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Affinity Purification of Chloroplast Translocon Protein Complexes using the TAP Tag

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TITLE:

Affinity Purification of Chloroplast Translocon Protein Complexes using the TAP Tag

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KEYWORDS:

Chloroplast, TOC-TIC Complex, TAP tag, TOC159, IgG binding, TEV protease, affinity purification.

SUMMARY:

We here present a proven and tested protocol for the purification of chloroplast protein import complexes (TOC-TIC complex) using the TAP-tag. The one-step affinity-isolation protocol can potentially be applied to any protein and be used to identify new interaction partners by mass spectrometry.

ABSTRACT:

Chloroplast biogenesis requires the import of thousands of nucleus-encoded proteins into the plastid. The import of these proteins depends on the translocon at the outer (TOC) and inner (TIC) chloroplast membranes. The TOC and TIC complexes are multimeric and probably contain yet unknown components. One of the main goals in the field is to establish the complete inventory of TOC and TIC components. For the isolation of TOC-TIC complexes and the identification of new components, the preprotein receptor TOC159 has been modified N-terminally by the addition of the tandem affinity purification (TAP) tag resulting in TAP-TOC159. The TAP-tag is designed for two sequential affinity purification steps (hence “tandem affinity”). The TAP-tag used in these studies consists of a N-terminal IgG-binding domain derived from *Staphylococcus aureus* Protein A (ProtA) followed by a calmodulin-binding peptide (CBP). Between these two affinity tags, a tobacco etch virus (TEV) protease cleavage site has been included. Therefore, TEV protease can be used for gentle elution of TOC159-containing complexes after binding to IgG beads. In the protocol presented here, the second Calmodulin-affinity purification step was omitted. The purification protocol starts with the preparation and solubilization of total cellular membranes. After the detergent-treatment, the solubilized

membrane proteins are incubated with IgG beads for the immunoisolation of TAP-TOC159-containing complexes. Upon binding and extensive washing, TAP-TOC159 containing complexes are cleaved and released from the IgG beads using the TEV protease whereby the *S. aureus* IgG-binding domain is removed. Western blotting of the isolated TOC159-containing complexes can be used to confirm the presence of known or suspected TOC and TIC proteins. More importantly, the TOC159-containing complexes have been used successfully to identify new components of the TOC and TIC complexes by mass spectrometry. The protocol that we present potentially allows the efficient isolation of any membrane-bound protein complex to be used for the identification of yet unknown components by mass spectrometry.

INTRODUCTION:

Plants depend on chloroplasts for photosynthesis and photoautotrophic growth¹. The vast majority of chloroplast proteins are encoded in the nucleus, synthesized in the cytosol and imported into the chloroplast via the TIC and TOC complexes in the envelope membranes². The core of the TOC complex consists of the Toc75 protein-conducting channel and the two receptor GTPases Toc33 and Toc159³⁻⁵. Toc159 is essential for the biogenesis of chloroplasts and mediates the massive accumulation of photosynthesis-associated proteins⁶. The TIC complex consists of the TIC20 protein-conducting channel as well as TIC110 and TIC40^{7,8}. Recently, a 1MD protein translocation complex at the inner envelope membrane was isolated and contained previously unidentified components⁹, one of which was TIC56. We recently co-isolated TIC56 of the 1 MDa complex using the TAP-TOC159 affinity purification protocol. The data indicated a structural overlap between the “canonical” TOC/TIC complexes and the 1 MDa complex¹⁰. Previously unknown components such as KOC1 were also identified as new interaction partners of the TOC and TIC complexes using the TAP-method described here^{10,11}.

Thus, the TAP-tag purification is an efficient method for the isolation of protein complexes and the identification of interacting partners by subsequent mass spectrometric analysis¹². The TAP tag consists of two IgG binding repeats separated from a calmodulin-binding peptide (CBP) by a tobacco etch virus (TEV) protease cleavage site. The original method, consisting of an IgG-affinity purification step followed by TEV cleavage and subsequent calmodulin-affinity chromatography permitted the native purification of large and highly pure protein complexes^{13,14}. We have simplified the procedure and demonstrated that the TOC159-containing protein complexes can also be purified efficiently using only the IgG-affinity purification step followed by TEV-cleavage for elution¹⁵. In our experience, the omission of the calmodulin-affinity step resulted in higher yields and may therefore be appropriate for low abundance proteins.

In short, we engineered stable transgenic *A. thaliana* lines expressing TAP-TOC159 in the *ppi2* (*toc159* KO mutant) background and established it as a reliable source for the purification of TOC-TIC complexes. The protocol for the isolation of the TOC-TIC complex starts with the homogenization of the plant material in a detergent-free buffer. After centrifugation, the supernatant is discarded. The pellet containing the total membrane fraction is solubilized using a detergent-containing buffer. After an ultra-centrifugation step, the supernatant containing solubilized TOC-TIC complexes is applied to the IgG-resin for affinity purification. After several washing steps, elution is carried out using a TEV protease-containing buffer to selectively cleave

downstream of the IgG-binding domains and gently release native TOC159-containing complexes. The TEV eluate can be analyzed directly by Western blotting or mass spectrometry to identify the interaction partners of TOC159^{10,11,15}. The method has also been used to identify post-translational modifications of TOC159¹⁵. In the future, native TOC159-containing complexes may be used for structural studies using cryoelectron microscopy.

PROTOCOL:

1. Preparation of Arabidopsis Plants

1.1. Prepare 1 L of half strength of Murashige and Skoog (MS) medium including vitamins with 1% sucrose and adjust the pH to 5.7 with potassium hydroxide (KOH). Add 0.8% of phytoagar and autoclave for 20 min at 120 °C.

1.2. Pour 75 mL of MS medium per Petri plate (diameter 14.5 cm, height 3 cm) and allow the solidification for 1 h.

1.3. Add 30 mg of *Arabidopsis thaliana* seeds to a 1.5 mL microfuge tube, and surface sterilize with 70% (v/v) ethanol containing 0.05% (v/v) Triton X-100 for 5 min, followed by 100% (v/v) ethanol for 10 min. Remove the ethanol.

1.4. Transfer the seeds to sterile filter paper for drying. Sprinkle the sterilized seeds evenly onto a MS plate (each genotype requires at least 8-10 plates), and seal each plate with surgical tape.

1.5. Incubate the plates at 4 °C for two days in the dark to synchronize seed germination. Transfer to a growth chamber with the following light cycle: 8 h of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, and 16 h of dark at 22-20 °C.

2. Preparation of HsIgG Agarose Beads

2.1. Suspend CNBr-activated agarose beads (4 g) in 100 mL of 1 mM HCl and incubate for 30 min at room temperature.

2.2. Transfer the agarose beads into a sintered glass filter, wash under vacuum with 100 mL of 100 mM HCl and repeat 2-3 times.

Note: Precool the wash solution to 4 °C.

2.3. Resuspend the agarose beads in 1 mL of cold 100 mM HCl and transfer to a 50 mL conical centrifuge tube. Wash the sintered glass filter with 3 mL of 100 mM HCl and transfer the liquid to the same tube.

2.4. Spin at 400 $\times g$ for 5 min at 4 °C and remove the supernatant with a pipette.

Note: Do not decant the tube.

2.5. Resuspend the beads in 50 mL of coupling buffer (**Table 1**); mix briefly, and then spin at $400 \times g$ for 5 min at 4 °C.

2.6. Dissolve 50 mg of lyophilized *Homo sapiens* IgG (Hs-IgG) in 10 mL of coupling buffer, mix the agarose beads gently, and incubate in rotary shaker overnight at 4 °C.

2.7. Spin at $400 \times g$ for 5 min at 4 °C and remove the supernatant with a pipette.

2.8. Wash the IgG-agarose beads with 50 mL of coupling buffer and spin at $400 \times g$ for 5 min at 4°C.

2.9. Resuspend the IgG-agarose beads with 50 mL of blocking buffer (**Table 1**) and spin at $400 \times g$ for 5 min at 4 °C. Repeat once.

2.10. Resuspend IgG-agarose beads in 50 mL of blocking buffer, rotate the mixture for 2 h at RT and spin at $400 \times g$ for 5 min at 4 °C.

2.11. Remove the supernatant and resuspend the IgG-agarose beads in 200 mL of NaCl coupling buffer (**Table 1**).

2.12. To block any remaining cross-linking CNBr groups, wash the IgG-agarose beads with 100 mL of 0.1 M glycine-HCl, pH 2.8. Spin at $400 \times g$ for 5 min at 4 °C and remove the supernatant. Wash with 0.2 M glycine-HCl, pH 2.8, spin at $400 \times g$ for 5 min at 4 °C and remove the supernatant. Finally, wash the IgG-agarose beads with 200 mL of ultrapure water.

2.13. Wash the IgG-agarose beads with 200 mL of ultrapure water and then 200 mL of PBS buffer (**Table 1**).

2.14. Resuspend the IgG-agarose beads in 20 mL of PBS buffer with 0.01% NaN₃ (sodium azide) and store at 4 °C.

3. Isolation and Solubilization of the Membrane Fraction

3.1. Grind 10 g of three weeks old *A. thaliana* seedlings in liquid nitrogen using a cold mortar and pestle with sand.

3.2. Add 20 mL of cold grinding buffer (**Table 1**) to 10 g of ground tissue, mix well, and thaw on ice.

3.3. Filter the homogenate through two layers of quick filtration material into a 50mL conical centrifuge tube. Soak the quick filtration material with grinding buffer before filtration.

3.4. Centrifuge the filtrate for 10 min at $1,500 \times g$ at 4°C and transfer the supernatant to a chilled 50 mL conical centrifuge tube, repeat the same step one more time and collect the supernatant. Retain a 200 μL sample of the “total fraction” and store at -80°C for later analysis

3.5. Transfer the supernatant to cold 38.50 mL ultracentrifuge tubes and top up to 35 mL with cold grinding buffer. Centrifuge for 1 h at $100,000 \times g$ at 4°C in an ultracentrifuge.

3.6. Transfer the supernatant to a 50 mL conical centrifuge tube. Retain a 200 μL sample of the “soluble fraction” and store at -80°C for later analysis.

3.7. Re-suspend the green pellet using a glass teflon homogenizer, in grinding buffer. Centrifuge for 1 h at $100,000 \times g$ at 4°C in an ultracentrifuge and discard the supernatant.

3.8. Resuspend the green pellet in 18.75 mL of 1x grinding Buffer using a glass teflon homogenizer and add 9.375 mL of 1x grinding buffer.

3.9. Add 9.375 mL of 4x solubilisation solution (**Table 1**) (containing the TX-100 detergent) to give 37.5 mL and incubate for 30 min on a “rotating shaker” at 4°C .

3.10. Centrifuge for 1 h at $100,000 \times g$ at 4°C in an ultracentrifuge. Transfer the supernatant to a 50 mL conical centrifuge tube. Retain a 200 μL sample of the supernatant (future “load fraction”) and store at -80°C for later analysis.

3.11. Resuspend the pellet in 37.5 mL of grinding buffer. Retain the sample of insoluble part and store at -80°C

4. Immunoprecipitation

4.1. Equilibrate 100 μL of packed IgG-agarose resin in Buffer A (**Table 1**) and spin at $100 \times g$ for 5 min at 4°C .

4.2. Remove the supernatant and resuspend the IgG-agarose resin in 100 μL of Buffer A at 4°C .

4.3. Transfer the washed IgG-agarose resin to the 37.5 mL of solubilized membranes and incubate overnight on a rotating shaker in a 50 mL conical centrifuge tube at 4°C .

4.4. Sediment the IgG-agarose resin at $100 \times g$ for 5 min at 4°C and remove the supernatant with a pipette. Retain a 200 μL sample of the “flow through” and store at -80°C for later analysis.

4.5. Wash the IgG-agarose resin in 37.5 mL of Buffer A and incubate for 10 min on a rotating shaker.

4.6. Sediment the IgG-agarose resin at $100 \times g$ for 5 min at 4 °C, remove the supernatant with a pipette, add 5 mL of grinding Buffer A to a 15 mL centrifuge tube and centrifuge at $100 \times g$, 4 °C, 5 min. Retain a 200 µL sample of the “Wash 1” and store at -80 °C for later analysis.

4.7. Repeat the 5 mL washing step three times with Buffer A.

4.8. Carry out the last two washing steps without the inhibitors (NaF, Protease inhibitor and PMSF)

Note: Inhibitors are no longer required at this stage and omitted to reduce cost. Retain a 200 µL sample of the last washing step “final wash (W6)” and store at -80 °C for later analysis.

4.9. Transfer the IgG-agarose resin to a 500 µL spin column with a 35 µm filter and wash the beads twice with 300 µL of TEV elution buffer (**Table 1**) (not containing AcTEV) for equilibration. Close the spin column.

4.10. Add 300 µL of TEV elution buffer containing 50 units (10 µL) of AcTEV. Prepare the mix in a tube before adding to the resin.

4.11. Incubate the spin column with beads in a thermomixer at 16 °C, 350 rpm for 2 h. Invert the column gently several times every 30 min.

4.12. Open the spin column, place it in a new clean 1.5 mL tube and spin at $100 \times g$ for 5 min at 4 °C to collect the eluate.

4.13. Dry (for instance) 10% (v/v) of the eluted sample (~25 µL) in a centrifugal evaporator and use for mass spectrometry or other further analysis.

4.14. Aliquot the remaining eluate (~275 µL) divided in to eleven 1.5 mL centrifuge tubes (25 µL each), dry in a centrifugal evaporator, and store at -80 °C.

4.15. Analyze the samples retained throughout the procedure as well as the eluates by standard SDS-PAGE followed by immunoblotting. For sample preparation, precipitate 50 µg of Total (T), Load (L), Flow through (FT), Wash 1 (W1) and 200 µL of Wash 6 (W6) by chloroform/methanol extraction¹⁶.

4.16. Add 10 µL of 2x SDS-PAGE loading buffer (**Table 1**) to the precipitated samples and 25 µL of eluted dried sample. Vortex and boil for 10 min at 95 °C in a heat block.

4.17. Analyze Total (T), Load (L), Flow through (FT), Wash 1 (W1), Wash 6 (W6) and Elution (E) by immunoblotting using anti-TOC159 antibodies to determine the efficiency of the isolation procedure.

REPRESENTATIVE RESULTS:

We here described a protocol for the purification of a TAP-tagged chloroplast envelope protein complex from transgenic *A. thaliana* plants. As shown in **Figure 1A**, plants expressing the 35S:NTAP-TOC159 construct were used to isolate this complex while plants expressing the 35S:NTAP construct were used as a control. **Figure 1B** shows detailed steps of the purification of TAP-TOC159 protein complexes followed by mass spectrometry to allow the identification of interacting proteins. The immunoblot analysis (**Figure 2**, right side) confirms that isolated TAP-TOC159 interacts with TOC75 and TOC33 of the TOC complex. The chloroplast outer membrane kinase KOC1 known to phosphorylate TOC159 also co-isolated with the TAP-TOC159 complex (**Figure 2B**, right side). The presence of TIC110 reveals that the isolated TAP-TOC159 complex also contains the components of the TIC complex. The FBN1A antibody raised against a plastoglobule protein unrelated to protein import did not recognize the TAP-TOC159 complex indicating the absence of contaminations. In the negative control, immunoprecipitated NTAP protein did not co-isolate with any of the TOC-TIC proteins (**Figure 2**, left side) and confirms the specificity of the TAP-TOC159 purification.

FIGURE AND TABLE LEGENDS:

Figure 1. Scheme of the constructs and affinity purification procedure. (A). The 35S:NTAP-TOC159 construct, encoding NTAP-TOC159 was stably expressed in *Arabidopsis thaliana*. Negative control plants expressed the 35S:NTAP construct, encoding the TAP-tag alone. (B). The step wise affinity purification protocol consists of membrane isolation and solubilization, IgG agarose immuno-purification, washes, TEV protease cleavage and elution.

Figure 2. Tandem affinity purification of the TOC159 protein. N-terminally TAP-tagged TOC159 was purified from NTAP-TOC15:ppi2 *Arabidopsis* plants and TAP protein purified from TAP:WT plants was used as a negative control. Total protein extracts (T), solubilized membrane proteins = load fraction (L), flow through (FT), first and last wash fractions (W1 and W6) and 10% of the TEV eluate were analyzed by Western blotting. The membrane was probed with the antibodies against TOC159 (AT4G02510), TOC75 (AT3G46740), TOC33 (AT1G02280), KOC1 (AT4G32250), TIC110 (AT1G06950), and FBN1A (AT4G04020).

Table 1. Buffer recipes.

DISCUSSION:

We demonstrated that a single step TAP tag purification is a specific and efficient method for the purification of the *Arabidopsis* TOC-TIC complex. Although the TAP-tag would allow two successive purification steps (IgG- followed by calmodulin affinity purification after TEV protease-cleavage), we believe that in many cases the first affinity step followed by TEV protease cleavage will be sufficient. Our target, TOC159, is low abundant and the inclusion of the second calmodulin-affinity step excessively diminished the protein yield which was the main reason for excluding it from our present protocol. The TEV-elution in itself is a highly specific and gentle purification step that will only release the TAP-tagged target together with associated interaction partners while contaminating the proteins sticking non-specifically to the IgG resin will remain there. Thus, in combination with the IgG-affinity step, the TEV elution results in a sufficiently efficient purification for our and probably many others' purposes.

Care must be taken that the TAP-tagged construct is fully functional *in vivo*. TAP-tagging could have potentially deleterious effects on protein activity, stability or localization. TOC159 consists of three domains, the N-terminal acidic domain, the central GTP-binding domain and C-terminal membrane domain, that anchors TOC159 in the outer chloroplast membrane². To avoid potential interference with membrane insertion, we fused the TAP-tag to the N-terminus of TOC159. Fusion to the N-terminus is rather the exception than the rule. Many proteins contain N-terminal targeting information and should therefore be tagged at the C-terminus. We assured that TOC159 is functional *in vivo* by complementation of the *ppi2* mutant (an albino mutant lacking TOC159) with TAP-TOC159. The rescue of the green, wild type phenotype indicated that TAP-TOC159 was functional¹⁵.

The purification protocol was used to identify new interaction partners of TOC159, that included KOC1 and TIC56^{10,11}. In total, around 30 proteins were associated with the complex suggesting that new interaction partners will be isolated and characterized in the near future. While we highly recommend the TAP-tag for complex purification, we would still like to point out that additional versions to the one presented here exist and may be very useful for some applications.

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DISCLOSURES:

The authors declare no competing interests.

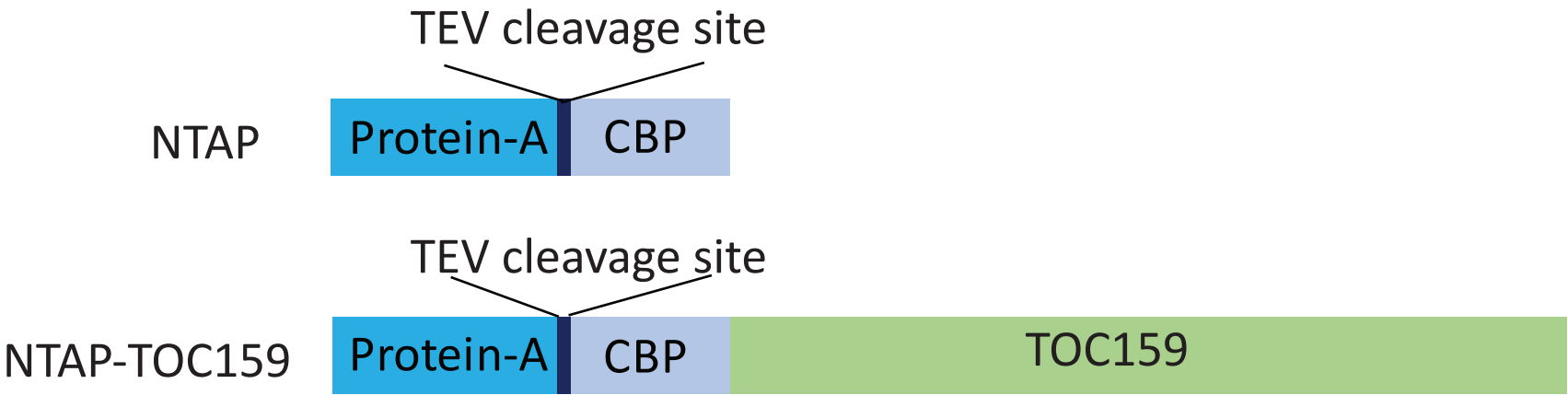
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Figure 1:

A



B

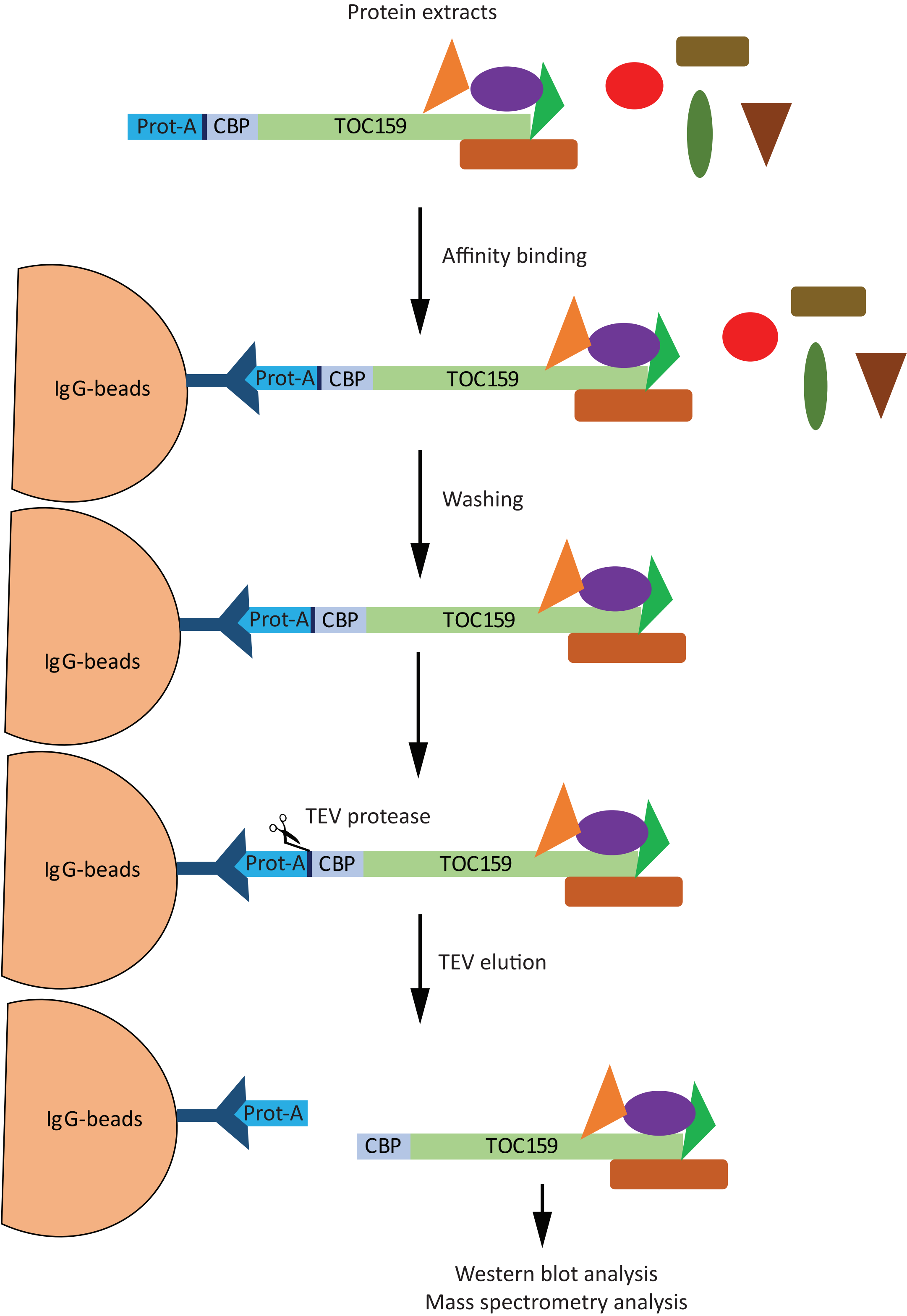


Figure 2:

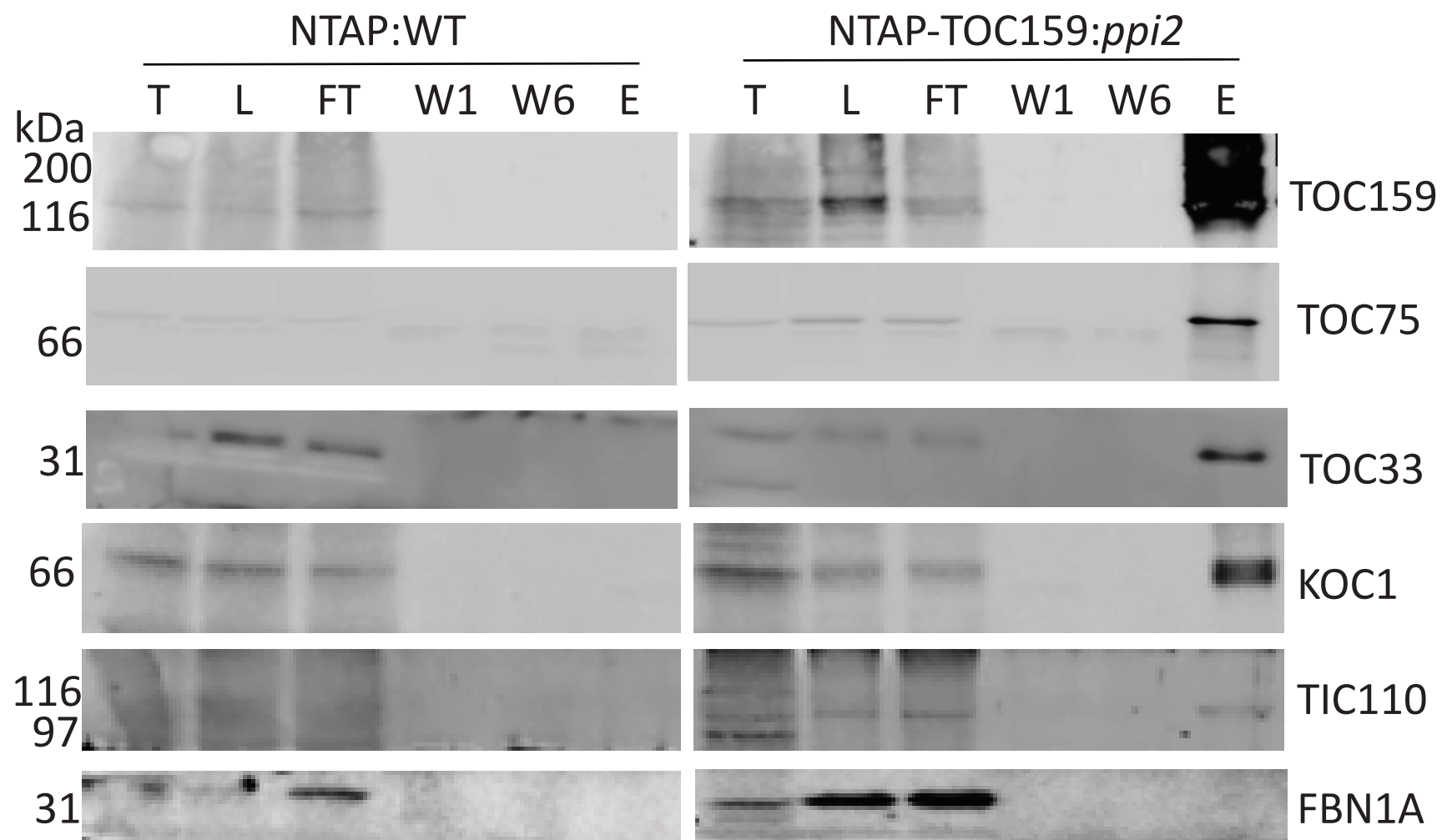


Table of buffers

Coupling Buffer	NaHCO ₃ Adjust to pH 8.5 adjust pH with 0.1 M Na ₂ CO ₃	100 mM
Blocking Buffer	Tris-HCl Adjust to pH 8 with HCl.	100 mM
PBS Buffer	Na ₂ HPO ₄ KH ₂ PO ₄ NaCl KCl Adjust to pH 7.3 with HCl.	4.3 mM 1.4 mM 137 2.7
NaCl coupling Buffer	Coupling buffer NaCl	1 M
2X grinding Buffer	Tris-HCl, pH 7.5 with HCl. NaCl NaF PMSF Plant protease inhibitor cocktail	100 mM 200 mM 5 mM 1 mM 0.20%
4X solubilisation solution	Glycerol Triton-X100	40% 3%
Buffer A	2X grinding Buffer 1X grinding Buffer 4X solubilisation solution	100 ml 50 ml 25 ml 25%
TEV elution Buffer	Tris-HCl, pH 8 with HCl EDTA, pH 8 with HCl NaCl Glycerol Triton-X100 DTT	50 mM 0.5 mM 100 mM 10% 0.75% 1 mM
2x SDS-PAGE loading Buffer	Tris-HCl pH 6.8 with HCl SDS Glycerol Bromophenol blue DTT	0.1 M 4% 20% 0.20% 0.2 M

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
NaHCO ₃	PanReac AppliChem	A0384	
Tris	Biosolve	0020092391BS	
Na ₂ HPO ₄	Sigma	S7909	
KH ₂ PO ₄	PanReac AppliChem	A2946	
NaCl	PanReac AppliChem	A2942	
NaF	Sigma	S1509	Toxic
PMSF	PanReac AppliChem	A0999	Toxic
Plant protease inhibitor cocktail	Sigma	P9599	
Glycerol	PanReac AppliChem	A0970	
Triton X100	Roche	10789704001	
Glycine	PanReac AppliChem	A1067	
EDTA	Carl Roth	8043	
Dithiothreitol (DTT)	PanReac AppliChem	A1101	
SDS	PanReac AppliChem	A0675	
Bromophenol blue	Sigma	B0126	
HCl	VWR	20252-290	Corrosive
Sucrose	PanReac AppliChem	A3935	
Murashige and Skoog medium with vitamins	Duchefa Biochemie	M0222	
Phytoagar	Duchefa Biochemie	P1003	
Petri plate	Greiner Bio-one	639102	
1.5 mL microfuge tubes	Sarstedt	72.706	
Ethanol	Fisher chemical	E/0600DF/15	
Filter paper	Whatman	10311804	
Surgical tape	3M	1530-1	
CNBr-Activated agarose beads (sepharose 4B)	GE Healthcare	17-0430-01	
Sintered glass filter	DURAN	2515703	
Centrifuge tube 50 mL	TPP	91050	
Purified immunoglobulin G (HsIgG)	MP Biomedicals	855908	
Rotating shaker	IKA	4016000	
NaN ₃ (sodium azide)	Sigma	71290	Toxic
Quick filtration material (Miracloth)	Merk-Millipore	475855	

Ultracentrifuge XNP-80	Beckman Coulter	A99839	
Rotor SW 32 Ti	Beckman Coulter	369650	
Ultracentrifuge tubes	Beckman Coulter	326823	
Glass teflon homogenizer (Potter)	Wheaton	357426	
Spin column (Mobicol)	Mo Bi Tec	M1003	
Filter 35µm for spin column (Mobicol)	Mo Bi Tec	M513515	
AcTEV	Invitrogen	12575-015	
Centrifugal evaporator (Speed Vac)	Eppendorf	5305000100	
FBN1A(PGL35) antibody	AgriSera	AS06 116	RRID:AB_2247012
Sand, Fontainebleau	VWR	27460.295	



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Dear Dr. Bing Wu,

Thank you for providing us with your comments. We have now improved the manuscript and reply your comments in this document.

We hope that the improvements of our manuscripts are satisfactory.

Yours sincerely,



Felix Kessler

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