FLAG® 96-well Immunoprecipitation System for High-Throughput Protein-Protein Interaction Studies

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Application Notes

- High-throughput 96-well plate configuration for validation of protein-protein interactions
- Rapid streamlined protocol for handling large numbers of samples (< 4 hours)
- Sensitive assay system with quantitative results
- Flexible for user-defined assays

Introduction

Comprehensive studies of protein-protein interaction (PPI) are critical in understanding the vast networks of protein complexes that mediate biological processes. Co-immunoprecipitation (co-IP) is the most widely used *in vitro* method for PPI discovery and verification of interactions seen in other systems such as the yeast two-hybrid system (*in vivo* method). This affinity-based molecular pull-down method, facilitated by epitope tagging of recombinant proteins, has enabled rapid and detailed studies of expression, function and interaction of proteins that may fuel discovery of new drug targets and therapeutic molecules. However, traditional co-IP using affinity resins involves laborious and time-consuming steps that limit sample throughput. A 96-well plate-based immunoprecipitation platform, based on the FLAG® epitope tag (DYKDDDDK), has been developed to address concerns encountered in traditional co-IP. The ANTI-FLAG® M2 antibody coated 96-well plate provides a rapid system for quantitative analysis of captured FLAG-tagged fusion protein complexes from crude mammalian cell lysates via an ELISA format.

Overview of the system

The FLAG® 96-well Immunoprecipitation System (Product Codes HT-COIP-1, COIP-P, and COIP-D) provides a streamlined procedure for handling large numbers of PPI candidates (Figure 1). Genes for proteins with unknown or suspected interaction partners may be cloned into the pFLAG®-CMV-2 (Product Code E 7398) and pc-Myc-CMV-2 expression vectors for transient expression in mammalian cells such as CHO, COS-7 or HeLa. The bait-prey approach is used in that a protein of interest is tagged with FLAG while a suspected interacting protein may be tagged with c-Myc. The FLAG-tagged bait protein may also be used to pull-down complexes of unknown interacting proteins for downstream analysis. Following transfection and expression of the FLAG and c-Myc constructs, the cells are lysed and the crude cell lysate is incubated in the wells of the ANTI-FLAG M2 affinity capture plate (Product Code P 2983). The FLAG fusion is captured by the M2 antibody on the surface of the plate. Any interacting proteins (tagged or untagged) are co-immunoprecipitated. Unbound protein is washed away and the captured interacting proteins can be analyzed using epitope-specific or protein-specific antibody-enzyme conjugates such as monoclonal Anti-c-Myc alkaline phosphatase conjugate (Product Code A 5963). The results are determined by standard ELISA detection methods. Activity of bound protein may be determined in-well or samples may be eluted for downstream assays or for analysis by Western blotting. Compared to traditional resin-based IP, the 96-well format permits the study of large numbers of samples and numerous variables such as time points, ranges of cell lysate, side-by-side analysis of controls, replicate samples, and optimization of reaction conditions. Covalent coupling of the M2 antibody to the surface of the plate allows for complete removal of wash buffer without concern of removing sample to be assayed. Removal of resin at wash steps using traditional IP makes resin a less quantitative method compared to the highly quantitative plate-based method. Based on these features, the system provides a unique streamlined method for study of protein-protein interaction.

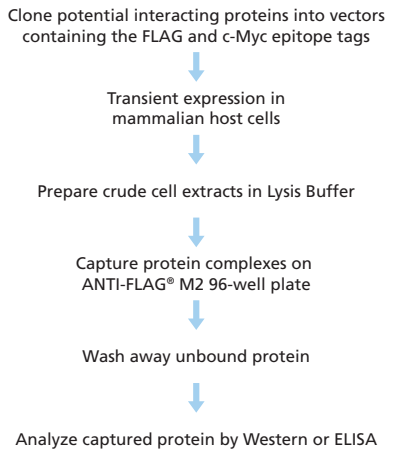

Figure 1. Overview of the FLAG® 96-well Immunoprecipitation System.

Protein-protein interaction of p53 and large T antigen

The streamlined capabilities of the system were demonstrated using two known interacting pairs. The first, also provided as controls in the Immunoprecipitation Vector Kit (Product Code COIP-P), is based on the interaction of the tumor suppressor p53 with the SV40 large T antigen. The p53 gene was cloned into the pFLAG®-CMV-2 expression vector while the gene for SV40 large T antigen was cloned into pc-Myc-CMV-2. The constructs were expressed in Cos-7 cells and PPI using the system is demonstrated in Figure 2. For further verification of PPI, captured protein complexes were eluted from the plate using 2x-SDS-Sample Buffer and analyzed by Western blotting alongside samples eluted from traditional ANTI-FLAG M2-Agarose (Product Code A 2220; data not shown). Both results demonstrated c-Myc-large T antigen was specifically co-immunoprecipitated with FLAG-p53.

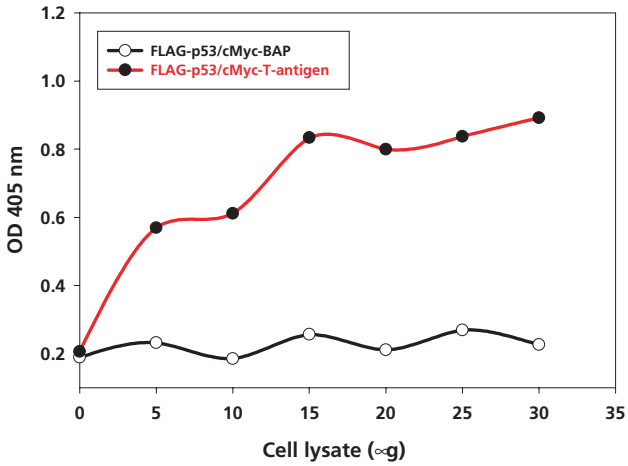

Figure 2. ELISA analysis of protein-protein interaction by co-transfection of protein pairs. *COS-7 cells were co-transfected with pFLAG-CMV-2-p53 and pc-Myc-CMV-2-large T antigen. For a negative control, pc-Myc-CMV-2-BAP was co-transfected with pFLAG-CMV-2-p53. Cell lysates were incubated in the wells of the ANTI-FLAG M2 96-well affinity capture plate for thirty minutes. FLAG-p53 and any interacting proteins were captured on the plate. Detection of the c-Myc-tagged interacting partners was performed in-well using Anti-cMyc-AP monoclonal antibody (1:100) followed by colorimetric ELISA analysis. Interaction was measured for 0-30 µg of cell lysate. The interaction of FLAG-p53 with c-Myc-large T antigen was detected in as little as 5 µg of cell lysate with little non-specific interaction of the negative control c-Myc-BAP.*

Detection of endogenous interacting proteins

The second model system used is based on the interaction of p65 of the nuclear factor NF-κB complex and the NF-κB inhibitor IκB-α. p65 was expressed as a FLAG-tagged fusion protein in HeLa cells and the ANTI-FLAG M2 affinity capture plate was used to capture the FLAG-tagged p65 along with any endogenous interacting proteins in the crude cell lysate. After washing away unbound protein, the captured protein complex was probed with a specific antibody against IκB-α. Detection of the endogenous co-immunoprecipitated IκB-α was detected by colorimetric ELISA analysis (Figure 3) and validated by western blot analysis of bound protein (data not shown). Thus, the platform is capable of detecting co-transfected interacting pairs as well as pull-down of endogenous complexes.

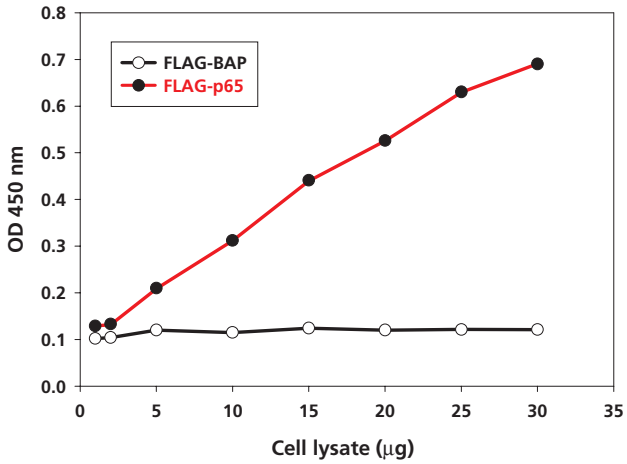

Figure 3. ELISA analysis of protein-protein interaction using a single FLAG-tagged "bait" protein. *HeLa cells were transfected with FLAG-p65 or FLAG-BAP (negative control). Cell lysates were incubated in the wells of the ANTI-FLAG M2 96-well affinity capture plate for thirty minutes. FLAG-tagged proteins and any other interacting proteins were captured on the plate. The samples were probed for interaction with endogenous IκB-α using a polyclonal anti-IκB-α antibody (1:500), followed by peroxidase conjugated anti-rabbit IgG secondary antibody (1:10,000). Endogenous co-immunoprecipitated IκB-α was detected in as little as 5 µg of the FLAG-p65 lysate and not in the negative control FLAG-BAP.*

Powerful tool, unique high-throughput platform

The FLAG® 96-well Immunoprecipitation System is a powerful tool for molecular interaction studies. The system is not only useful for analysis of stable protein-protein interactions (Figures 2 and 3), but is also useful for the analysis of transient protein interactions such as protein kinase activity.* The method overcomes drawbacks of traditional resin-based co-IP while providing an alternative or validation platform for the yeast two-hybrid system. In addition, the platform can easily be adapted to a variety of applications such as analysis of PPI by *in vitro* transcription and translation, inhibitors and activators of protein interaction, mapping of protein interaction domains, and other molecular interactions for potential drug target identification.* Thus, the system provides a unique high-throughput platform for rapid identification of protein-protein interactions and for sensitive bioassay development.

*For citations 1 and 2, visit our website at **www.sigma-aldrich.com**.

Ordering Information

Product	Description	Unit
HT-COIP-1	FLAG 96-Well Immunoprecipitation	1 kit
COIP-P	Immunoprecipitation Vector Kit	1 kit
COIP-D	Immunoprecipitation Detection Kit	1 kit