Chapter 3 1

Activity-Based Protein Profiling of Infected Plants 2

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Abstract 4

Activity-based protein profiling (ABPP) is a powerful analytical method to detect and compare the activity 5
of proteins in proteomes. This is achieved using specific activity-based probes that are often derived from 6
inhibitors and are linked to reporter groups like rhodamine or biotin for fluorescence detection and/or 7
affinity purification, respectively. The probes react with the active site residue of proteins and become 8
covalently and irreversibly attached, facilitating the separation, detection and identification of the labelled 9
proteins. In this protocol we describe all the steps required for labelling, purification and identification of 10
labelled proteins from gels and show how activities in two proteomes can be compared. The identification 11
of serine hydrolases from Arabidopsis plants infected with *Botrytis cinerea* using the trifunctional probe 12
TriFP is used as an example. 13

Key words: Activity-based protein profiling, In-gel digest, ABPP, Serine hydrolases, Fluorophos- 14
phonate, *Botrytis cinerea*, *Arabidopsis thaliana* 15

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1. Introduction 16

Activity-based protein profiling (ABPP) is a diagnostic tool developed 17
to record and study the changes in activity of proteins in proteomes 18
(1–3). Recently, we introduced this approach in plant science (4). 19
The key to successful ABPP is the probe (Fig. 1a). This is often a small 20
molecule inhibitor of the enzyme class to be investigated which is 21
chemically modified with a reporter group (e.g. rhodamine for 22
fluorescent detection and/or biotin for affinity purification). 23
Trifunctional FP (TriFP, Fig. 1b) is an example of an activity-based 24
probe for serine hydrolases. The probe will react with the serine in the 25
active site of serine hydrolases by forming an irreversible, covalent bond 26
(Fig. 2a). This covalent bond facilitates the purification and detection 27
of the labelled proteins under denaturing conditions (Fig. 2b). 28
The ABPP methodology allows us to compare changes in the activity 29
of proteins after different treatments (comparative ABPP, Fig. 2b). 30

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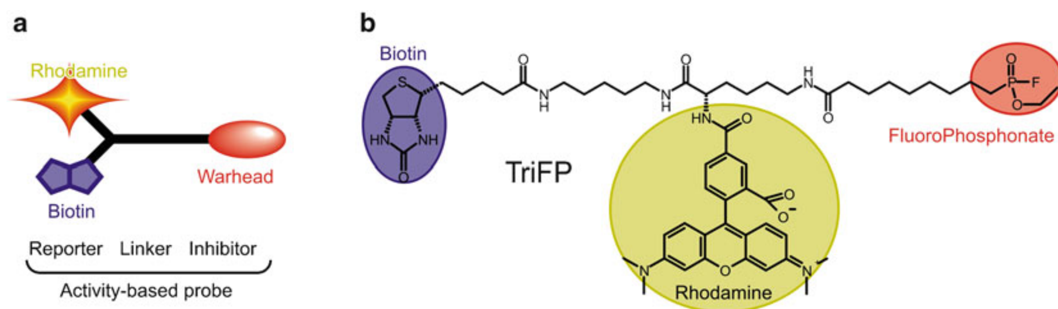

Fig. 1. Trifunctional activity-based probes. (**a**) Components of a trifunctional probe. The trifunctional probes carry a fluorescent group (e.g. rhodamine) for detection, a biotin group for affinity purification and a warhead that confers selective, covalent and irreversible labelling of the catalytic residue of the tageted protein class. (**b**) TriFP, an example of a trifunctional probe. TriFP contains biotin, rhodamine and a fluorophosphonate (FP) group that reacts with the catalytic serine of serine hydrolases.

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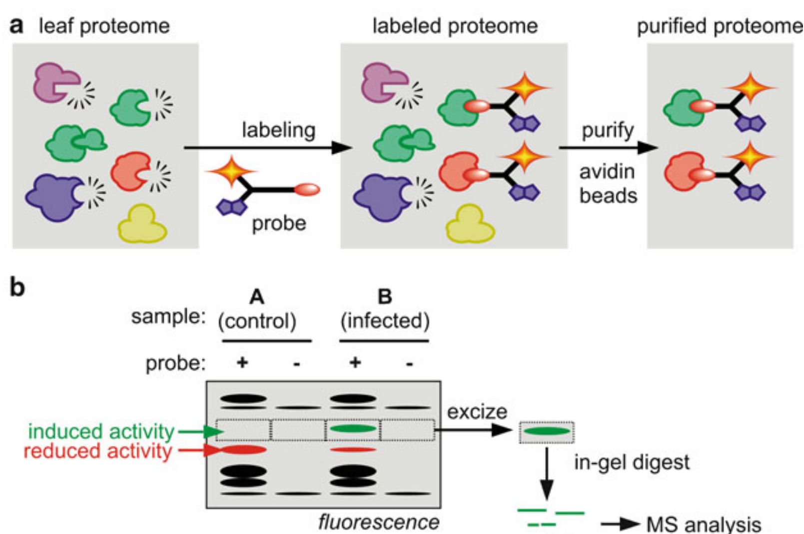

Fig. 2. ABPP principle and procedure. (**a**) General principle of ABPP. Proteomes are labelled with a trifunctional probe in an activity-dependent manner. Labelled proteins are purified, detected and identified. (**b**) Procedure of ABPP analysis. Probe-labelled proteins are purified and separated by gel electrophoresis and detected by fluorescent scanning. Fluorescent signals are excised and proteins digested in-gel with trypsin. Tryptic peptides are eluted and analysed by mass spectrometry to identify the labelled protein. Comparison with the no-probe control ("–" lane) confirms that the identified protein is not a background contamination. Comparison between probe-labelled samples will display proteins with differential activities.

The impact of ABPP has been particularly large in the field of plant-pathogen interactions (5–8). Comparative studies where changes in the activity of e.g. cysteine proteases (5, 6), serine hydrolases (7) or the proteasome (8) were displayed with the help of specific probes, revealed exciting new functions for these proteins.

The success of ABPP experiments relies to a large extent on the identification of the targeted proteins. The following protocol contains all the information to perform a comparative ABPP experiment on 1D protein gels, followed by affinity purification

and preparation of samples for identification by mass spectrometry (MS). As an example we explain the procedure for serine hydrolase profiling of *Arabidopsis thaliana* infected with the necrotrophic pathogen *Botrytis cinerea* using TriFP (7, 9, 10).

2. Materials

Prepare all solutions immediately before use unless otherwise stated. Always wear gloves to reduce keratin contamination. Use chemicals and water suitable for mass spectrometry when preparing solutions.

2.1. Components for the ABPP Reaction

1. Bovine serum albumin (BSA) protein standard (2 mg/mL).
2. Amber glass threaded vials (4 mL) with screw cap.
3. Phosphate buffered saline (10× PBS): 10.6 mM KH_2PO_4, 1,552 mM NaCl, 30 mM Na_2HPO_4, pH 7.4. In a 1-L glass beaker mix 1.44 g KH_2PO_4, 90.70 g NaCl and 4.26 g Na_2HPO_4. Add 800 mL water and stir the initial suspension with the help of a magnetic stirrer until all the salts are completely dissolved. Then adjust the pH (if necessary) to pH 7.4 by adding hydrochloric acid. Transfer the solution to a graduated cylinder and adjust the volume to 1 L with water. Sterile filter the buffer using a 0.22-μM filter unit. The solution can be stored at RT indefinitely.
4. Activity-based probe: for large scale TriFP labelling experiments the probe is diluted to a concentration of 1 mM with dimethyl sulfoxide (DMSO) and the stock stored at −20°C. Handle the stocks always at room temperature. This reduces the likelihood of water getting into the stock by condensation.
5. Phosphate buffered saline (1× PBS): 1.1 mM KH_2PO_4, 155 mM NaCl, 3 mM Na_2HPO_4, pH 7.4. Transfer 100 mL 10× PBS to a 1-L measuring cylinder and add water up to the 1 L mark. Transfer the solution to a 1-L bottle and mix well by inverting the bottle several times. The solution can be stored at RT indefinitely.

2.2. Affinity Purification Components

1. Prepacked desalting column.
2. 0.22-μM filter unit attached to a syringe.
3. Sodiumdodecylsulfate (10% SDS): 350 mM SDS. Dissolve 10 g SDS in 80 mL sterile water (see Note 1). Stir gently until all the SDS is dissolved and the solution is clear. Then adjust volume to 100 mL. The solution can be stored at RT indefinitely.
4. Avidin beads: Sigma-Aldrich, St. Louis, MO, USA (see Note 2).
5. Sodiumdodecylsulfate (1% SDS): 35 mM SDS. Transfer 20 mL 10% SDS solution to a measuring cylinder and add water up to the 200 mL mark. Store this solution at room temperature.

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2.3. Elution and Separation of Proteins

1. Tris (tris(hydroxymethyl)aminomethane) (1 M Tris pH 6.8): dissolve 60.57 g Tris in 400 mL sterile water. Adjust the pH to 6.8 with concentrated HCl. Then transfer the solution to a 500 mL measuring cylinder and add water up to the 500 mL mark. Transfer the solution to a 500-mL bottle and autoclave it. The solution can be stored at RT indefinitely.
2. SDS-PAGE gel-loading buffer (4× GLB): 280 mM SDS, 400 mM Tris, 40% glycerol, 1.4 M β-mercaptoethanol, 0.6 mM Bromophenol Blue, pH 6.8. Add the following components to a 50-mL Falcon tube: 2 g SDS, 10 mL 1 M Tris (pH 6.8) and 10 mL glycerol (see Note 3). Tumble this solution until all the components are dissolved. Then add 2.5 mL 14.2 M β-mercaptoethanol (see Note 4) and bring the volume up to 25 mL with water. Finally, add a pinch (~10 mg) of Bromophenol blue (see Note 5). Make 1 mL aliquots and store these at –20°C.
3. Tris (tris(hydroxymethyl)aminomethane) (1.5 M Tris pH 8.8): dissolve 90,85 g Tris in 400 mL sterile water. Adjust the pH to 8.8 with hydrochloric acid (HCl). Then transfer the solution to a 500-mL measuring cylinder and add water up to the 500 mL mark. Transfer the solution to a 500-mL bottle and autoclave it. The solution can be stored at RT indefinitely.
4. Ammoniumpersulfate (10% APS): 400 mM APS. Add 1 g APS ($(NH_4)_2S_2O_8$) to a 15-mL tube and add sterile water up to 10 mL. Mix well until the chemical is completely dissolved. The solution can be used for at least 1 week when stored at 4°C.
5. Ready-made Acrylamide-Solution (30% Acrylamide/Bis): 29% Acrylamide, 1% Bisacyrlamide (see Note 6).
6. Resolving gel (12%): Mix the following components in a clean glass beaker on ice: 33 mL water, 40 mL 30% Acrylamide/Bis solution, 25 mL 1.5 M Tris (pH 8.8), 1 mL 10% SDS, 1 mL 10% APS, 40 μL TEMED. Pour the gel between the glass plates and overlay the surface with 2-Butanol. You have to be quick. Once the TEMED has been added the gel starts to polymerize within minutes when left at room temperature. Once the resolving gel is polymerized (~20–60 min), pour the 2-Butanol off and rinse the gel surface with water. Dry the gel surface by tipping (not sliding!) the gel with Whatman paper.
7. Stacking gel (6%): Mix the following components in a glass beaker on ice: 19.6 mL water, 6 mL 30% Acrylamide/Bis solution, 3.8 mL 1.0 M Tris (pH 6.8), 300 μL 10% SDS, 300 μL 10% APS, 30 μL TEMED. Pour the stacking gel solution onto the resolving gel until it reaches the brim of the gel-cassette and add the comb. Let the gel rest for 1 h at RT. Then wrap the gel-cassette in saran wrap and store it over night at 4°C. This is essential to complete the polymerization.

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8. SDS Running Buffer (10×): 248 mM Tris, 2 M glycin, 35 mM SDS. Mix 30 g Tris, 144 g glycin and 10 g SDS in a 1-L glass beaker with 800 mL water. Stir gently until all the components are dissolved. Then transfer the solution to a 1-L measuring cylinder and adjust the volume to 1 L with water. Store the solution at RT in a 1-L bottle. It is not necessary to sterilise this solution. 126 127 128 129 130 131 132

2.4. In-Gel-Digestion (IGD)-Components

1. Disposable steel blades. 133
2. Ammonium bicarbonate solution (100 mM): Dissolve 395 mg ammonium bicarbonate ($(NH_4)CO_3H$) in 50 mL MS-water. 134 135
3. TCEP solution (10 mM): Dissolve 28.7 mg Tris(2-carboxyethyl) phosphine (TCEP) in 10 mL MS-water. Prepare immediately before use. 136 137 138
4. Iodoacetamide (IAA, 55 mM): Dissolve 40.7 mg IAA in 4 mL MS-water. Keep in darknes; IAA is sensitive to light. Prepare immediately before use and store at a dark place. 139 140 141
5. Acetonitrile (ACN). 142
6. ACN and 100 mM ammonium bicarbonate (50:50 solution): mix 25 mL 100 mM ammonium bicarbonate with 25 mL ACN. 143 144 145
7. Ammonium bicarbonate (25 mM): mix 1 mL 100 mM ammonium bicarbonate with 3 mL water. 146 147
8. Trypsin (10 ng/μL): Dissolve 20 μg lyophilised trypsin in 200 μL of trypsin resuspension buffer (provided by manufacturer, usually 50 mM acetic acid). Leave on the bench for 15 min to activate trypsin. Then dilute with 25 mM ammonium bicarbonate solution to a final concentration of 10 ng/μL (1:10 dilution). 148 149 150 151 152
9. Formic acid (FA, 5%): 1.33 mM FA. Mix 50 μL 100% FA (HCOOH) in 950 μL MS-water. Formic acid is volatile and corrosive. Handle always in a chemical hood. 153 154 155
10. Formic acid (FA, 0.1%): 0.27 mM FA. Mix 1 μL FA with 999μL water. 156 157

3. Methods 158

3.1. Sample Preparation

1. Collect the material of interest from non-infected (sample A) and infected plants (sample B).Collect enough material corresponding to at least 6 mg protein for each sample. 159 160 161
2. Grind the tissues in a mortar. Add enough water (see Note 7) to the sample to obtain a homogenous solution. Transfer the extract to a centrifugation tube and clear it by centrifugation (13 k, 5 min, RT). Transfer the supernatant to a fresh tube. 162 163 164 165

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3. Determine the protein concentration (see Note 8).
4. Prepare 2 mg/mL stock solutions of each proteome in 1× PBS (see Note 9). Bring the equivalent of 10 mg protein up to a volume of 4.5 mL with water and add 500 μL 10× PBS. Vortex for 5 s.

3.2. Labelling of Plant Extracts with Activity-Based Probes

1. Label four 4-mL amber glass vials (or equivalent, see Note 10) with A+, A–, B+and B–.
2. Transfer 1 mL of the 2 mg/mL protein solutions to the different vials. The extract from non-infected plants is added to vials labelled A– and A+, and the extract from infected samples is added to vials labelled B– and B+(Fig. 3.2b).
3. Then add 5 μL of the 1 mM probe to those vials labelled with "+" and 5 μL DMSO to vials labelled with "–" (see Note 11). Vortex for 5 s.
4. Incubate at room temperature in the dark for 1–2 h (see Notes 10 and 12).
5. Meanwhile, equilibrate a prepacked desalting column with 1× PBS. Snap off the bottom lip of the column and discard the buffer solution. Equilibrate the columns by passing twice 15 mL PBS through the column by gravity.

3.3. Large Scale Affinity Purification of Labelled Proteins

1. After 1 h incubation dilute the reaction mix with 1× PBS to a final volume of 2.5 mL and mix well. The reaction mix should be clear (see Note 13).
2. Apply the diluted reaction mix onto the 1× PBS equilibrated prepacked desalting column and allow the solution to enter by gravity (see Note 14). Discard the flow-through.
3. Place a 15-mL tube containing 183 μL 10% SDS solution (see Note 15) under the desalting column, add 3.5 mL 1× PBS to the desalting column and collect the flow-through in the SDS-containing tube. Mix well by inverting the tube several times. Then incubate the tube for 5 min at 90°C in a water bath (see Note 16).
4. Immediately afterwards place the falcon tube on ice for 1 min and invert the tube a couple of times. Do not leave your samples on ice for too long otherwise SDS and proteins may precipitate. Then add 5 mL 1× PBS (see Note 17).
5. Equilibrate the avidin beads by adding the equivalent of a 100 μL aliquot (50 μL bed volume) of the avidin beads (see Note 18) to a 1.5-mL Eppendorf tube and wash the beads twice with 1 mL 1× PBS (see Note 19). Re-suspend each aliquot in 100 μL 1× PBS.

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6. Add one aliquot of the washed avidin bead suspension to the diluted protein solution and incubate for 1 h at RT by gently inverting the tube (see Note 20). 207 208 209
7. Collect the beads by centrifugation (400 × *g*, 3 min, swinging out rotor; see Note 21) and remove the supernatant with a 10-mL disposable pipette (see Note 22). 210 211 212
8. Add 10 mL 1% SDS to the beads and re-suspend them by inverting the tube several times. Incubate at RT for 10 min by gently inverting the tube (see Note 20). 213 214 215
9. Collect the beads by centrifugation (400 × *g*, 3 min, swinging out rotor; see Note 21) and remove the supernatant with a 10-mL disposable pipette (see Note 22). 216 217 218
10. Repeat the washes with 1% SDS (see steps 8 and 9) five more times (see Note 23). 219 220
11. Transfer the beads to a new 1.5-mL tube. Collect the beads by centrifugation (1 min, 16,000 × *g*) and remove the supernatant carefully. 221 222 223

3.4. Separation of Biotinylated Proteins

1. Add 30 μL of the 4× GLB to the tube containing the avidin beads with the captured proteins. Mix well. 224 225
2. Incubate this mix for 10 min at 90°C. Agitate every 2 min by ticking the bottom of the tube. 226 227
3. Place the tubes on ice for 10 s and centrifuge briefly (10 s, 16,000 × *g*) to spin down the water that has condensed in the lid. Vortex briefly again and centrifuge (5 min, 16,000 × *g*). 228 229 230
4. Assemble the protein gel device using previously prepared gels. Fill the reservoirs with 1× SDS running buffer and make sure the apparatus is not leaking. 231 232 233
5. Load the pre-stained fluorescent marker in the first and the last lane (see Note 24). Then load 35–50 μL of the eluted proteins. Leave one lane empty between samples to prevent cross-contaminations. Fill the empty lanes with 2× GLB. Separate the proteins by SDS-PAGE (see Note 25). 234 235 236 237 238

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3.5. In-Gel-Digestion Protocol

1. After electrophoresis carefully transfer the gel to a plastic box containing water suitable for MS. Wash the gel with several changes of water for at least 1 h. 239 240 241
2. Next, place the gel on a fluorescent scanner and detect TriFP-targets in-gel using the appropriate settings (see Note 26). 242 243
3. Printout the scan and put an overhead transparency sheet on top of your printout. Place the gel on the transparency and superimpose it with the gel. Use the marker bands as guide. Excise the regions where you see fluorescent signals on the printout with a new disposable steel blade. Excise the corresponding region from the no-probe control ("–" samples) for 244 245 246 247 248 249

250 background control. Cut the gel slices into at least eight pieces
251 and transfer them to a properly labelled, low protein binding
252 Eppendorf tube (or equivalent). Indicate on the printout which
253 gel fragment was removed and in which tube this fragment was
254 transferred. Do not squash the gel pieces. After the bands of
255 interest have been excised, re-scan to verify that the right part
256 of the gel has been removed.

257 4. Wash the gel pieces twice with 500 μL water for 15 min while
258 vigorously shaking (see Note 27). Briefly centrifuge (10 s,
259 16,000 × *g*) and discard the supernatant.

260 5. Wash the gel pieces twice for 15 min with 500 μL 100 mM
261 ammonium bicarbonate (see Note 28). Briefly centrifuge (10 s,
262 16,000 × g) and discard the supernatant.

263 6. Add 200 μL 10 mM TCEP or enough to completely cover the
264 gel slices. Incubate at 62°C for 30 min (see Note 29). Briefly
265 centrifuge (10 s, 16,000 × *g*) and discard the supernatant.

266 7. Add immediately 200 μL 55 mM IAA (see Note 30). The gel
267 slice has to be completely covered by the IAA solution. Tumble
268 gently in the dark for 30 min at RT. Briefly centrifuge (10 s,
269 16,000 × *g*) discard the supernatant.

270 8. Wash the gel slices three times15 min with 500 μL 50:50 ACN:
271 100 mM ammonium bicarbonate (see Note 31). Briefly centri-
272 fuge (10 s, 16,000 × *g*) and discard the supernatant.

273 9. Add 50 μL 100% ACN to dry gel slices (they become com-
274 pletely white). Remove ACN and dry the samples in a vacuum
275 concentrator (see Note 32).

276 10. Add 20 μL of the 10 ng/μL trypsin solution to the gel slice
277 and incubate for 10 min at RT (see Note 33). Then completely
278 cover the slices with 25 mM ammonium bicarbonate and seal
279 the tubes with parafilm. Incubate over night under constant
280 shaking at 37°C (see Note 34).

281 11. After 16 h, briefly centrifuge (10 s, 16,000 × *g*) and transfer the
282 supernatant to a new Eppendorf tube (low protein binding or
283 equivalent). Do NOT discard this solution as this is the major
284 peptide fraction!

285 12. Add sufficient 5% formic acid to the gel slices to cover them
286 (~100–200 μL) and incubate at RT for 15 min (see Note 35).

287 13. Combine this supernatant with the fraction obtained from the
288 overnight digestion (see step 11).

289 14. Add sufficient ACN to cover the gel slices and incubate for
290 15 min at RT while vigorously shaking. Combine this superna-
291 tant with the other supernatants. Repeat this step three times
292 until the gel slices have become opaque. (e.g. use 100 μL
293 ACN, then 70 μL and finally 50 μL).

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15. Reduce the volume of the combined supernatants in a vacuum concentrator (~3–5 h at 30°C) to a final volume of 10 μL (see Note 36).
16. Use this peptide concentrate for MS analysis (see Note 37).

4. Notes

1. When handling SDS powder always wear a dust mask, gloves and protective clothing.
2. The protocol has been thoroughly tested with these beads. Beads from other manufacturers may also work. The avidin or streptavidin on the beads should be able to bind biotinylated proteins at 0.2% SDS and the interaction with the biotinylated proteins should not be affected during washes with 1% SDS and 6 M Urea.
3. Glycerol is a viscous liquid that can be hard to handle. To dispense this liquid, always calculate the respective weight (using the density of glycerol), place your tube on a balance and directly pour the required amount in. In this case add ~12.5 g of glycerol directly to your Falcon tube.
4. β-mercaptoethanol is a strong reducing agent. It is toxic (11) and smells bad. Wear protective clothing and gloves when handling this chemical. Always work in chemical hood.
5. Very little of Bromophenol Blue is required. It is added as a pH indicator. As long as the pH is higher than 4.6 it will be violet-blue. If the pH drops to a lower pH the colour of the indicator will change to yellow (12).
6. To reduce the health hazard of acrylamide we use a commercially available ready-made solution, which is always stored at 4°C. Acrylamide is a neurotoxin (13) and causes cancer in rats (14). The powder tends to form dust clouds. When you opt to make your own 30% Acrylamide/Bis always work in a chemical hood and wear a dust mask, gloves, and protective clothing.
7. You can also use a buffer like 1× PBS or 1× TBS to extract your proteins as long as it will be compatible with your following ABPP reaction.
8. We use the RCDC kit from Bio-Rad for measuring the protein concentration. The changes in absorbance are measured at 750 nm which reduces interference of chlorophyll at this wavelenght. Expect protein concentrations for leaf-extracts to be around 2–5 mg/mL.
9. PBS is the standard buffer for labelling reactions with FP-derived probes. Other probes may require a different buffer. Adjust this step accordingly.

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10. The fluorescent group rhodamine on TriFP is light sensitive. Therefore, samples should be kept in the dark as much as possible. The protection from light can also be achieved by wrapping the sample tubes in aluminium foil.
11. It is very important to include a no-probe control. It will allow us later to identify proteins that bind non-specifically to the affinity purification matrix. This negative control increases confidence for identified targets.
12. If you plan to profile the activity of a different enzyme class, keep in mind that the probe concentration, reaction times and buffer components have to be optimised individually for each probe and for each plant extract.
13. The reaction mix should be clear. In case a precipitate has formed remove it by passing the reaction mix through a 0.22-μM filter unit, attached to a syringe.
14. The desalting step removes non-reacted, excess probe and other small molecules. This will increase the yield for targeted, biotinylated proteins in the following affinity purification steps.
15. SDS is added to unfold proteins (15). Activity-based probes react with the active site residue of an enzyme. Since these are often buried deep inside the protein, affinity tags like biotin on TriFP may not be accessible to affinity purification matrices. The SDS treatment and the subsequent incubation at 90°C will permanently unfold the proteins and therefore significantly increase the yield of affinity purification. At the same time this SDS treatment will also abrogate the activity of proteases and other degradative enzymes which might interfere with efficient affinity purification.
16. If you do not have a traditional water bath at hand: place 1 L water in a 2-L glass beaker and bring it to the boil in a microwave oven. Let the water cool down for 10 min. Then place the closed tubes upright into the hot water (~90°C) for 5 min. Invert the tubes from time to time. Wear heat protective gloves when you handle the hot tubes.
17. The sample is diluted at this step with 1× PBS to reduce the SDS concentration from ~0.5 to 0.2%. The reduced SDS concentration is compatible with the subsequent affinity purification with avidin beads.
18. We use avidin beads from Sigma (A9207). The beads are delivered as 1 or 5 mL aliquots in 50% glycerol. Please make 100 μL aliquots and store at −20°C to reduce freeze–thaw cycles. With a cut-off yellow tip, transfer 100 μL of the slurry to a new Eppendorf tube. Because the beads tend to settle down quickly, it is necessary to vortex before pipetting. The aliquots are stored at −20°C. One aliquot is sufficient for one affinity purification.

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19. Add 1 mL PBS to the beads and mix them well. Then spin the beads down (5 min, 800–1,000 × *g*) and gently remove 900 µL of the supernatant. Do not attempt to remove all the supernatant. The closer you get to the beads the more beads you will remove. This will reduce your capture efficiency. Repeat this step once more.
20. During incubation and washes it is important to constantly agitate the beads. Otherwise they will settle down quickly. It does not matter how this is achieved (horizontal, by rolling or overhead) but it should be a gentle process.
21. It is important to pellet the beads at low *g*-values. If the centrifugal force is too high the beads will stick together with precipitating proteins, thereby compromising the quality of the purification. For optimal settings consult the manufacturer's instructions.
22. Remove only 90% of the supernatant (e.g. of 10 mL supernatant only remove 9 mL). Do not attempt to remove all the supernatant. The closer you get to the beads the more beads you will remove, which will reduce the yield of your purification.
23. If you know that you are dealing with sticky contaminants that are not removed by SDS alone you may consider washing the beads twice with 10 ml 6 M Urea (Dissolve 36 g Urea in 80 mL water. Then adjust the volume with water to 100 mL). Wash first twice with 10 mL 1% SDS then twice with 10 mL 6 M Urea and finally twice with 10 mL 1% SDS.
24. Fluorescent protein markers are expensive and not essential. Many of the regular protein ladders that contain red marker bands are fluorescent under the settings used to detect rhodamine-labelled proteins.
25. We have successfully used both homemade gels and commercial pre-cast gels for the separation and identification of probe-labelled targets. When making your own gels please keep in mind that keratin is a frequent contamination that will have a drastic effect on the success of the downstream MS identification. You can minimise the risk of keratin contamination by always using fresh solutions. Also use MS pure ingredients (particularly MS-water) whenever possible. Avoid wearing clothes that contain wool. The choice in length of the gel and acrylamide percentage depends on the number of targets you expect and how close they migrate on the gel. The more targets you expect the longer the gel should be. For TriFP target identification we usually run proteins for 350 V-h on a 10 or 12% 20 × 18 cm gel.
26. We detect fluorescent proteins in protein gels using the Typhoon 8600 (GE Healthcare, Munich, Germany). Any other scanner or camera system that can excite at 532 nm (green laser) and

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that has a TAMRA filter (580 nm BP30) for detection of rhodamine can be used. These systems can, however, differ significantly in sensitivity. For other fluorescent tags the settings might be different. Please check before proceeding.

27. It is necessary to remove all the excess SDS from the gel. Small traces of residual SDS may inhibit the following alkylation reaction (16) and may interfere with the separation of peptides later.
28. The following alkylation step requires a basic pH (16). This is achieved by washing the gel slices with the ammonium bicarbonate solution.
29. TCEP will reduce disulfide-bonds (R^1–S–S–R^2 → R^1–SH + HS-–R^2) which is essential for the subsequent alkylation of the cysteines (17).
30. The IAA treatment is necessary to irreversibly alkylate the thiol-group of cysteins (R–SH + I–CH_2–C(O)–NH_2 → R–S–CH_2–C(O)–NH_2 + HI) which are prone to oxidation (18). Through this chemical modification all thiol groups (–SH) are uniformly modified with a 57 Da carbamidomethyl-rest, which facilitates the automated detection of cysteine-containing peptides by MS.
31. With every washing step the gel slices become more opaque/white. This is because acetonitrile extracts water from the gel slices. What remains is solid polyacrylamide.
32. It is essential to use a vacuum centrifuge. Simply using a desiccator attached to a pump will dry your samples nicely but retardation of boiling may cause your gel pieces to jump out of their Eppendorf tubes. This may lead to a loss of your samples and cause contaminations.
33. The polyacrylamide flakes will start to swell once the trypsin mix is added. This way they absorb trypsin.
34. It is important to seal the tubes with parafilm so they will not open accidentally. A nice trick is to put the tubes in 200-mL plastic beaker and stuff the beaker with paper towels so the tubes cannot move. This beaker is then placed in a 37°C shaker for bacteria and left there shaking over night.
35. The formic acid treatment inactivates trypsin (19) and protonates all the peptides (the peptides will become positively charged).
36. Do not dry samples completely as this causes loss of peptides. If samples have dried accidentally then add 10 µL 0.1% formic acid solution to bring the peptides back in solution.
37. The preparation of the samples for the subsequent MS analysis is dependent on the mass spectrometer you use in your lab. We usually load the samples directly on a 10 cm 100-µM equilibrated C_{18}-column (7), which is directly attached to a LTQ or Velos (Thermo Fisher Scientific Inc., Waltham, MA, USA). Please check with your MS department for details.

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Uncorrected Proof