage in the MS dataset, enabling better resolution and improved insight into post-translational modifications.

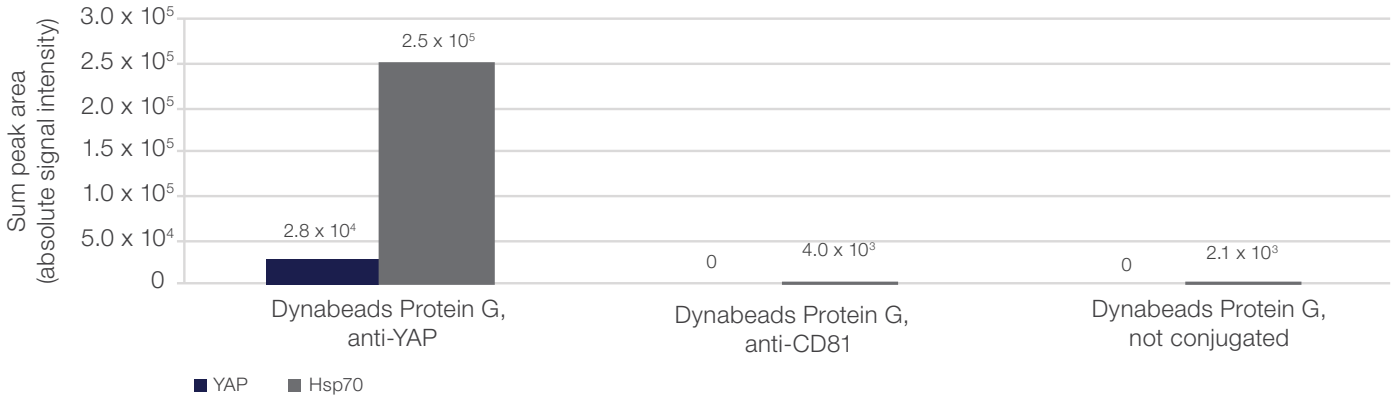

Figure 6. Dynabeads Protein G pull-down of YAP enables co-capture of an interacting partner: Hsp70. Using Dynabeads Protein G conjugated with anti-YAP antibody, Hsp70 is also pulled down from the cell lysate. The protocol can be further optimized for co-IP. Low nonspecific binding to Dynabeads Protein G conjugated with anti-CD81 or beads without antibody was also evident.

Table 2. Heatmap intensities of identified Hsp70 peptides.

Rank	Peptide	Area Dynabeads Protein G, not conjugated, rep 1	Area Dynabeads Protein G, not conjugated, rep 2	Area Dynabeads Protein G, not conjugated, rep 3	Area Dynabeads Protein G, anti-CD81, rep 1	Area Dynabeads Protein G, anti-CD81, rep 2	Area Dynabeads Protein G, anti-CD81, rep 3	Area Dynabeads Protein G, anti-YAP, rep 1	Area Dynabeads Protein G, anti-YAP, rep 2	Area lysate
1	K.VIAVYDLGGGTFDISILEIQK.G									
2	K.SEDKVIAVYDLGGGTFDISILEIQK.G									
3	K.LYSPSQIGAFVLMK.M									
4	K.SQVFSTAADGQTQVEIK.V									
5	K.GAVVGIDLGTTNSCVAVMEGK.Q									
6	K.LLGQFTLIGIPPAPR.G									
7	R.VINEPTAAALAYGLDK.S									
8	R.VEAVNMAEGIIHDTETK.M									
9	K.STNGDTFLGGEDFDQALLR.H									
10	K.ERVEAVNMAEGIIHDTETK.M									
11	R.ASNGDAWVEAHGK.L									
12	K.MKETAENYLGHTAK.N									
13	K.KSQVFSTAADGQTQVEIK.V									
14	R.EQQIVIQSSGGLSK.D									
15	R.VINEPTAAALAYGLDKSEDK.V									
16	R.QAVTNPNNTFYATK.R									
17	K.SDIGEVILVGGMTR.M									
18	K.AVNPDEAVAIGAAIQGGVLAGDVTDVLLLDVTPLSLGIETLGGVFTK.L									
19	R.GVPQIEVTFDIDANGIVHVSAK.D									
20	R.ETGVDLTKDNMALQR.V									
21	K.MEEFKDQLPADECNK.L									
22	K.ETAENYLGHTAK.N									
23	R.TTPSVVAFTADGER.L									
24	K.VQQTVQDLFGR.A									
25	R.AQFEGIVTDLIR.R									
26	K.AMQDAEVSKSDIGEVILVGGMTR.M									
27	K.DAGQISGLNVLR.V									
28	K.NAVITVPAYFNDSQR.Q									
29	K.SDIGEVILVGGM(+15.99)TR.M									
30	K.GAVVGIDLGTTNSCVAVM(+15.99)EGK.Q									
31	R.EGSGSSGTGEQKEDQKEEK.Q									
32	R.KDSETGENIR.Q									
33	R.QAASSLQQASLK.L									
34	K.AKCELSSSVQTDINLPYLTMDSSGPK.H									
35	K.M(+15.99)EEFKDQLPADECNK.L									
36	R.EQQIVIQSSGGLSKDDIENMVK.N									
37	R.VINEPTAAALAYGLDKSEDKVIAVYDLGGGTFDISILEIQK.G									
38	R.YDDPEVQK.D									
39	K.LYSPSQIGAFVLM(+15.99)K.M									
40	R.RYDDPEVQK.D									
41	R.ETGVDLTKDNM(+15.99)ALQR.V									
42	K.LFEMAYKK.M									
43	R.VEAVNM(+15.99)AEGIIHDTETK.M									
44	K.ERVEAVNMAEGIIHDTETKMEEFKDQLPADECNK.L									
45	K.DQLPADECNK.L									

Table 2. Heatmap intensities of identified Hsp70 peptides. (continued)

Rank	Peptide	Area Dynabeads Protein G, not conjugated, rep 1	Area Dynabeads Protein G, not conjugated, rep 2	Area Dynabeads Protein G, not conjugated, rep 3	Area Dynabeads Protein G, anti-CD81, rep 1	Area Dynabeads Protein G, anti-CD81, rep 2	Area Dynabeads Protein G, anti-CD81, rep 3	Area Dynabeads Protein G, anti-YAP, rep 1	Area Dynabeads Protein G, anti-YAP, rep 2	Area lysate
46	R.YDDPEVQKDIK.N									
47	K.RQAVTNPNNTFYATK.R									
48	R.VEAVNMAEGIIHDTETKMEEFKDQLPADECNK.L									
49	K.ERVEAVNM(+15.99)AEGIIHDTETK.M									
50	K.RETGVDLTK.D									

Discussion

The analysis of mass spectrometry data is a complex interdisciplinary procedure. It requires knowledge in the fields of mass spectrometry, proteomics, statistics, biology, and molecular biology, among others.

Numbers of reported proteins can vary depending on a plethora of variables, such as the selection mass error tolerances allowed during the database search or the selected significance and false discovery rates that can be chosen on the protein and peptide levels. Various data analysis tools provide different algorithms for identification of peptides and proteins. The search space is also influenced by selection of the database, as well as the allowed number of potential fixed and variable modifications and the maximum missed cleavage sites. These are just a few examples of the additional variables that must be considered during the data analysis. Furthermore, different MS instruments and the selection of their operation modes influence the resulting data. Therefore, it is important to analyze the samples consistently to ensure comparability across the analysis. In this study, bead samples were analyzed in a 1 hr LC/MS analysis to allow alignment with the substantially more complex full cell lysate. For some studies, a shorter analysis method could be chosen for bead-based proteomics samples.

Nevertheless, the most significant findings can typically be retrieved by many methods, and it is the weaker signals that need validation by an orthogonal method like western blots or repetition with more targeted approaches in which specific peptides are selected to be observed in the MS instrument.

This study highlighted the suitability of Dynabeads Protein G in combination with the KingFisher Flex Purification System for co-IP to be used in a downstream MS analysis. None of the samples using Dynabeads Protein G showed signs of leaching in the form of polymer traces in the MS raw data, demonstrating the suitability of Dynabeads Protein G for MS-based proteomics applications.

We successfully demonstrated the isolation of YAP and identified a co-IP of Hsp70. Hsp70 is a high-abundance protein and was the 15th most abundant protein in the cell lysate when ranked by intensity. Consequently, Hsp70 has not been enriched in the co-IP relative to the cell lysate. Furthermore, a few Hsp70 peptides have also been identified in the negative controls, which is not surprising given the abundance of this protein. Nevertheless, the co-IP interaction is an interesting finding as the data clearly suggest a protein-protein interaction with 100-fold increased signal intensities in the target sample compared to the negative controls (Figure 6 and Table 2). This demonstrates the high performance of the Dynabeads Protein G—while enabling the enrichment of low-abundance proteins (YAP), their low nonspecific binding allows for discrimination between nonspecific binding and actual co-IP partners, even of very abundant proteins (Hsp70).

The data analysis led to a shortlist of additional proteins that would need follow-up experiments for confirmation.

Summary

The results provided here demonstrate the speed and reliability of Dynabeads Protein G, combined with automation using the KingFisher Flex Purification System, to co-immunoprecipitate intracellular targets and binding partners for protein-protein interaction studies. The Dynabeads Protein G product demonstrates excellent compatibility with downstream MS analysis applications. No contamination of PEG (polyethylene glycol) or other potential polymer signatures were detected in the MS raw data, indicating that Dynabeads Protein G beads are suitable for MS-based proteomic workflows.

Using the workflow described herein and automation scripts can promote faster and standardized MS and western blots of oncogenic proteins for multiomic analysis.

References

1. Baldan S et al. (2021) The Hsp70-Bag3 complex modulates the phosphorylation and nuclear translocation of Hippo pathway protein Yap. *J Cell Sci* 134(23).
2. Cheng Y et al. (2022) The biology of YAP in programmed cell death. *Biomark Res* 10:34.
3. Zanconato F et al. (2016) AP/TAZ at the roots of cancer. *Cancer Cell* 29(6):783-803.
4. McGowan M et al. (2017) NSCLC depend upon YAP expression and nuclear localization after acquiring resistance to EGFR inhibitors. *Genes Cancer* 8(3–4):497-504.

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