

Product Information

FLAG® HA Tandem Affinity Purification Kit

Catalog Number **TP0010**

Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

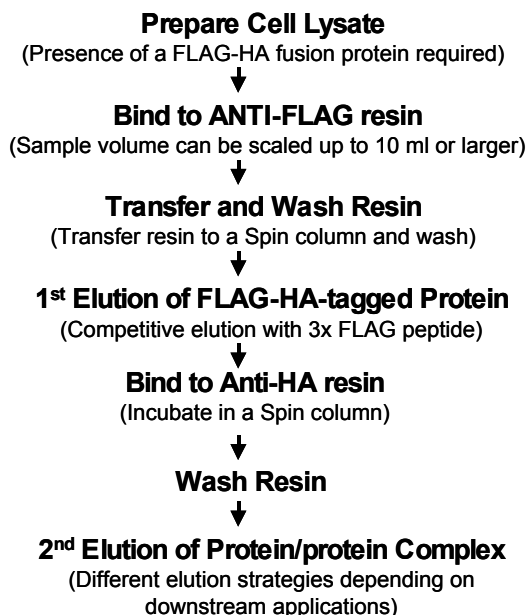
The FLAG HA Tandem Affinity Purification Kit is designed for the isolation of high purity FLAG-HA dual-tagged fusion proteins from complex matrices, such as cell lysates and tissue homogenates. The kit is particularly suitable for isolation of protein complexes using TAP (Tandem Affinity Purification) technology.

TAP technology is a recent development in protein purification. This technology incorporates tandem-linked affinity tags into genes of interest so that fusion proteins can be isolated through two consecutive affinity purification steps. The technology is particularly useful for the isolation and identification of protein complexes by adding a tandem affinity tag to a known “bait” protein to pull down endogenous proteins that interact with the targeted protein of interest. Protein complexes isolated using TAP technology have a much higher purity compared to single purification and can be used for mass spectrometry (MS) analysis.^{1,2} Although existing TAP systems using combinations of GST, calmodulin, nickel binding peptides, streptavidin, and Protein A have been used in various experimental systems, they suffer from several limitations: interference with complex assembly or protein function due to relatively large TAP tags (20 kDa and larger), high rate of cross reactivities with non-targeted endogenous proteins, and/or elution requirement of TEV protease.

The FLAG HA tandem epitope tagging system eliminates or minimizes these concerns. Both FLAG (DYKDDDDK) and HA (YPYDVPDYA) are small, non-eukaryotic derived tags. These features minimize interference with protein functions and provide superior specificity. The hydrophilic character of the FLAG HA peptides increases the likelihood that the dual epitope will be located on the surface of the fusion protein where it is accessible for antibody-antigen interaction. Antibodies against FLAG and HA tags and affinity resins derived from them have demonstrated a high specificity and affinity in pull-down assays compared to other existing epitope-tagging systems.

As shown below, the protein purification procedure using the FLAG HA Tandem Affinity Purification Kit is simple and fast. The kit can be used for isolation of single tandem-tagged fusion proteins or for isolation of protein complexes by co-immunoprecipitation using tandem-tagged “bait” proteins.

Outline of FLAG HA Fusion Protein Purification



Precautions and Disclaimer

FLAG HA Tandem Affinity Purification kit is for R&D use only, not for drug, household or other uses. Consult the MSDS for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Upon receipt, store the spin columns and collection tubes at room temperature. Store the remaining reagents at 2-8 °C.

Table 1

Components	Product Code	Quantity
EZview™ ANTI-FLAG® M2 affinity resin	F2426	1ml
Anti-HA agarose affinity resin	A2095	0.5 ml
RIPA (150 mM NaCl, 1.0% Igepal® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0)	R0278	1 X 50 ml
3XFLAG peptide	F4799	1mg
Urea	U4883	25 ml
SigmaPrep™ Spin column	H6912*	10 columns, caps and tubes
Spin column caps	C6739*	
2 ml collection tubes	T7813*	

*To reorder please use Product Code MC1000

Additional Reagents and Equipment Not Supplied

- FLAG HA TAP Tag Generation Kit, Product Code TP0020
- Immunoprecipitation compatible extraction buffer
- Protease inhibitor cocktail (see Table 2)
- Conical vials and microcentrifuge tubes
- Pipettes and tips
- Refrigerated microcentrifuge
- Shaker/agitator
- Tris Buffered Saline TBS (0.138 M NaCl, 0.003 M KCl, 0.05 M Tris; pH 8.0) Product Code T6664
- Water, Molecular Biology Reagent, Product Code W4502
- HA peptide, Product Code I2149
- 2x Laemmli Sample Buffer (LSB), Product Code S3401
- Ammonium bicarbonate, Product Code A6141
- C18 micro spin column (Vivapure® from Vivascience)
- Chemiluminescent Peroxidase Substrate-1, Product Code CPS1120
- Monoclonal ANTI-FLAG® M2-Peroxidase (HRP), Product Code A8592
- Monoclonal Anti-HA-Peroxidase (HRP), Product Code H6533
- ProteoSilver™ Plus Silver Stain Kit, Product Code PROTSIL2

★ required

☞ optional

General Notes

Please read the entire protocol before proceeding with the procedure.

Extract Preparation

The appropriate amount of starting material depends on the downstream protein analysis technique intended. The abundance of the tagged protein and the interacting endogenous complexes will also determine the necessary amount of starting material. As a frame of reference, 15-50 g of transgenic *Arabidopsis* seedlings (fresh weight) and 1 X10⁷ transfected COS-7 cells have been used successfully to isolate tandemly tagged protein for visualization and identification.

The concentration of protein in the extracts should be 1-100 mg/ml. For protein identification by mass spectrometry (MS) and Edman sequencing, the protein concentration is measured in picomoles (pmols) and not micrograms (μg). The following formula can be used for picomole/microgram conversion:

$$1000/\text{MW of the protein in kDa} = \text{pmol}/\mu\text{g}$$

Extracts should be prepared in an immunoprecipitation-compatible buffer with added protease inhibitors (see table below). Removal of cellular debris by multiple filtration steps or by centrifugation is necessary. If protein-protein interactions mediated by phosphorylation are being investigated, suitable phosphatase inhibitors (such as P5726 and/or P2850) should be included. When performing experiments, samples should remain at 4 °C when possible.

Table 2

Specialized Protease Inhibitor (PI) Cocktails	Product Code
Plant Cells	P9599
Tissue Culture Media	P1860
Fungal/Yeast Cells	P8215
Bacterial Cells	P8465
General Use	P2714

EZview ANTI-FLAG M2 and anti-HA affinity resins

The ANTI-FLAG and anti-HA affinity resins have been created by covalently linking monoclonal antibodies to resin and are supplied as a 50% slurry suspension in phosphate buffered saline with an anti-microbial agent. The initial immunoprecipitation volume is relatively large and dependent on the experimental design. EZview ANTI-FLAG resin is provided to enhance visibility during incubation and transfer manipulations. The red color of EZview resin does not affect binding capacity. Using epitope tagged proteins for quantification, ANTI-FLAG and anti-HA resins were found to have binding capacities greater than or equal to 0.6 mg/ml and 0.4 mg/ml, respectively.

There are many variations in immunoprecipitation procedures. The initial incubation volume affects the amount of ANTI-FLAG resin used. The user may need to adjust the amount of resin used to satisfy the specific experimental requirements. In general, 100 μ l of ANTI-FLAG resin slurry is appropriate for 10 ml of extract. Approximately 300 to 500 μ l of ANTI-FLAG resin is recommended for 30 ml of extract. Larger extract volumes (>30 ml) should be divided for processing.

Carefully mix resins for homogeneous suspension before aliquots are taken. Resins should be washed 3x with the supplied RIPA buffer. The volume of each wash should be at least 4X the packed-resin volume. In case of numerous immunoprecipitation samples, wash the resin needed for all the samples together. After washing, divide the resin according to the number of samples. Use a pipette tip with a wide bore to dispense slurry. Centrifugation should be done at 3,000 $\times$ g.

Using SigmaPrep spin columns with breakable tips

The use of spin columns ensures minimal loss of the affinity resins during washing. Break off the end tip of the spin column and plug column with tip. See Figure 1. The column has a capacity of 750 μ l. Keep the tip inserted in the bottom of the column and the red screw cap secure on the column during the incubation steps. Remove the tip from the column during centrifugation and save for later steps. The column should be placed in a 2 ml collection tube for the washing step, or a microcentrifuge tube for the elution step. The column and red screw cap should be labeled.

Figure 1



Break off tip, invert, and plug column

Procedure

Step 1. Binding to ANTI-FLAG M2 resin

A. Prepare RIPA + protease inhibitor (PI) cocktail (see Table 2 for suitable inhibitors). Each sample needs approximately 4 ml of RIPA + PI (for Step 2.A, 2.B, 4.B, and 5.B).

B. Aliquot an appropriate amount of ANTI-FLAG M2 resin into a clean tube or vial. Wash three times with RIPA buffer. For each of the washes, gently agitate the resin in the buffer then centrifuge at 3,000 $\times$ g for 30 seconds. Decant as much of the remaining final wash volume as possible without losing any resin. (See General notes on affinity resins for amount of ANTI-FLAG resin/sample and resin-washing directions.)

C. At this point, a FLAG HA fusion protein must be present in the extract. It is worthwhile to confirm the presence of a FLAG HA protein with an appropriate immunodetection technique. If the FLAG HA protein is not endogenously expressed in the tissue or species being investigated, it is necessary to add purified FLAG HA protein to experimental samples to serve as a “bait” protein.

Add washed ANTI-FLAG[®] M2 resin (from Step B) to the sample extract (See Extract Preparation under General Notes). Conical vials may be used as incubation containers.

D. Incubate two hours to overnight at 4 °C with gentle rocking or agitation. The resin must remain suspended during the incubation.

Step 2. Removing unbound protein.

A. Spin the conical vial (from Step 1.D.) at 3,000 x *g* at 4 °C for two minutes. The supernatant contains unbound protein. Remove as much of the supernatant as possible with a pipette without disturbing the resin. Discard the supernatant. Again, using a pipette, carefully transfer the remaining supernatant and resin into a spin column that has been placed into a collection tube. For adequate washing, the spin column's packed-resin volume should not exceed 150 μ l. Use multiple spin columns if needed. Add 500 μ l RIPA + PI cocktail to each spin column being used. Spin sample in microcentrifuge at 3,000 x *g* at 4 °C for 30 seconds. (See page 3 for notes on using SigmaPrep spin columns).

B. Reinsert tip into column(s) (See Fig. 1). Add 500 μ l of RIPA + PI cocktail. Agitate samples for several minutes at 4 °C.

C. Remove tip from column(s). Spin column(s) in the same 2 ml collection tube at 3,000 x *g* at 4 °C for 30 seconds. Discard the column washings which contains unbound protein

D. Repeat wash steps (B-C) two more times.

Step 3. First elution of the protein complex (using 3XFLAG peptide)

A. Reinsert tip into column(s). Place column(s) into clean microcentrifuge tube(s).

B. Preparing the 3XFLAG peptide:

Prepare a 5mg/ml 3XFLAG peptide stock solution by adding TBS, Product Code T6664, to the product vial and dissolve powder by vortexing. Aliquot and store at -20 °C. Repeated freezing and thawing is not recommended. For elution, add 3 μ l of 5 μ g/ μ l 3XFLAG peptide stock solution to 100 μ l of TBS (150 ng/ μ l final concentration).

C. Elute the tandem tagged FLAG HA protein with 2.5 times the packed-resin volume of 150 ng/ μ l 3XFLAG peptide (see Table 3). Incubate resin/elution volume for at least 10 minutes. The resin must remain suspended during the incubation.

D. Remove the tip and spin the column in a clean microcentrifuge tube at 3,000 x *g* for 1 minute. **Keep the eluate**, which contains the eluted protein.

E. Perform a second elution (Steps C-D) and pool the eluates.

Table 3

Anti-FLAG resin slurry used (μ l)	Packed-resin amount (μ l)	Total 3XFLAG elution volume (μ l)	# of columns needed/sample (Step 4)
100	50	250	1
300	150	750	1
500	250	1250	2

Step 4. Binding to anti-HA resin

A. Determine the number of new spin columns needed from Table 3. Use 40 μ l of washed anti-HA resin slurry for each column. See General notes on affinity resins for resin-washing directions.

Final elutions can be concentrated by not splitting larger volumes (≥ 750 μ l) into multiple spin columns and not increasing resin amount. During the anti-HA resin incubation (Step 4.B.), use a microcentrifuge tube instead of a spin column. After incubation with anti-HA resin, the sample needs to be sequentially added to the spin column (Step 5.A.).

B. Break the tip(s) off new column(s), invert, and plug column(s) (See Fig. 1). Add *x* μ l RIPA +PI, 20 μ l packed-resin volume of washed anti-HA resin, and the 3XFLAG peptide elution (from Step 3). The maximum volume of the column is 700 μ l. Incubate the column from 30 minutes to 2 hours at 4 °C with gentle rocking or agitation. The resin must remain suspended during the incubation.

Step 5. Removing unbound protein

A. Remove tip and place the spin column(s) into a new 2 ml collection tube. Spin at 3,000 x *g* for 30 seconds without tip. Discard column washings..

B. Reinsert column tip. Add 500 μ l of RIPA + PI cocktail. Agitate samples for several minutes. If the eluted sample will be assayed directly by mass spectrometry without running PAGE (see information under Step 6.A.3.) wash in TBS instead of RIPA. RIPA contains detergents that interfere with MS results; however, residual amounts of RIPA enhance urea elution.

C. Remove the tip and spin column(s) in the same 2 ml collection tube at 3,000 x *g* for 30 seconds. Discard column washings, which contains unbound protein.

D. Repeat wash steps (B-C) two more times.

Step 6. Final elution of the protein complex

Three elution methods are recommended according to downstream applications and preference.

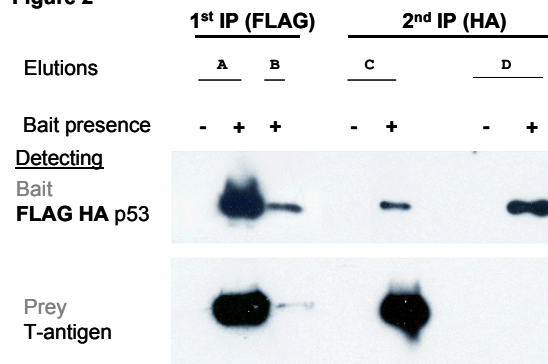
A. Elution with urea

For MS analysis of the protein, elute the sample with 8 M urea. Urea will preferentially elute the prey proteins that interact with the “bait” (dual tagged) protein. See Figure 2. This maximizes the detection sensitivity of unknown proteins by reducing contamination by the “bait” protein. The protein is denatured. Urea elution is applicable to PAGE gel and direct MS submission

1. Add 16 ml of water (W 4502) to the bottle containing urea and vortex. The resulting solution will be 25 ml of 8 M urea.
2. Insert tip into column outlet. **Put the column with resin sample into a new microcentrifuge tube.** Elute 20 µl of packed-resin from Step 5.D. with 50 µl of 8 M urea at room temperature. Incubate resin/elution volume for a minimum of 10 minutes. The resin must remain suspended during the incubation. Remove tip from column. Spin at 3,000 x g for 1 minute. **Sample can now be prepared to load on a PAGE gel.**
3. For direct MS submission, samples needed to be washed with TBS in Step 5.B. Protein needs to be at a picomole level (See General Notes on Extract Preparation on page 2). Dilute the eluate (as in Step 6.A.2.) with 100 mM ammonium bicarbonate until the final urea concentration is $\leq 1\text{M}$. Sample is then ready to be digested with trypsin. For MS analysis, the sample can be concentrated using a C18 micro spin column (such as Vivapure from Vivascience).

Figure 2: Urea preferentially elutes the prey complex from the bait protein attached to the resin. p53 and large T-antigen interact in COS-7 cells (a mammalian cell line). FLAG HA was incorporated into p53 using overlapping PCR (see FLAG HA Tandem TAP Tag Generation Kit TP0020), placed into an expression vector driven by the CMV2 promoter, and transiently expressed. Transfected and non-transfected samples were processed using ANTI-FLAG resin followed by anti-HA resin. Protein was eluted with 3XFLAG peptide (A-once, B-twice) from the ANTI-FLAG resin then urea (C) followed by LSB (D) from the anti-HA resin. Resins were washed with RIPA through out the procedure. The prey protein is predominantly eluted in urea, while the bait protein is not.

Figure 2



B. Elution with HA peptide (I 2149).

This elution strategy is the mildest elution, suitable for recovery of single dual-tagged proteins or protein complexes. HA peptide competitively elutes the tandem tagged protein from the HA resin with minimum interference to protein function.

1. Prepare the HA peptide (I2149, not supplied) according to the product instructions. The final working dilution should be 1 µg/µl.
2. Insert tip into column outlet. **Put the column with resin sample into a new microcentrifuge tube.** Elute 20 µl of packed-resin from Step 5.D. with 50 µl of 1 µg/µl HA peptide. Incubate for at least 10 minutes. The resin must remain suspended during the incubation. Remove tip from column. Spin at 3,000 x g for 1 minute. Save eluate in a microcentrifuge tube. Repeat elution. Combine the eluates.

C. Elution with 2X Laemmli Sample Buffer (LSB) **Product Code S3401**

This is a harsh elution condition that elutes everything, including the antibody from the resin. It is useful for analysis of tagged proteins and protein complexes by PAGE gel and Western blotting.

1. Insert tip into column outlet. **Put the column with resin sample into a new microcentrifuge tube.**
 Elute 20 μ l of packed resin (from Step 5.D) with 50 μ l 2X LSB. Incubate and mix for 5 to 10 minutes. The resin must remain suspended during the incubation. Remove plug from column. Spin at 3,000 x *g* for 1 minute. The eluate contains the sample. Heat sample at 95 °C for 5 minutes.
2. Run on a PAGE gel. Heavy and light chains of the antibody (from the HA resin) are present and may interfere with visualizing the proteins of interest.

References

1. Honey, S., *et al.*, A novel affinity purification tag and its use in identification of proteins associated with a cyclin-CDK complex. *Nucleic Acids Res.*, **29**, e24 (2001).
2. Rigaut, G., *et al.*, A generic protein purification method for protein complex characterization and proteome exploration. *Nat. Biotechnol.* **17**, 1030 - 1032 (1999).
3. Rose, J. K. C., *et al.*, Tackling the plant proteome: practical approaches, hurdles and experimental tools. *Plant J.*, **39**, 715-733 (2004).
4. Shevchenko, A., *et al.* Deciphering protein complexes and protein interaction networks by tandem affinity purification and Mass Spectrometry. *Mol. Cell. Proteomics*, **1**, 204-212 (2002).
5. Hall, D. B., and Struhl, K., The VP16 Activation Domain Interacts with Multiple Transcriptional Components as Determined by Protein-Protein Cross-linking *in vivo*. *J. Biol. Chem.*, **277**, 46043-46050 (2002).

Troubleshooting Guide

Problem	Possible cause	Solution
The FLAG HA bait protein is not seen in the ANTI-FLAG resin elution.	The extraction buffer is not compatible with immunoprecipitation	A positive control protein (amino-terminal FLAG-BAP™ P7582) should be tried. Consider using RIPA as the extraction buffer.
	The starting material does not contain an appropriate level of FLAG HA (TAP) tagged protein	Increase expression by screening more transgenic lines, use more starting material, or add more exogenously expressed FLAG HA protein.
	Protease activity removed the FLAG HA (TAP) tag from the protein of interest	Verify by Western analysis. Add more protease inhibitor cocktail or create a different TAP tag fusion.
	The pI of the FLAG HA (TAP) tagged protein >10	Highly basic proteins may be problematic with general immunoprecipitation procedures. ⁴
	The immunoprecipitation volume is larger than the ideal conditions for the resin amount used	Increase resin amount or perform parallel incubations with smaller volumes (see general notes on resins). Use a FLAG tagged protein (see above) as positive IP control.
The FLAG HA bait protein is not seen in the Anti-HA resin elution.	Eluted with urea.	Urea does not efficiently elute the TAP tag bait protein from the resin. However, protein interactions associated with the TAP-protein of interest should be disrupted and interacting proteins eluted. For bait protein presence, look at the follow-up elution with 2X LSB, see Figure 2.
Protein complexes are not seen in the anti-HA resin elutions.	Buffer conditions are not ideal for specific interactions to be maintained.	Salt concentrations, pH, and phosphorylation/phosphatase inhibitors may need to be altered or added to the extraction and wash buffers.
	Interaction of the heterocomplex is very weak.	Cross-linking with formaldehyde prior to purification can maintain weak interactions. ⁵
	Interaction of the heterocomplex is very strong. Eluted with urea after washing the anti-HA resin with TBS (Step 5.B)	Will see protein in a follow-up elution with LSB. Some strong protein-protein interactions need the presence of detergents to facilitate their disruption. Use RIPA instead of TBS at Step 5.B. Not applicable for direct MS submission.
	Protein complexes are very low in abundance.	Increase the amount of starting material used.

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