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# TurboID-Based Proximity Labeling for In Planta Identification of Protein-Protein Interaction Networks

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## Abstract

Proximity labeling (PL) techniques using engineered ascorbate peroxidase (APEX) or *Escherichia coli* biotin ligase BirA (known as BioID) have been successfully used for identification of protein-protein interactions (PPIs) in mammalian cells. However, requirements of toxic hydrogen peroxide ($H_2O_2$) in APEX-based PL, longer incubation time with biotin (16–24 h), and higher incubation temperature (37 °C) in BioID-based PL severely limit their applications in plants. The recently described TurboID-based PL addresses many limitations of BioID and APEX. TurboID allows rapid proximity labeling of proteins in just 10 min under room temperature (RT) conditions. Although the utility of TurboID has been demonstrated in animal models, we recently showed that TurboID-based PL performs better in plants compared to BioID for labeling of proteins that are proximal to a protein of interest. Provided here is a step-by-step protocol for the identification of protein interaction partners using the N-terminal Toll/interleukin-1 receptor (TIR) domain of the nucleotide-binding leucine-rich repeat (NLR) protein family as a model. The method describes vector construction, agroinfiltration of protein expression constructs, biotin treatment, protein extraction and desalting, quantification, and enrichment of the biotinylated proteins by affinity purification. The protocol described here can be easily adapted to study other proteins of interest in *Nicotiana* and other plant species.

### Keywords

Immunology and Infection; Issue 159; proximity labeling; TurboID; protein interactions; desalting; quantification; purification; TIR

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Video Link
The video component of this article can be found at https://www.jove.com/video/60728/

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The authors have nothing to disclose.

## Introduction

PPIs are the basis of various cellular processes. Traditional methods for identifying PPIs include yeast-two-hybrid (Y2H) screening and immunoprecipitation coupled with mass spectrometry (IP-MS)[1]. However, both suffer from some disadvantages. For example, Y2H screening requires the availability of Y2H library of the target plant or animal species. Construction of these libraries is labor-intensive and expensive. Furthermore, the Y2H approach is performed in the heterologous single-cell eukaryotic organism yeast, which may not represent the cellular status of higher eukaryotic cells.

In contrast, IP-MS shows low efficiency in capturing transient or weak PPIs, and it is also unsuitable for those proteins with low abundance or high hydrophobicity. Many important proteins involved in the plant signaling pathways such as receptor-like kinases (RLKs) or the NLR family of immune receptors are expressed at low levels and often interact with other proteins transiently. Therefore, it greatly restricts the understanding of mechanisms underlying the regulation of these proteins.

Recently, proximity labeling (PL) methods based on engineered ascorbate peroxidase (APEX) and a mutant *Escherichia coli* biotin ligase $BirA^{R118G}$ (known as BioID) have been developed and utilized for the study of PPIs[2,3,4]. The principle of PL is that a target protein of interest is fused with an enzyme, which catalyzes the formation of labile biotinyl-AMP (bio-AMP). These free bio-AMP are released by PL enzymes and diffuse to the vicinity of the target protein, allowing the biotinylation of proximal proteins at the primary amines within an estimated radius of 10 nm[5].

This approach has significant advantages over the traditional Y2H and IP-MS approaches, such as the ability to capture transient or weak PPIs. Furthermore, PL allows the labeling of proximal proteins of the target protein in their native cellular environments. Different PL enzymes have unique disadvantages when applying them to different systems. For example, although APEX offers higher tagging kinetics compared to BioID and is successfully applied in mammalian systems, the requirement of toxic hydrogen peroxide ($H_2O_2$) in this approach makes it unsuitable for PL studies in plants.

In contrast, BioID-based PL avoids use of the toxic $H_2O_2$, but the rate of labeling is slow (requiring 18–24 h to complete biotinylation), thus making the capture of transient PPIs less efficient. Moreover, the higher incubation temperature (37 °C) required for efficient PL by BioID introduces external stress to some organisms, such as plants[4]. Therefore, limited deployment of BioID-based PL in plants (i.e., rice protoplasts, *Arabidopsis*, and *N. benthamiana*) has been reported[6,7,8,9]. The recently described TurboID enzyme overcomes the deficiencies of APEX and BioID-based PL. TurboID showed high activity that enables the accomplishment of PL within 10 min at RT[10]. TurboID-based PL has been successfully applied in mammalian cells, flies, and worms[10]. Recently, we and other research groups independently optimized and extended the use of TurboID-based PL for studying PPIs in different plant systems, including *N. benthamiana* and *Arabidopsis* plants and tomato hairy roots[11,12,13,14]. Comparative analyses indicated that TurboID performs better for PL in plants compared to BioID[11,14]. It has also demonstrated the robustness of TurboID-based PL

in planta by identifying a number of novel interactions with an NLR immune receptor[11], a protein whose interaction partners are usually difficult to obtain using traditional methods.

This protocol illustrates the TurboID-based PL *in planta* by describing the identification of interaction proteins of the N-terminal TIR domain of the NLR immune receptor in *N. benthamiana* plants. The method can be extended to any proteins of interest in *N. benthamiana*. More importantly, it provides an important reference for investigating PPIs in other plant species such as *Arabidopsis*, tomato, and others.

## Protocol

NOTE: An overview of the method is shown in Figure 1.

### 1. Plant material preparation

1. Grow *N. benthamiana* seeds in wet soil at a high density and maintain them in a climate chamber with a 16 h light (about 75 μmol/$m^2$s) and 8 h dark photoperiod at 23–25 °C.
2. About 1 week later, carefully transfer each young seedling to 4′ × 4′ pots and keep the seedlings in the same chamber.
3. Maintain the plants in the chamber for about 4 weeks until they grow to a leaf stage of 4–8 for subsequent agroinfiltration[15].

### 2. Construction of TurboID fusions

1. Use standard molecular cloning technique to generate fusion of the target protein with TurboID (PCR TurboID from Addgene plasmid #107177). Here, we used the N-terminal TIR domain of the NLR immune receptor as the target protein of interest and constructed TIR fused to TurboID under the control of Cauliflower mosaic virus 35S promoter (p35S::TIR-TurboID).

   NOTE: Fusion of the TurboID enzyme to the carboxyl-terminus or amino-terminus of the target protein will depend on the protein of interest. Usually, for a cytoplasmic protein, both termini should be acceptable, as long as the TurboID fusion does not affect the function of the target protein. However, for a membrane localized protein, the protein topology should be characterized in advance prior to determining which terminus is best for TurboID fusion.

2. Construct a TurboID-fused citrine under the same promoter to serve as the control for subsequent quantitative proteomic analysis.

   NOTE: It is important to construct a TurboID control for identifying the proteome proximal to the target protein. The TurboID fusion control should show an expression level similar to that of the TurboID-fused target protein. This can be empirically determined by adjusting the *Agrobacterium* concentration during agroinfiltration. In addition, it is important that the control protein has a subcellular localization pattern similar to that of the target protein of interest.

## 3. Agroinfiltration

1. Transformation of the plasmids to *Agrobacterium*

    1. Add 0.5 μg of the plasmids generated from steps 2.1 and 2.2 to 50 μL of *Agrobacterium tumefaciens* strain GV3101 competent cells, prepared as described previously[16].

        NOTE: Other *Agrobacterium* strains, such as GV2260, can also be used.

    2. Incubate on ice for 30 min.

    3. Heat shock in a water bath at 42 °C for 60 s.

    4. Add 400 μL of LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl; pH = 7.0) to the *Agrobacterium* and incubate at 28 °C for 90 min.

    5. Plate the entire content in the tube on LB agar supplemented with appropriate antibiotics to select the plasmid, as well as for the *Agrobacterium* (for GV3101: 50 mg/L gentamicin and 50 mg/L rifampicin).

    6. Incubate plates at 28 °C for 36–48 h until individual colonies are visible.

2. Pick and streak several individual colonies onto a fresh LB agar plate with antibiotics (see step 3.1.5) and grow at 28 °C overnight.

    NOTE: It is more optimal to perform the colony PCR to confirm the presence of the specific binary construct in the *Agrobacterium*. In our experience, over 95% of the colonies contain the introduced binary constructs.

3. Inoculate 3 mL of LB medium plus appropriate antibiotics (see step 3.1.4) with *Agrobacterium* colony harboring the construct of interest, and incubate by shaking overnight at 28 °C until the $OD_{600}$ of the *Agrobacterium* culture reaches 2.0.

4. Centrifuge the cells at 3,000 × *g* and resuspend them to $OD_{600}$ = 1.0 in agroinfiltration buffer (10 mM $MgCl_2$, 10 mM MES [pH = 5.6], 250 μM acetosyringone).

    NOTE: Although it is optimal to incubate the inoculum for 2 h at RT prior to agroinfiltration, in our experience with the GV3101 strain, there has not been a large difference between the target protein expression with *vs.* without incubation.

5. Use a 1 mL needleless syringe to infiltrate the inoculum in the (abaxial) epidermis of the fully mature *N. benthamiana* leaves.

    NOTE: To prepare a sufficient amount of leaf materials for three biological replicates: an entire leaf is usually infiltrated, three leaves are infiltrated per

plant, and three to four plants are used for each construct. For each leaf, 1.5–2.0 mL of resuspended agrobacteria is sufficient.

6. After 36 h post-infiltration (hpi), infiltrate 200 μM biotin (in 10 mM $MgCl_2$ solution) into the leaves pre-infiltrated with TurboID constructs.

7. Maintain the plant for additional 3–12 h before harvesting the leaf tissue as described in section 4.

   NOTE: The reason for choosing the 36 hpi for biotin infiltration is that the target protein expression peaks at this timepoint according to previous studies[11]. It is advised to determine the time required for optimal expression of the target protein of interest. The incubation time post-biotin infiltration depends on the experimental design and target protein under study. Usually, 3–12 h of biotin treatment allows the labeling of most proteins proximal to the target protein by the TurboID fusions.

## 4. Leaf sample collection

NOTE: For subsequent processing of the leaf samples, wear sterile gloves to avoid keratin contamination of the samples. All reagents should also be as keratin-free as possible.

1. Cut the infiltrated leaves at the base of the petiole, remove the leaf vein, then flash-freeze the leaf tissue in liquid nitrogen.

2. Grind the leaf tissue using a pestle and mortar and store the leaf powder in 15 mL or 50 mL falcon tubes at −80 °C for subsequent use.

   NOTE: Take three to four pieces of leaves for each of the three biological replicates from different plants. The protocol can be paused here. Prior to subsequent steps, it is recommended to assess protein expression and biotinylation of the target protein by immunoblot analyses. Typical western blots are shown in Figure 2.

## 5. Extraction of leaf total protein

1. Transfer about 0.35 g of leaf powder to a 2 mL tube. Prepare two tubes for each sample.

   CAUTION: Wear gloves when touching any object cooled by liquid nitrogen.

2. Add 700 μL of RIPA lysis buffer (50 mM Tris-HCl [pH = 7.5], 500 mM NaCl, 1 mM EDTA, 1% NP40 [v/v], 0.1% SDS [w/v], 0.5% sodium deoxycholate [w/v], 1 mM DTT, 1 tablet of protease inhibitor cocktail) to 0.35 g of leaf powder.

3. Vortex the tubes for 10 min.

4. Leave the samples on ice for 30 min.

5. Mix the contents every 4–5 min by turning the tubes upside down several times.

## 6. Removal of free biotin by desalting

NOTE: This section takes about 50 min.

1. Equilibrate the desalting column
    1. Remove the sealer at the bottom of the desalting column and put the column in a 50 mL tube.
    2. Remove the storage solution by centrifugation at 1000 × *g* and 4 °C for 2 min and ensure that that the cap is loosened.
    3. Put the desalting column in a 50 mL tube. Equilibrate the column 3× with 5 mL of RIPA lysis buffer, each time centrifuging at 1000 × *g* and 4 °C for 2 min and discarding the flowthrough.
    4. Transfer the desalting column into a new 50 mL tube and store temporarily at 4 °C for subsequent use.
2. Spin the tubes from step 5.4 at 16,500 × *g* and 4 °C for 10 min and transfer the supernatant from the two tubes into a new 2 mL tube.
3. Add 1,500 µL of protein extract to the top of the resin of the equilibrated desalting column (from step 6.1.4). When the protein extract enters the resin, add another 100 µL of RIPA lysis buffer.

    NOTE: Although 1,400 µL of RIPA lysis buffer was used for protein extraction from the leaves, the total volume after protein extraction and desalting was invariably increased to some extent relative to the original volume. Therefore, a combination of the samples from each group as described in steps 5.1 and 5.4 can result in at least 1,500 µL of protein extract per sample.
4. Centrifuge at 1000 × *g* and 4 °C for 2 min and leave the desalted samples on ice temporarily.

## 7. Quantification of the desalted protein extracts using a Bradford assay

1. Prepare 50 µL of each gradient BSA solution: 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1 mg/mL.
2. Dilute the desalted protein extract by mixing a 5 µL sample with 45 µL of $ddH_2O$.
3. Prepare 1× Bradford regent by diluting the 5× Bradford regent (100 mg of Coomassie brilliant blue G250, 47 mL of methanol, 100 mL of 85% phosphoric acid, 53 mL of $ddH_2O$).
4. Add 50 µL of the each gradient BSA solution and 50 µL of diluted protein extract to the 2.5 mL of 1× Bradford regent.
5. Incubate at RT for 10 min.
6. Add 200 µL of solution of each sample to one well of an ELISA plate (three technical replicates per sample).
7. Measure the $OD_{595}$ using a microplate reader.

8. Draw the standard curve based on the value of the gradient BSA solution and calculate the concentration of the desalted protein samples. Usually, the total protein concentration obtained from 0.7 g of leaves ranges from 3–6 mg/mL.
9. Prepare 6–8 mg of desalted protein extract for subsequent affinity purification.

## 8. Enrichment of biotinylated proteins

1. Take 200 μL of streptavidn-C1-conjugated magnetic beads into a 2 mL tube.
2. Equilibrate the streptavidn-C1-conjugated magnetic beads with 1 mL of RIPA lysis buffer for 1 min at RT.
3. After each washing, use the magnetic rack to adsorb the beads for 3 min and gently remove the solution by pipetting.
4. Repeat steps 8.2 and 8.3.
5. Transfer the desalted protein extract to the equilibrated streptavidn-C1-conjugated magnetic beads.
6. Incubate the tube at 4 °C for 12 h (or overnight) on a rotator to affinity-purify the biotinylated proteins.
7. Capture the beads on a magnetic rack for 4 min at RT until the beads collect at one side of the tube, then gently remove the supernatant by pipette.
8. Add 1.7 mL of wash buffer I (2% SDS in water) to the tube and keep it on the rotator at RT for 8 min. Repeat step 8.3.
9. Add 1.7 mL of wash buffer II (50 mM HEPES: pH = 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% deoxycholic acid [w/v], 1% Triton X-100) to the tube and keep it on the rotator at RT for 8 min. Repeat step 8.3.
10. Add 1.7 mL of wash buffer III (10 mM Tris-HCl: pH = 7.4, 250 mM LiCl, 1 mM EDTA, 0.1% deoxycholic acid [w/v], 1% NP40 [v/v]) to the tube and keep it on the rotator at RT for 8 min. Repeat the step 8.3.
11. Add 1.7 mL of 50 mM Tris-HCl (pH = 7.5) to remove the detergent, then repeat step 8.3.
12. Transfer the beads to a new 1.5 mL tube, and repeat steps 8.11 and 8.3.
13. Wash the beads 6× for 5 min each with 50 mM ammonium bicarbonate buffer at RT.
14. Add 1 mL of 50 mM ammonium bicarbonate buffer to the magnetic beads and mix well.
15. Remove 100 μL of beads for immunoblot analysis to confirm the enrichment of biotinylated proteins. A typical western blot is shown in Figure 3.
16. Flash-freeze the rest of the protein samples and stored at −80 °C or send them immediately for LC-MS/MS analysis on dry ice.

NOTE: Typical MS results are displayed in a previous publication (Zhang et al. 2019; Figure 2, Supplementary Data 1, and Supplementary Data 2)[11]. The whole datasets are available on MassIVE (found at <http://massive.ucsd.edu>) using the identifier: MSV000083018 and MSV000083019.

## Representative Results

The representative data, which illustrate the expected results based on the described protocol, are adapted from Zhang et al[11]. Figure 1 summarizes the procedures for performing TurboID-based PL in *N. benthamiana*. Figure 2 shows the protein expression and biotinylation in the infiltrated *N. benthamiana* leaves. Figure 3 shows that the biotinylated proteins in the infiltrated leaves were efficiently enriched for subsequent mass spectrometry analysis. It should be noted that after enrichment of the biotinylated proteins using streptavidn-C1-conjugated magnetic beads, different proteins with varied sizes were captured, and western blot analysis of the enriched proteins showed smeared bands (Figure 3). Similar observations have been made in several recently published studies[6,7,13,14].

## Discussion

The TurboID biotin ligase is generated by yeast display-based directed evolution of the BioID[10]. It has many advantages over other PL enzymes. TurboID allows the application of PL to other model systems, including flies and worms, whose optimal growth temperature is around 25 °C[10]. Although the PL approach has been widely used in animal systems, its application in plants is limited. The protocol described here provides a step-by-step procedure for establishing the TurboID-based PL in *N. benthamiana*, a model plant that has been widely used in plant-pathogen interaction studies. This protocol outlines leaf sample preparation, removal of free biotin, quantification of the extracted proteins, and enrichment of the biotinylated proteins.

Although free biotin in animal cell culture systems can be largely removed by washing the cells with PBS buffer[4], the free biotin in leaf tissue cannot be cleared by simple washing. A recent study indicated that the free biotin can severely impact subsequent enrichment of the biotinylated proteins[11]. In this protocol, a desalting column is utilized to successfully remove free biotin in the protein extracts, allowing the efficient binding of biotinylated proteins to the streptavidin beads.

Moreover, this protocol serves as an important reference for conducting PL in other plant systems. Recently, three reports have used TurboID-based PL in *Arabidopsis*[12,13,14] and tomato hairy root[12], besides the *N. benthamiana* described here. Similar to animal cell culture systems, the removal of the free biotin was achieved by washing the plant protoplasts prior to protein extraction[6]. However, lower amounts of free biotin molecules that were not integrated to the protein existed in the interior of the cell, which impacted the efficiency of subsequent enrichment of the biotinylated proteins. Therefore, a desalting procedure is recommended for complete removal of free biotin, thereby increasing the recovery efficiency of biotinylated proteins.

Two types of columns, PD-10 and Zeba, have been used for free biotin removal[11,12,13,14]. It may be of interest in the future to compare the efficiency of these two columns in removing free biotin in leaf protein extracts. Moreover, this protocol employs a canonical syringe-mediated agroinfiltration method to transiently express the target protein of interest in *N. benthamiana* leaves. Then, the biotin substrate is reinfiltrated into the leaves for labeling proteins that are proximal to the target protein. Similar operations have also been utilized in other two recently reported studies[12,13]. As an alternative method, vacuum-mediated infiltration of biotin is applicable for both *N. benthamiana* and *Arabidopsis*[14]. In addition, in plants that are not suitable for agroinfiltration, cell cultures can be transformed for target protein expression, followed by biotin treatment and proximity labeling assay[12]. These recent studies have underpinned the robustness of TurboID-based PL in studying PPIs and have laid the foundation for future applications of TurboID-based PL in different plant species.

In this protocol, well-established *Agrobacterium*-mediated transient expression in *N. benthamiana* is employed to identify PPIs of the target protein of interest. Transient expression may lead to overexpression of the fusion proteins. Therefore, a more optimal alternative is to engineer the TurboID fusion under the control of a native promoter and express it by agroinfiltration or generation of stable transgenic lines. With the development of genome editing technology, it is also possible to knock-in the TurboID fragment directly into the native genomic loci of genes of interest.

Another important factor to be considered is to ensure that the TurboID fusion does not alter the function of the target protein of interest. In a previous study, the function of the NLR immune receptor fused to TurboID was confirmed by testing its ability to induce the defense-mediated cell death in the presence of *Tobacco mosaic virus* p50 effector[11]. TurboID is relatively larger (i.e., 35 kDa) than GFP, and its fusion to a target protein can affect the function of a target protein. In such cases, the smaller miniTurboID[10] can be used.

It is also important to determine which terminus of the target protein is suitable for fusing with TurboID. A general approach for such functional tests is determining whether the TurboID fusion will complement the mutant plant line of the target gene. Alternatively, analysis of the interaction of the TurboID fusion with a previously known interaction partner of the target protein can be used. In most cases, for cytoplasmic proteins, N-terminal or C-terminal tagging of the TurboID can produce comparable datasets in subsequent mass spectrometry analysis. However, for membrane-localized proteins, it is important to determine the topology before vector construction. Otherwise, fusion of TurboID upstream or downstream of the coding sequence of genes of interest will generate completely different results. Therefore, it is important to determine the facing sides (e.g., cytoplasmic- or lumen-facing) of the N-terminus or C-terminus of the membrane-localized proteins. This ensures that the expected proximal proteome of the target protein can be obtained.

Although PL has several advantages over the traditional IP-MS approaches for detecting transient or weak PPIs, it has its own intrinsic limitations. First, identification of a candidate interaction proteins does not immediately imply a direct or indirect interaction with the bait protein, but it only reflects close proximity[2]. Therefore, independent in vivo assays (i.e., co-

immunoprecipitation, bimolecular fluorescence complementation [BiFC], or in vitro GST-pull down assay) can be carried out to further verify PPIs.

Second, false negatives or false positives may arise from PL assay due to various reasons. For example, false negatives can occur when the protein lacking accessible primary amines. In addition, recent studies of BIN2 interactors in plants showed a partial overlap between data from two experiments[13], indicating the existence of false negative results due to inadequate MS coverage. Some interactors of the target protein may also be weakly biotinylated by the TurboID-fused control, resulting in a fold enrichment below the cutoff threshold and eventually the loss of some true interactors[13,14]. Therefore, sufficient biological replicates with appropriate controls and cutoff values should be set to minimize the number of false negatives[11,14].

Moreover, a long biotin treatment period can increase the biotinylation of nonspecific proteins, resulting in false positives[10]. Therefore, it is important to optimize in vivo labeling time windows to reduce the nonspecific biotinylation, while also not affecting the production of biotinylated proteins for analysis[11,12,13,14]. In addition, harsh extraction and stringent wash conditions is recommended to reduce the false positives derived from nonspecific binding of proteins to the beads[17]. Besides the caveats described above, the design of a proper negative control is crucial to distinguish true interactors from false interactors and avoid missing of true interactors[11,12,13,14].

TurboID showed greatly improved labeling kinetics compared to that of BioID[10,11]. However, this can also lead to a higher background during PL analysis. Therefore, appropriate controls must be included to eliminate the background signals. We used the citrine-fused TurboID in this protocol[11], and a similar control has been utilized in previous studies[18]. For the target proteins that localize to a specific organelle membrane, TurboID fusing with a signal peptide that targets the same organelle membrane makes a better control than TurboID fusing with one that is expressed in the cytoplasm[14].

Furthermore, if information about the key domain or amino acids in the target protein that determine its interactions with other partners are known, fusing the TurboID with a target protein with a mutation in the key domain or amino acids should be the best control and will maximally reduce the background signals generated by the target protein. In addition, TurboID enzymes require specific temperatures as well as proper pH conditions. For most organelles in the cell such as the ER, nucleus, and mitochondria, TurboID successfully labeled the proximal proteins[10]. However, some special organelles (i.e., vacuoles in plant cells, whose pH is very low[19]) may be not suitable for studying PPIs using the PL approach. Moreover, changes in the pH levels or oxidation-reduction states of the subcellular environment during the stress response may affect the labeling efficiency of the TurboID.

In summary, the protocol described here provide the basis for investigating PPIs using TurboID in *N. benthamiana*, a model plant system that has been widely used in many aspects of plant biology[20]. With the recently described TurboID-based PL studies in *Arabidopsis* plants and tomato hairy roots[12,13,14], it is expected that this method will become applicable

to other plant species. It is anticipated that TurboID-based PL will play an important role in studying PPIs in plant biology research.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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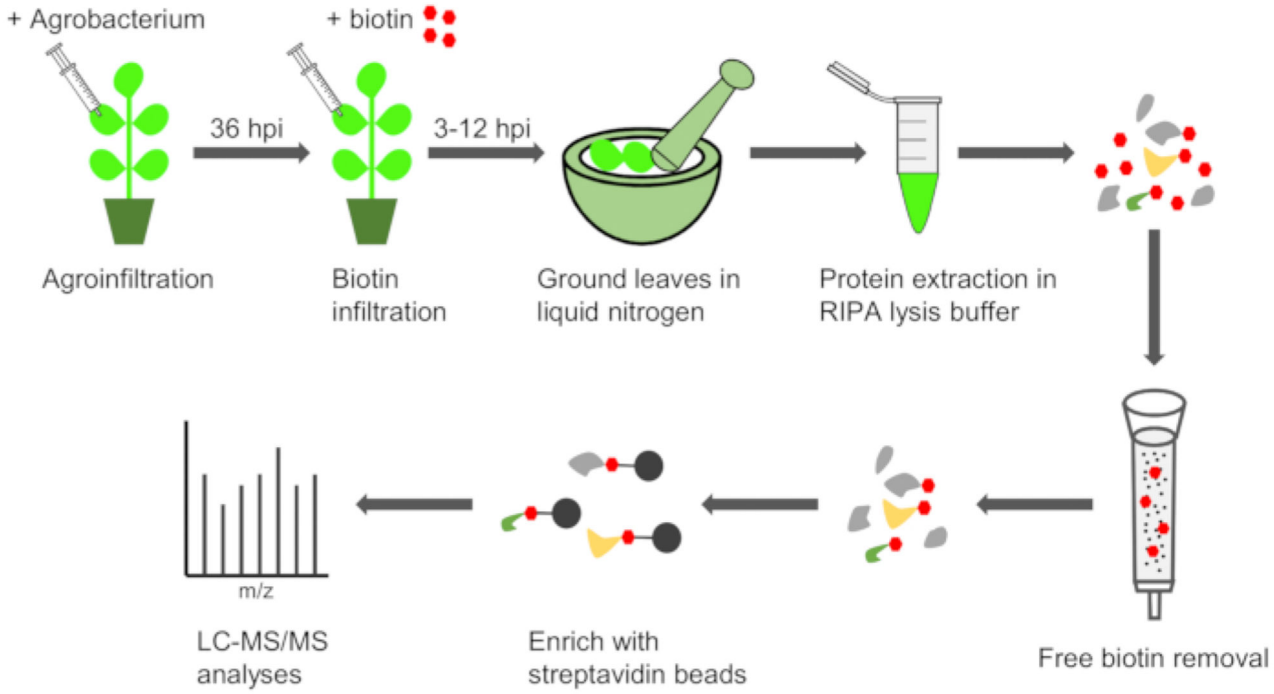


**Figure 1: Overview of the TurboID-based PL method in *N. benthamiana*.**

*Agrobacterium* harboring the TurboID-fusion constructs were infiltrated into *N. benthamiana* leaves. 36 h post-infiltration, 200 μM biotin is infiltrated to the same leaves to initiate biotinylation of the endogenous proteins that are proximal to the TurboID-fused target protein. The infiltrated plants are then incubated at RT for 3–12 h, followed by leaf harvesting and grinding in liquid nitrogen. Leaf powder is lysed in the RIPA lysis buffer, and a desalting column is employed to remove the free biotin in the protein extract. The biotinylated proteins were then affinity-purified with streptavidin-conjugated beads and identified by mass spectrometry. This figure is adapted from Supplementary Figure 3 from Zhang et al[11].

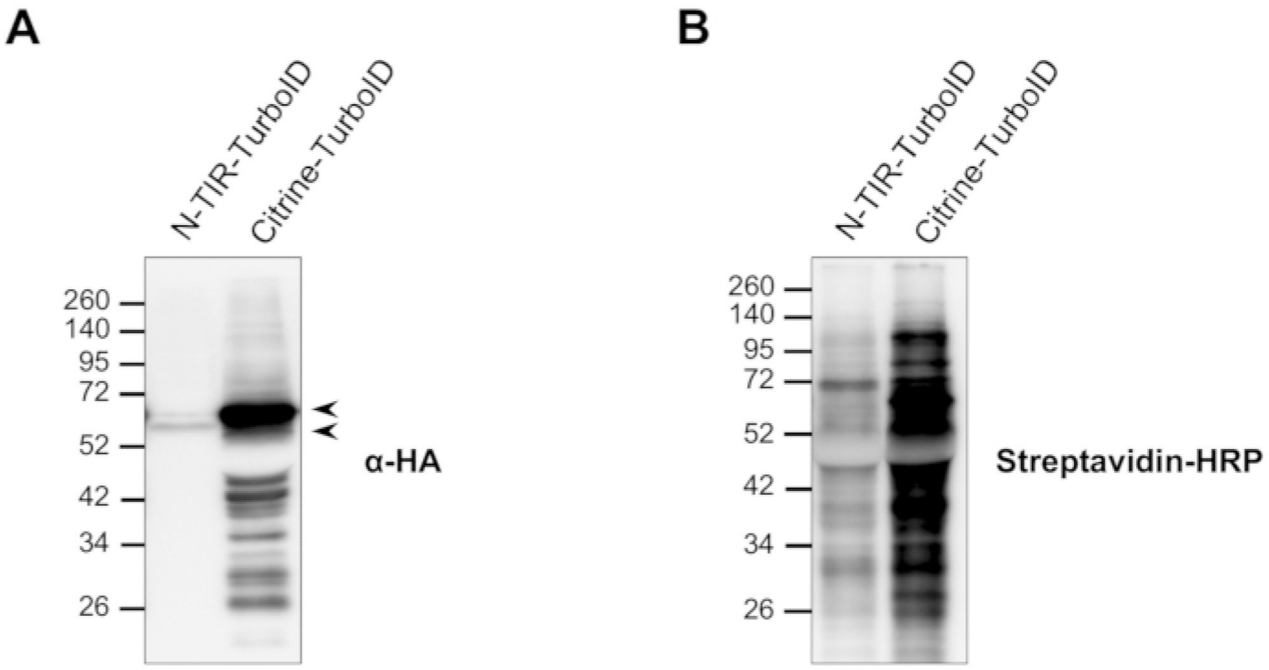


**Figure 2: Immunoblot analysis of protein extracts obtained in step 4.2.**

(**A**) Western blot analysis of proteins extracted from the agroinfiltrated leaves with antibody against HA tag. (**B**) Western blot analysis of biotinylated proteins in the agroinfiltrated leaves with streptavidin-HRP. This figure is adapted from Supplementary Figure 6B of Zhang et al[11].

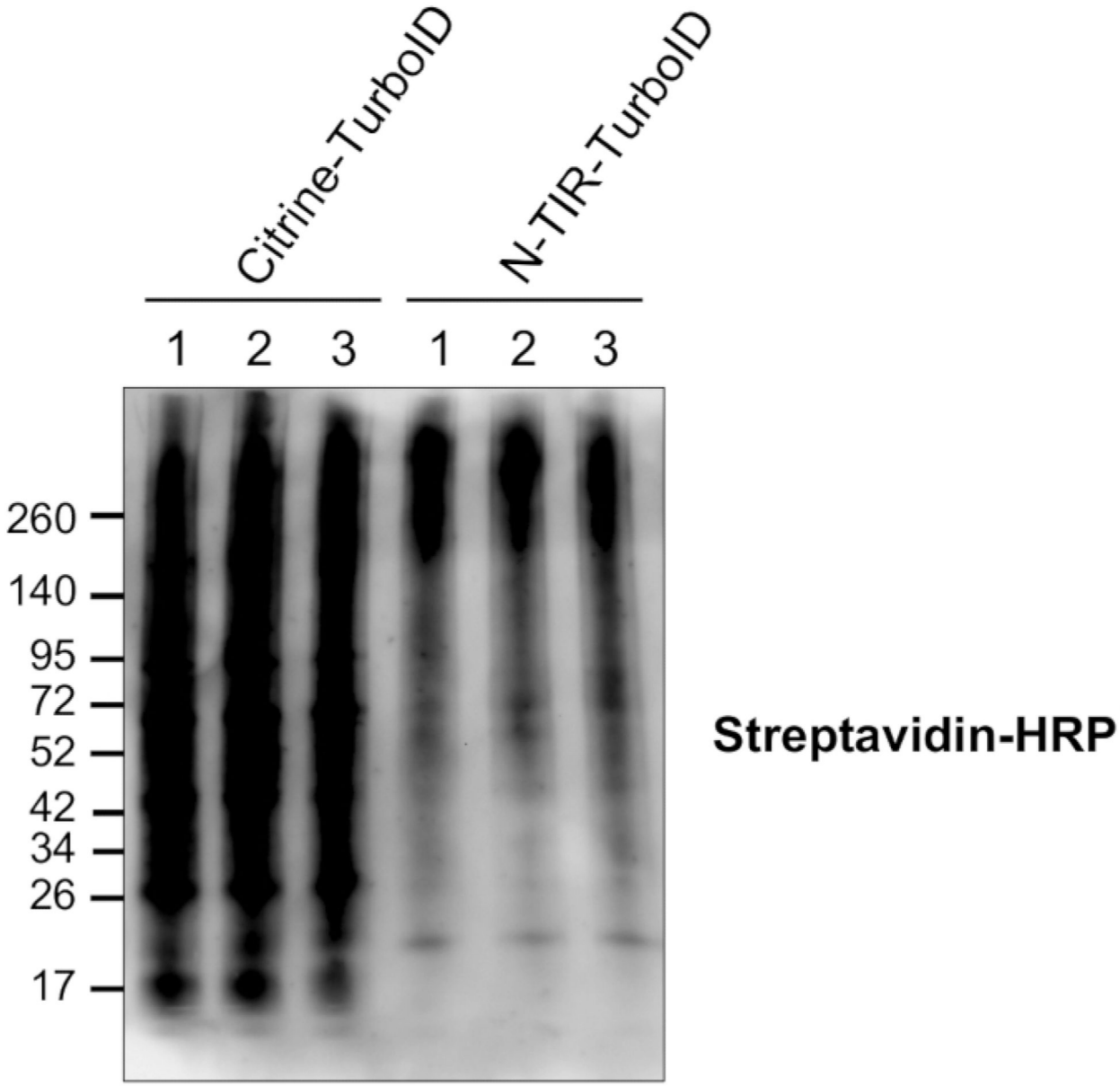


**Figure 3: Western blot analysis of beads obtained in step 8.15 to confirm the enrichment of biotinylated proteins.**

For each construct, there are three independent replicates (1, 2, and 3). Streptavidin-HRP was used for analysis of biotinylated proteins in different samples. This figure is adapted from Supplementary Figure 7 from Zhang et al[11].