Chapter 2

Fragment Complementation and Co-immunoprecipitation Assays for Understanding R Protein Structure and Function

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Abstract

Plant disease resistance (R) proteins confer protection against specific pathogens or pathogen isolates. R proteins function by recognizing pathogen-encoded avirulence (Avr) proteins and translating this recognition event into an initiation of downstream signaling pathways. Key to understanding this process is the study of the protein–protein interactions involving R proteins. Recognition and signaling mechanisms are mediated by both intramolecular interactions that take place between different domains of R proteins as well as intermolecular interactions between R proteins and additional plant proteins. These processes have been studied in part by using Agrobacterium-mediated transient expression of R protein fragments in *Nicotiana benthamiana* which allows for the rapid assessment of functionality. Furthermore, pairs of proteins or protein fragments can be transiently expressed as fusions with different epitope tags. One putative protein partner is subjected to immunoprecipitation. Subsequent immunoblotting is performed to determine whether the second protein has remained associated (or co-immunoprecipitated) with the first, indicating a protein–protein interaction. This technique has contributed substantially to structure–function analyses of R proteins and to the characterization of interactions between R proteins and other plant proteins.

**Key words:** *Agrobacterium*, Agroinfiltration, NB-LRR, Immunoprecipitation, Transient expression, *Nicotiana benthamiana*

1. Introduction

*Agrobacterium tumefaciens* transfers T-DNA to plant cells where-upon it migrates to the nucleus and becomes transcriptionally competent (1). In some species, this process is so efficient that most or all cells become transiently transformed within a leaf patch that has been syringe-infiltrated with a suspension of *Agrobacterium*. The use of *Agrobacterium*-mediated transient expression (agroexpression or agroinfiltration) has become a
widely adopted technique for functional genomics in plants. Agroinfiltration is highly efficient in the model tobacco species *Nicotiana benthamiana* and *N. tabacum* as well as most other *Nicotiana* species we have tested. Agroinfiltration can also be applied to a number of other plant species (2), albeit often with much lower efficiency that may preclude biochemical analyses of the transiently expressed proteins. The high efficiency of agroinfiltration in *N. benthamiana* allows for the ability to co-express multiple proteins in the same infiltrated patch by mixing *Agrobacterium* suspensions. Furthermore, protein expression levels can be modulated by using native promoters versus strong constitutive or inducible promoters, or by co-expression with suppressors of gene silencing (3). Agroinfiltration has been very informative in the study of the recognition of pathogen-encoded Avirulence (Avr) proteins by plant disease resistance (R) proteins, particularly those encoding NB-LRR proteins (4). Since co-expression of matching R and Avr proteins is sufficient to induce a form of cell death known as the hypersensitive response (HR) agroinfiltration allows for rapid functional analysis of R and Avr protein derivatives (5–7).

One of the first insights into the molecular mechanisms by which plant NB-LRR proteins function was the observation that the Rx and Bs2 CC-NB-LRR proteins could undergo fragment complementation. Agroinfiltration-mediated co-expression of fragments encoding different domains of these proteins, either CC-NB plus LRR or CC plus NB-LRR, recapitulated the function of the full-length protein in conferring an Avr-dependent HR (8). The ability of these fragments to undergo functional complementation was subsequently shown to be due to the fact that these fragments physically interacted as demonstrated by co-immunoprecipitation (co-IP) of the transiently expressed fragments (8, 9). Subsequent use of these techniques has allowed researchers to fine-map the intramolecular interactions that take place between the different domains of CC-NB-LRR proteins and propose molecular models of NB-LRR protein function (10, 11).

Structure–function analyses of protein–protein interactions between known proteins or protein fragments can be undertaken rapidly by generating fusion proteins with epitope tags and making use of the commercially available antibodies that recognize them. Due to their small size, a single epitope tag can often be simply incorporated into a PCR primer allowing for rapid construction of protein derivatives for study. For example, we have co-expressed a series of differentially tagged fragments of the Rx protein and derivatives thereof in *N. benthamiana* leaves to undertake structure–function analyses of the physical interactions that take place within the Rx protein (8, 10, 11). In this method, protein is
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extracted from leaves co-expressing two differentially tagged fragments and antibodies recognizing one epitope are used to immunoprecipitate one of the fragments. Subsequent immunoblotting with antibodies recognizing the other epitope tag can then be used to probe for the presence of the second fragment in the immunoprecipitate, indicating a physical association between the two fragments. Similar immunoprecipitation methods can be adapted to the identification of unknown proteins using transiently expressed R protein fragments and candidates can be validated using epitope-tagged versions of such candidates (12, 13). The efficiency of agroinfiltration in N. benthamiana allows for the co-expression of multiple proteins (up to five in our experience). As such, the co-IP of multiprotein complexes is possible as well as the investigation of how a given protein–protein interaction is affected by the presence of a third protein. Below is a method for investigating the interaction between HA-tagged and FLAG-tagged proteins or protein fragments.

2. Materials

2.1. Agroinfiltration

1. A. tumefaciens strains containing a binary vector (see Note 1) encoding epitope-tagged (see Note 2) versions of the proteins or protein fragments of interest.
2. Luria–Bertani (LB) broth and agar plates supplemented with appropriate antibiotics for binary vector selection in Agrobacterium.
3. Incubators and shakers at 28°C.
4. High-speed table top or floor model centrifuge.
5. Infiltration buffer (see Note 3): 10 mM MgCl₂, 10 mM MES pH 5.6, 100 μM acetylsyringone (added immediately prior to use from a 100 mM stock in DMSO).
6. Spectrophotometer for measuring optical density of Agrobacterium cultures.
7. Slip tip syringes, 1 or 2 mL according to preference.
8. Four- to six-week-old N. benthamiana plants, grown at 20–23°C, 50% humidity, 16 h/8 h light/dark cycle.

2.2. Protein Extraction

1. Pre-chilled mortars and pestles.
2. Extraction buffer: GTEN (10% (v/v)glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl), 10 mM DTT, 2% (w/v) PVPP (polyvinylpolypyrrolidone), 1× protease inhibitor cocktail (see Note 4).
3. Refrigerated microcentrifuge.
4. Desalting matrix (Bio-Gel P6 DG; Bio-Rad, 150-0738), screening columns (Fisher, 11-387-50) and 15-mL snap-cap tubes (optional; see Note 5).

2.3. Immuno-precipitation

1. 10% (v/v) Nonidet P-40 (NP-40, sold as Igepal CA-630 by Sigma; should be less than 1 week old). Should be prepared at least 1 h beforehand as NP-40 takes time to mix with water.
2. IP buffer: GTEN, 0.15% (v/v) NP-40, 0.5 mM DTT.
3. Agarose conjugated Goat IgG (Rockland, 005-0050; see Note 6).
4. EZview™ Red anti-HA and anti-FLAG affinity gels (Sigma, E6779 and F2426; see Note 6).
5. Rotating microtube mixer.
6. 1-mL syringe with 25 Ga needle.
7. 1× SDS–PAGE loading buffer: 100 mM DTT, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM Tris–HCL pH 6.8, 0.02% (w/v) bromophenol blue.

2.4. Immunoblotting

1. Any suitable SDS–PAGE and proteins transfer blotting apparatus and appropriate buffers (see Note 7).
2. 100% methanol.
3. PVDF membrane (Bio-Rad, 162-0177).
4. ANTI-FLAG® M2 monoclonal antibody peroxygenase conjugate (Sigma, A8592) and Anti-HA (3F10) antibody peroxygenase conjugate (Roche, 12013819001).
5. TBST: 25 mM Tris–HCL pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20.
6. Blocking buffer: TBST, 5% (w/v) skim milk powder.
7. Amersham ECL Plus™ western blotting detection reagents (GE healthcare).
8. Saran Wrap, X-ray film, and developing machine.

3. Methods

Prior to co-IP the proteins or protein fragments of interest should be functionally validated. For R protein fragments, this may involve their co-expression with their cognate Avr protein. For example, co-expression of the Rx CC and NB-LRR fragments plus the coat protein of potato virus X results in a macroscopic HR in infiltrated patches within 2–3 days (8). Protein accumulation should be verified and an optimal time point determined for
protein extraction. Likewise, optimal extraction buffers should be determined before initiating co-IPs (see Note 4). In addition, appropriate controls for the co-IP should be identified. Ideally, these should be proteins similar to those under study but that do not interact with the protein(s) of interest. For example, the Rx CC domain interacts with the Rx NB-LRR fragment, whereas the Bs2 CC domain does not (8) make the latter an appropriate negative control when demonstrating an interaction between the Rx CC and NB-LRR fragments. Likewise, the RanGAP2 protein interacts with the Rx CC domain, whereas the similar RanGAP1 protein does not, and neither RanGAP1 nor RanGAP2 interact with the Bs2 CC domain (12, 13). In the absence of a highly similar noninteracting protein, it should at least be demonstrated that the protein of interest does not simply interact with the antibody-conjugated beads or any randomly tagged protein. As such, a tagged version of a protein that would not be expected to interact, such as GUS or GFP, can be used as a negative control.

Ideally, it is desirable to perform a co-IP in both “directions.” That is, to immunoprecipitate an HA-tagged protein followed by investigating the presence of the putative FLAG-tagged partner as well as performing an anti-FLAG immunoprecipitation followed by investigating the presence of the HA-tagged protein. When comparing the interaction properties of different proteins or protein derivatives, this also allows for the demonstration that equal amounts of proteins have been expressed and immunoprecipitated. Once the initial interaction is well established, however, it may be sufficient to perform the co-IP in only one direction to investigate the interaction under varying conditions or using protein derivatives.

3.1. Agroinfiltration

1. Germinate *N. benthamiana* seeds by spreading on top of wetted Metro-Mix 360 (Sun Gro) topped with a layer of vermiculite. Seeds must remain exposed to the light for germination. Two weeks later, transplant seedlings to individual 10 cm pots containing Metro-Mix 360 with growth conditions as outlined above. Plants should be fertilized weekly with Peters Excel “CalMag” Grower (15-5-15; 100 ppm N) (Scotts). Plants can be used for transient expression 2–4 weeks later.

2. Grow *Agrobacterium* cultures overnight at 28°C in LB broth with appropriate selection. 5–10 mL is usually sufficient to fully infiltrate several leaves. For repeat usage, make a glycerol stock (25–30% glycerol) from an overnight culture inoculated from a plate of freshly transformed bacteria and store at −70°C. As needed, streak out stocks onto LB plates with appropriate selection (50 μg/mL of kanamycin for pBIN19 and 5 μg/mL of tetracycline for pCH32). A freshly inoculated plate should be used as starting material for overnight
(O/N) cultures to allow for synchronous growth of different cultures. When setting up O/N cultures, inoculate with a generous amount of Agrobacterium in order to get a dense culture in 16 h.

3. After O/N growth, pellet Agrobacterium cultures by spinning for 15 min at 4,000 rpm in a Sorvall SH-3000 rotor. Resuspend in an equal volume of infiltration buffer (see Note 3). Leave at room temperature for 2–24 h.

4. Take an OD$_{600}$ of each Agrobacterium culture to be infiltrated. The cultures should have all grown to roughly similar ODs if they were all started from fresh plates. Combine Agrobacterium suspensions into the desired combinations for protein co-expression. It is important to infiltrate the same amount of Agrobacterium for each protein to be expressed. If one co-IP calls for the expression of three proteins, and another only two; make up the difference with Agrobacterium carrying empty vector. Each Agrobacterium culture should be infiltrated at an OD = 0.1–0.2. To achieve this, simply dilute the resuspended O/N cultures into a final volume of 10 mL of infiltration buffer. For example, if three cultures have an OD = 1.0, combine 2 mL of each culture plus 4 mL of infiltration buffer which will result in a final volume of 10 mL and an overall OD = 0.6. Avoid using combinations with a final ODs greater than 1.0.

5. Infiltrate Agrobacterium mixtures into N. benthamiana leaves with a syringe. For the easiest infiltration, use a razor blade or pin to make a slight scratch in the underside of the leaf without piercing all the way through. Lightly press the syringe to the scratch and infiltrate. Either mark the infiltrated area with a marker or ensure that the entire leaf has been infiltrated (see Note 8). For each combination of co-expressed proteins, we routinely infiltrate two leaves on two different plants.

### 3.2. Protein Extraction

1. Harvest leaves at 1–3 days post agroinfiltration. Cut leaf material off on either side of the large middle vein with a razor blade. Weigh out 1 g of tissue for each combination and place in a prechilled mortar.

2. Extract protein in the cold room using prechilled materials. Add 2.5 mL of extraction buffer to each mortar. Grind tissue in extraction buffer for 1–2 min until it becomes a consistent slurry.

3. Pour the slurry from each sample into 2-mL Eppendorf tubes and spin at full speed in a refrigerated microcentrifuge for 2 min. Transfer supernatant to a 1.5-mL Eppendorf tube and spin for an additional 10 min (see Note 9).

4. Run 1 mL of the supernatant through a desalting column (optional; see Note 5).
3.3. Immunoprecipitation

1. For each combination of co-expressed proteins add 200–1,200 µL of extract to two separate 1.5-mL Eppendorf tubes (depending on abundance of the proteins and whether or not it has been diluted by desalting. If larger amounts are required, increase extraction volumes accordingly). One tube is used for the anti-HA immunoprecipitation and one for the anti-FLAG immunoprecipitation. Bring up the total volume to 1.4 mL with GTEN and add 10% NP-40 to bring the final concentration (v/v) to 0.15% and DTT to 1 mM. At this point, detergent is usually beneficial in order to keep protein from “sticking” to the beads and from aggregating.

2. Perform a preclearing step by adding 25 µL of agarose-conjugated IgG beads. This is to eliminate nonspecific binding and can also eliminate some proteins that become unstable and precipitate during incubation. Incubate end-over-end in a rotating microtube mixer at 4°C for 30 min. Spin 1 min, full-speed in a microcentrifuge. Add 1.3 mL of the supernatant to a new tube for the specific immunoprecipitation.

3. To this supernatant, add 25 µL of slurry of either the anti-HA or anti-FLAG affinity gel agarose beads using a pipette tip that has had the bottom ~3 mm cut off with a razor blade.

4. Incubate end-over-end for 1–16 h at 4°C.

5. Spin full-speed in a microcentrifuge for 5 s. Discard supernatant and add 1 mL of fresh IP buffer. Repeat four more times but always leave ~50 µL at the bottom of the tube to avoid removing the beads. After the last wash, spin again to remove any liquid on the sides of the tube and aspirate the remaining liquid with a 1-mL syringe with a very fine (25 Ga) needle. Point the open side of the needle to the wall of the tube to avoid aspirating beads.

6. Resuspend beads in 100–150 µL of 1× SDS–PAGE loading buffer. Use immediately or store at −70°C.

3.4. Immunoblotting

1. Boil samples for 10 min. Vortex lightly and spin in microfuge for 5 s.

2. Load 10–50 µL (or up to ~1/3rd of the total) of the sample onto two different SDS–PAGE gels (see Note 7) for immunoblotting analysis; one for anti-HA and one for anti-FLAG immunoblotting.

3. Pre-wet PVDF membrane in 100% methanol followed by equilibration in transfer buffer for 10 min.

4. Transfer proteins to PVDF membrane (see Note 7).

5. Incubate PVDF membranes in blocking buffer for 1–16 h.

6. Place membranes in a tray containing 5–10 mL of TBST. Add primary antibody HRP conjugate (see Note 10) at the
manufacturer’s specified dilution. Incubate for 1 h at room temperature or O/N at 4°C.

7. Wash blots in excess TBST three times 15 min at room temperature.

8. Remove membranes, blotting off excess liquid, and transfer to a piece of Saran Wrap.

9. Perform ECL Plus™ reaction (or similar product) as per the manufacturer’s instructions.

10. Wrap membranes in Saran Wrap and expose immediately (in dark room) to film. Multiple exposure times (from 1 s to 5 min) should be tested, depending on the abundance of the proteins. Develop films.

4. Notes

1. Since agroinfiltration depends only on the ability of the T-DNA to be transferred to the host, most binary vectors can be used for this purpose (1, 14). We routinely use binary vectors based on pBIN19. One of the most effective \textit{Agrobacterium} strains in \textit{Nicotiana} spp. is C58C1 carrying the pCH32 plasmid. The pCH32 plasmid (tetracycline selection) carries two \textit{vir} genes (\textit{vir-E} and \textit{vir-G}) which increase virulence, and consequently the efficiency of T-DNA transfer and expression (15). However, most common strains of \textit{Agrobacterium} can be used effectively in \textit{Nicotiana} spp., including GV3101, LBA4404, and AGL1.

2. Epitope tags should be incorporated at the C terminus of CC-NB-LRR proteins as N-terminal fusions often interfere with function. With other proteins, potential interference with function by fusions at either end of the protein must be determined empirically. We routinely use single HA (YPYDVPDYA) or FLAG (DYKDDDDK) epitope tags on CC-NB-LRR protein derivatives. Many groups report difficulties detecting a single c-Myc epitope tag (EQKLISEEDLNE), and this epitope is commonly used as three to six consecutive tags. The GFP protein can also be added as a tag. GFP can add size in the case of a very small interaction motif and in some cases can stabilize proteins.

3. Acetosyringone induces virulence in \textit{Agrobacterium} at low pH. However, when using C58C1 plus pCH32 in \textit{N. benthamiana}, this is not strictly necessary. 10 mM MgCl$_2$ is sufficient and \textit{Agrobacterium} can be infiltrated immediately.

4. Inclusion of DTT is very important as protein from \textit{N. benthamiana} extracts is particularly prone to oxidation.
Proteins extracted without DTT often run as one or more higher molecular weight bands in SDS–PAGE. PVPP absorb some of the polyphenols which are abundant in *Nicotiana* spp. Although PVPP is insoluble, it is recommended that the PVPP be allowed to hydrate for several hours for maximum efficiency. Thus, one can simply make up the extraction buffer the night before. However, do not add the DTT or the protease inhibitors until just before use. Our extraction buffer functions for a number of different proteins; however, individual proteins may require extraction buffer optimization. Detergents may also be included, particularly for membrane proteins. Experiment with a number of detergents with different properties at first. For example, on the one hand, extraction with ionic detergents increases extraction of Rx, but can be denaturing. On the other hand, extraction of Rx with nonionic detergent results in a complete loss of Rx protein, possibly due to increased protease extraction. Most other proteins we have worked with, however, are fine when extracted with 0.1% NP-40 or Tween-20. Many commercial protease inhibitor cocktails are available, including Sigma plant protease inhibitor cocktail (P9599) which comes as a 100× stock solution in DMSO. If planning on performing IPs on a regular basis, however, it is considerably cheaper to make a protease inhibitor cocktail as follows:

<table>
<thead>
<tr>
<th>Stocks</th>
<th>Preparation</th>
<th>(Stock)</th>
<th>(Working)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEBSF</td>
<td>100 mg in 2.1 mL DMSO</td>
<td>0.02 M</td>
<td>1 mM</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>2 g in 10 mL ethanol</td>
<td>0.1 M</td>
<td>1 mM</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>5 mg in 5 mL methanol</td>
<td>1 mg/mL</td>
<td>1 µg/mL</td>
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<tr>
<td>E64</td>
<td>5 mg in 1 mL DMSO</td>
<td>14 mM</td>
<td>5 µM</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 mg in 200 µL water</td>
<td>50 mg/mL</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Bestatin</td>
<td>5 mg in 2.9 mL DMSO</td>
<td>5 mM</td>
<td>1 µM</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.174 g in 10 mL isopropanol</td>
<td>0.1 M</td>
<td>1 mM</td>
</tr>
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5. Passing protein extracts through a small desalting column is optional but can help reduce nonspecific modification of proteins during the IP. G25 Sephadex can be substituted for Bio-Gel P6, both of which act as size exclusion matrices that eliminate a good deal of small molecules, including harmful phenolic compounds. The difference can be seen after extended incubation of extracts: the desalted extract remains clear or greenish while the untreated extracts turn brownish. In addition, passage through a desalting column eliminates particulates that are often present even after centrifugation. Desalting columns can be used either by gravity or by spinning (similar to
a G-50 column used to clean up DNA reactions). Gravity is more effective, but spinning is sufficient for most purposes and is much more convenient when dealing with numerous samples. To make the desalting column, allow the desalting matrix to swell for at least 2 h in extraction buffer. At this point, it is not necessary to include protease inhibitors, and DTT concentration can be decreased to 1 mM. Do not include PVPP. Use about 10 mL of buffer per column. Add powder slowly to the liquid until saturation appears to be reached. If too much matrix is added, simply add more buffer. Aim to have as little liquid as possible at the top, once the slurry has settled (not more than half a centimeter). Place a screening column into a 15-mL round bottom snap cap tube. Pour slurry into the top of the column. Spin method: Spin in a clinical centrifuge for 1 min at 2,000 rpm to get rid of excess liquid. Transfer column to a new dry snap cap tube. There should be about 1 cm of space at the top of the tube. Load up to 25% of the column volume of protein extract (~1 mL) and spin again. Keep the flow-through and discard the rest. Gravity method: Allow the excess liquid to drip through until the column is settled and no more liquid is coming through. Add 1.2 mL of extract and allow it to drip through. Add 0.25 mL of buffer and allow it to drip through. Discard flow-through and transfer column to a new dry snap cap tube. Add 1.75 mL of buffer and collect what drips through. This contains the protein. This method results in dilution of the sample and/or partial loss compared to the spin method. However, this is usually not critical and gives a cleaner extract. A trial run is recommended to optimize collection with individual versions of this technique (i.e., differing columns or sample sizes). To determine what volumes to use to desalt, use a dilute solution of bromophenol blue (small molecule; is retained in the column) and dextran blue (very large molecule; is excluded from the column and remains in the void volume with the proteins). Add small amounts (0.25–0.5 mL) of buffer to the column to determine when the dextran blue elutes. Protein should elute in the same fractions as dextran blue. Fractions containing bromophenol blue (and other small molecules) should be avoided.

6. Agarose conjugated IgG is used for a nonspecific preclearing step and can be from any source and any species. Using antibodies preconjugated to agarose beads is convenient and economical if immunoprecipitating epitope-tagged proteins on a regular basis. Alternatively, nonconjugated antibodies can be used in conjunction with either protein A or protein G conjugated beads.

7. Use standard procedures (16) for SDS–PAGE and transfer of proteins to PVDF (or similar product such as nitrocellulose membrane).
8. *N. benthamiana* leaves are easy to infiltrate in their entirety under optimal conditions. Ensure that the plants are not drought stressed. Watering the plants an hour before infiltration can help. If plants are grown in a glasshouse, the best time to infiltrate is in the late afternoon or evening to avoid the effects of hot afternoon sun which can make infiltration more difficult and lead to leaf temperatures detrimental to *Agrobacterium*. Alternatively, if conditions are not optimal in the glasshouse, bring the plants to the lab a couple of hours before infiltrating. After infiltrating, leave on the bench overnight (or even for the next 1–3 days). Growth conditions can have a large effect on protein expression. Temperatures of 19–24°C and the absence of intense light are best, and the lab benchtop is often the optimal area for transient expression. Leaf position can also affect protein expression. Try to avoid the lowest leaves. These are easy to infiltrate but give lower and patchier expression. Newly emerging leaves are more venous and harder to infiltrate in large patches. The best expression and extraction can be obtained by using the two youngest fully expanded leaves. As a general rule, if the infiltration takes a lot of effort and requires more than three to six infiltration sites per leaf, conditions are nonoptimal and may yield poor results. Before initiating co-IP experiments, try experimenting with a few different aged plants and different leaf positions. It can be helpful to infiltrate with a binary vector expressing some version of mGFP and observing the plants under long-wave UV light to become familiar with which leaves give the best expression.

9. This is appropriate for soluble proteins but should be adjusted accordingly for membrane-associated proteins. An alternative approach often used is to grind in liquid nitrogen and then incubate with an extraction buffer, particularly if the extraction buffer contains detergent. This is probably fine for most proteins and protein complexes, but may make it more difficult to ensure the use of exactly the same amount of starting material for each sample.

10. This use of primary antibodies directly conjugated to HRP avoids cross-reaction with the antibodies used in the co-IP and reduces the time required for immunoblotting. Alternatively nonconjugated primary antibodies can be used, followed by incubation with an appropriate HRP-conjugated secondary antibody. However, care should be taken to use combinations of antibodies that will avoid using a secondary antibody that recognizes the antibody used for the co-IP. The latter is present in the final protein extracts and subsequently on the PVDF membranes.
References