

A New TAP System for Isolation of Plant Protein Complexes and Subsequent Mass-Spec Analysis



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Summary

Isolation and analysis of protein complexes has become a hot topic and remains a major technical challenge in functional proteomics research. Co-immunoprecipitation is the most widely used method for isolation of protein complexes. The TAP (Tandem Affinity Purification) technology is the latest development in the field. This technology incorporates tandem-linked affinity tags into a known "bait" protein to pull down and isolate endogenous interacting proteins. Protein complexes isolated using TAP technology have a much higher purity compared to single purification and, thus, could be subjected to Mass Spectrometry analysis.¹⁻² Existing TAP systems, including combinations of GST, calmodulin, nickel binding proteins, streptavidin, and Protein A have suffered from several limitations, such as interference with complex assembly or protein function due to relatively large TAP tags (20 KD and larger); high rate of contamination with non-targeted endogenous proteins; and requirement of TEV protease treatment for elution which adds additional bacterial contaminants.

The FLAG[®] HA tandem epitope tagging system eliminates or minimizes these concerns. Both FLAG (DYKDDDDK) and HA (YPYDVPDYA) are small epitope tags and are not eukaryotic derived tags. These features minimize interference with protein functions and provide superior specificity. The hydrophilic character of the FLAG HA peptide increases the likelihood that the dual epitope will be located on the surface of the fusion protein where it is accessible for the 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 (Figure 4).

A requirement of this system is the initial generation of the dual-tagged bait protein. Here we describe a novel method for the rapid generation of the TAP tags into the gene of interest and the subsequent cloning of those inserts into expression vectors. The TAP tag generation strategy combines unique PCR and directional cloning methods with the FLAG and HA epitopes for the synthesis of highly specific dual-tagged bait proteins (Figures 2 & 3).

As shown below, the FLAG[®] HA Tandem Affinity Purification method has been characterized using several model systems. The results indicated that this new TAP system provides a superior specificity, high efficiency of protein recovery, compatibility with different buffer systems, and minimum interference to protein function. Protein complexes were isolated from a mammalian cell line (Figure 5) and Arabidopsis (Figure 6) and were suitable for PAGE and Mass Spectrometry analysis.

Main Features of the FLAG[®] HA TAP System

- Rapid generation of TAP tag into the gene of interest
- The affinity resins have high specificity and pull down less plant contaminants compared to existing systems.
- The TAP tag does not sterically affect the bait proteins' interactions.
- No need for protease elution.
- Affinity resin for initial large immunoprecipitation volume is easily visible for ease of transfer.
- Spin columns for aid in resin washing.
- Efficient and mild elution for the first IP and flexible elution strategies for the second IP.

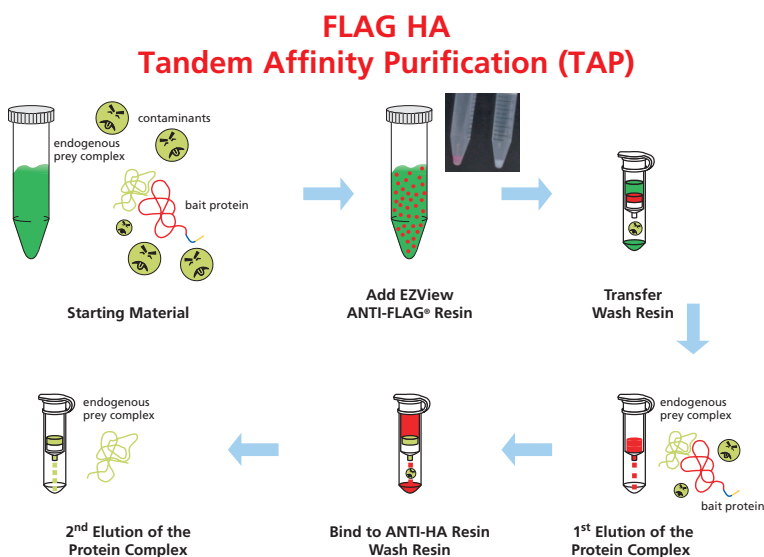


Figure 1. Schematic representation of the FLAG HA Tandem Affinity Purification system. The protein of interest is tandem tagged with FLAG HA epitopes and referred to as the bait protein. Endogenous protein complexes (referred to as prey complex) interact with the tandem tagged bait protein and will be sequentially co-purified. The whole procedure involves six simple steps: 1) Prepare the starting material that contains FLAG HA tagged bait protein; this is normally conducted in 15 mL conical tubes. The bait protein can be expressed *in vivo* (Figure 5) or expressed in bacteria, purified, and added exogenously to the extract (Figure 6); 2) Add EZview ANTI-FLAG resin directly to the lysate. The red color of the resin provides better visibility during incubation and for transfer (see sub-panel); 3) Transfer the resin to a spin column (~700 ul) and wash; 4) Conduct 1st elution with 3× FLAG peptide. This elution strategy is very efficient and mild and would not interrupt protein complex; 5) Transfer the 1st eluate directly to a spin column containing anti-HA affinity resin, bind and wash; and 6) Conduct the 2nd elution with different elution buffers (urea, HA peptide, or Laemmli Sample Buffer) depending on downstream manipulations. The advantages of the urea elution buffer are its compatibility with MS analysis (data not shown) and that it preferentially elutes the prey proteins so that contamination from the bait protein is minimized (Figures 5 & 6).

FLAG HA TAP Tag Generation Kit- Universal Cloning Method

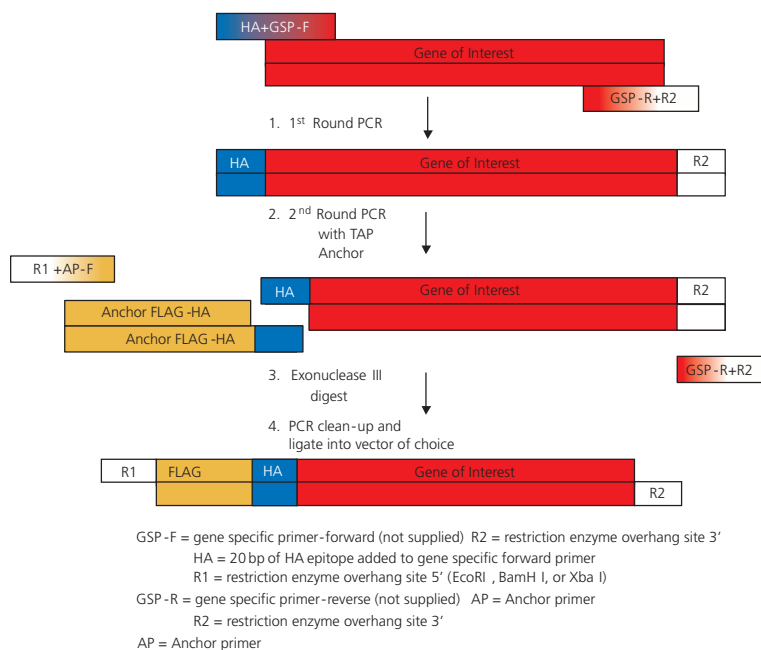


Figure 2. The TAP Tag Generation System allows for the rapid generation of ligation-ready DNA inserts that can be used with any expression vector to produce N-terminal FLAG-HA fusion proteins. The whole process of generating DNA inserts is completed in four simple steps. The first step is using gene-specific primers to amplify the gene of interest. Two unique additions are required for these gene-specific primers: one is to add a 20-base pair sequence corresponding to the HA tag to the gene-specific forward primer (GSP-F). The other addition is a 5 base pair sequence corresponding to the restriction site of choice to the gene-specific reverse primer (GSP-R). The restriction site is required for cloning of DNA inserts into expression vectors. The second step is to conduct PCR using the FLAG-HA anchor, an anchor primer, and the GSP-R. The FLAG-HA anchor is a double-stranded oligonucleotide containing FLAG and HA sequences and an overhang that is complementary to the HA overhang in the GSP-F. The anchor primer contains a complementary sequence to the FLAG-HA anchor and a restriction enzyme overhang. The choice of three overhangs, BamH I, EcoR I, and Xba I, are included in the kit to provide flexibility in choosing expression vectors. The third step is to digest PCR product with Exonuclease III to create cohesive ends for cloning. Using Exonuclease III digestion instead of conventional restriction enzyme digestion alleviates the concern about internal restriction sites in DNA inserts. To prevent over-digestion of Exonuclease III, a proprietary dNTP mix that contains dATPaS and dGTPaS is provided in the kit for PCR. This specially formulated dNTP mix has been optimized so that dATPaS and dGTPaS can be randomly incorporated into PCR products in a ratio that provides protection from Exonuclease III digestion and ensures consistency in creation of cohesive ends. The final step is to ligate dual-tagged DNA inserts to a double-digested expression vector of choice, which contains overhangs complementary to those in the DNA inserts. **Figure 3** shows the creation of and immunoprecipitations with FLAG HA protein of interest.

Creation and Immunoprecipitations of a FLAG HA Tandem Tagged Fusion Protein

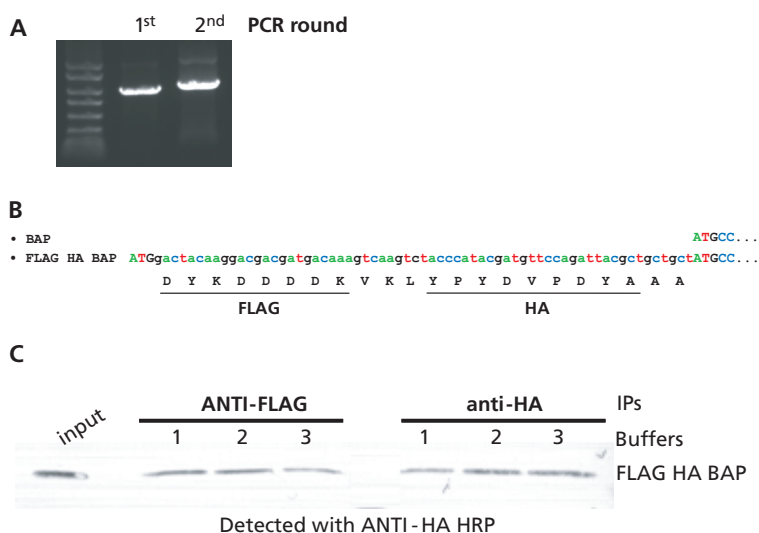


Figure 3. Both ANTI-FLAG and anti-HA resins reproducibly capture a high percentage of tandem tagged protein with different buffer systems. **Panel A** FLAG HA tandem tag was incorporated into bacterial alkaline phosphatase (BAP) using the FLAG HA TAP Tag generation Generation Kit (**Figure 2**) and the amplicon ligated into a bacterial expression vector (MAC, RDCLIG1-1KT). **Panel B** shows the sequence lineup of the created FLAG HA BAP fusion protein to BAP. The FLAG and HA epitopes are underlined. **Panel C** shows the dual tagged protein was expressed in and purified from the bacteria host, BL21. Purified FLAG HA BAP was incubated in three different extraction buffers (1–3) and immunoprecipitated using ANTI-FLAG and anti-HA affinity resins, separately. Protein recovery from both ANTI-FLAG and anti-HA affinity resins is greater than or equal to 70% of input protein. 150 ng/mL 3×FLAG peptide does not interfere with anti-HA IP (data not shown). Detection was shown with Anti-HA-Peroxidase conjugated antibody.

Specificity of FLAG and HA Epitope Tags/Resins and the Advantage of Consecutive Immunoprecipitations

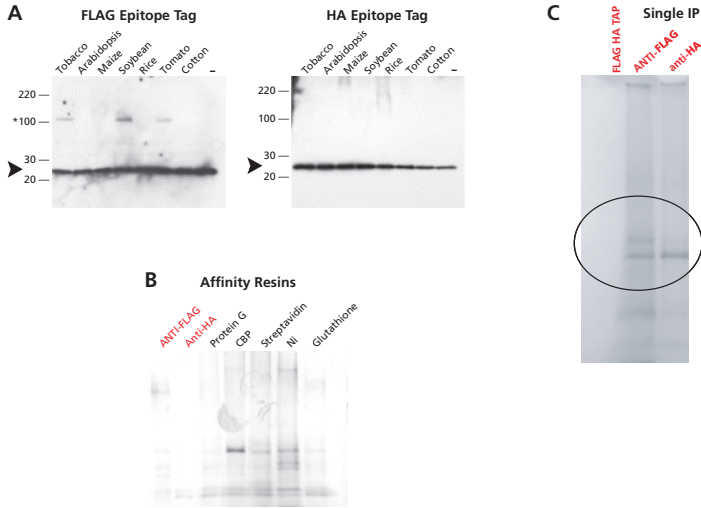


Figure 4. Specificity analysis of FLAG and HA tags and affinity resins using plant materials. **Panel A** shows high specificity of the ANTI-FLAG and anti-HA antibodies to FLAG- and HA-tagged fusion proteins, respectively, in 7 different plant extracts. 0.1 mg of epitope-tagged proteins (FLAG GST or GST HA) were spiked into 20 µg of total leaf proteins extracted from tobacco, Arabidopsis, maize, soybean, rice, tomato, and cotton. Immunoblot detection was with ANTI-FLAG M2[®]-AP and anti-HA-HRP antibody conjugates. **Panel B** shows specificity of ANTI-FLAG and anti-HA affinity resins in comparison with existing resins. 40 µL of resin slurries (ANTI-FLAG, anti-HA, Protein G, CBP, Streptavidin, Nickel, and Glutathione) were incubated with 6 mg total leaf protein from light grown Arabidopsis. After washing, proteins that were non-specifically bound to the resins were eluted, resolved on PAGE gel, and detected by silver stain. Both ANTI-FLAG and anti-HA resin showed minimal cross-reactivity compared to other resins. **Panel C** illustrates consecutive immunoprecipitations provide a higher purity of sample compared to a single immunoprecipitation. 8 mg of Arabidopsis leaf protein was immunoprecipitated with FLAG, then HA (FLAG HA TAP) or FLAG and HA alone. After incubation, resins were washed 3× with RIPA, eluted, and silver stained on a PAGE to see contaminants.

B

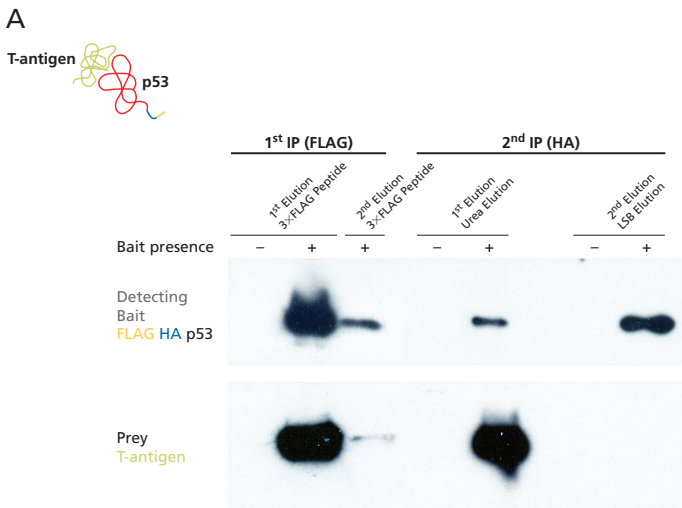
Large tumor antigen T; Sequence Coverage: 29%
Matched peptides shown in **Bold Red**

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1 MDKVLNREES LQLMDLLGLE RSAWGNIPLM RKAYLKKCKE FHPDKGGDEE
51 KMKMNTLYK KMDGVVYAH QPDFGGFWDA TEIPTYGTDE WEQWVNAFNE
101 ENLFCSEEMP SSDEATADS QHSTPPKRR KVEDPKDFPS ELLSFLSHAV
151 FSNRTLACFA IYTTKEKAAL LYKKIMEKYS VTFISRHSY NHNILFFLTP
201 HRHRVSAINN YAQKLCTFSF LICKGVNKEY LMYSALTRDP FSVIEESLPG
251 GLKEHDFNPE EAEETKQVSW KLVTEYAMET KCDDVLLLLG MYLEFQYSFE
301 MCLKCIKKEQ PSHYKHEKH YANAIFADS KNQKTCQQA VDTVLAKKRV
351 DSLQLTREQM LTNRFNDLLD RMDIMFGSTG SADIEEWMAG VAWLHCLLPK
401 MDSVVYDFLK CMVYNIPKR YWLFKGPIDS GKTTAAALL ELCGGKALNV
451 NLPLDRLNFE LGVAIDQFLV VFEDVKGTG ESRDLPSGQG INNLDNLRDY
501 LDGSVKVNLE KKHlnkrtQI FPPGIVTMNE YSVPKTLQAR FVKQIDFRPK
551 DYLKHCLERS EFLLEKRI IQ SGIALLLMLI WYRPVAEFAQ SIQSRIVEWK
601 ERLDKEFSLS VYQKMKFVNA MGVGVLDWLR NSSDDDDSDQ ENADKNEDKN
651 MEDSGHETGI DSQSQGSFQA QPSQSSQSVH DHNQPYHICR GFTCFKPPPT
701 PPFPEPT
  
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Figure 5. A well-known protein-protein interaction system in mammalian cells, p53 and T-antigen, was used to characterize the FLAG HA TAP system. FLAG HA tandem tag was incorporated into p53 (see FLAG HA Tandem Tag Generation Kit TP 0020) and transiently expressed in COS-7 cells (a mammalian cell line). The tagged p53, used as the bait protein, interacts with the endogenous T-antigen to form a complex. The protein complex was isolated using the TAP procedure. **Panel A** illustrates two major features: one is that FLAG HA tandem tag does not interfere with the interaction between the bait and the prey, and the other is that the urea solution preferentially elutes the prey protein (T-antigen) and leaves the bait protein (p53) on the affinity resin. The first immunoprecipitation was conducted using ANTI-FLAG resin and the protein complex was eluted with 3×FLAG peptide (once or twice). Both the bait and the prey were eluted off the resin equally. The second IP was conducted using anti-HA resin and eluted by urea solution followed by LSB. Almost all prey protein was eluted by urea, whereas the bait protein remains on the resin and only elutes with the addition of Laemmli Sample Buffer. Sample volumes loaded on each gel were normalized. Blots were probed with ANTI-FLAG[®] M2-Peroxidase or anti-T-antigen biotin (BD Biosciences)/streptavidin peroxidase. In **Panel B**, the TAP procedure was performed on COS-7 cells expressing FLAG HA p53 and the final elution was with urea. The elution was run on a PAGE gel and silver stained. The protein band cut out was identified with MS/MS as T-antigen, the targeted “prey”.

Isolation of Prey Protein using p53/T-antigen Interaction Model



Isolation of Prey Protein Using IAA1/TIR1 Interaction Model

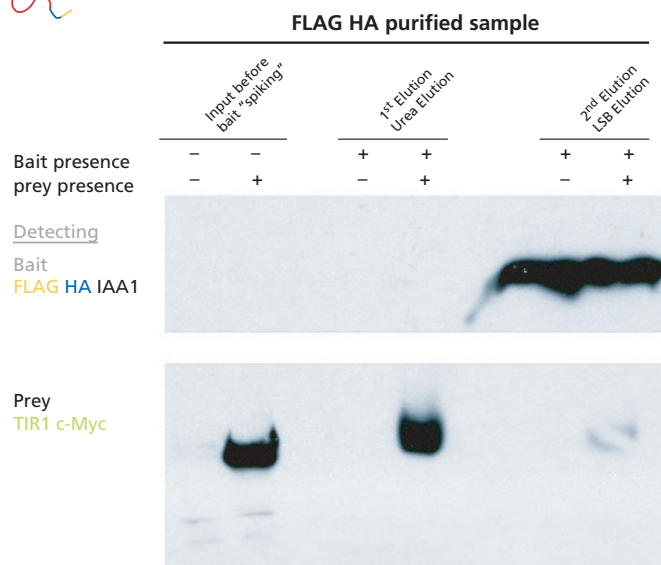


Figure 6. Further characterization of the FLAG HA TAP system using a protein-protein interaction model in plants, the Aux/IAA—TIR1 interaction.^{3,4} TIR1 is an auxin receptor.⁵ Auxin increases the affinity TIR1 has for Aux/IAAs (IAA1 is a family member).³ FLAG HA IAA1 (bait protein) was expressed in bacteria and purified using ANTI-FLAG resin and 3×FLAG peptide (data not shown). The purified FLAG HA IAA1 protein was incubated with protein extracts from wild type Arabidopsis or from transgenic Arabidopsis plant expressing TIR1 c-Myc in the presence of auxin. Samples were then purified using ANTI-FLAG followed by anti-HA resin. Anti-HA resin was eluted with urea (to elute the prey protein) then LSB (to elute the bait protein). Blots were probed with ANTI-FLAG[®] antibody to detect FLAG HA tagged IAA1 (the bait) or anti-c-Myc antibody to detect cMyc-tagged TIR1 (the prey). Similar to p53/T-antigen model, the prey protein is predominantly eluted with the urea elution, while the bait protein is not.

Future Work

- Further optimize the condition of FLAG HA TAP protein purification system.
- Expand the study to multi-component protein complexes.

References

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Sigma Products Used

FLAG[®] HA Tandem Affinity Purification Kit [TP0010](#)
 FLAG[®] HA TAP Tag Generation Kit [TP0020](#)
 ProteoSilver[™] Plus Silver Stain Kit [PROTSIL2](#)
 Chemiluminescent Peroxidase Substrate-1 [CPS1120](#)
 ANTI-FLAG[®] M2-Peroxidase (HRP) [A8592](#)
 Anti-HA-Peroxidase (HRP) [H6533](#)
 Streptavidin peroxidase, [S5512](#)
 Anti-rabbit peroxidase, [A8102](#)

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