Preface

The diagnosis of infections in plants has changed immeasurably over the years. Early references to disease in barley crops date back to the mid-1500s and probably represent the first true records that we have of symptoms being noted. Early records merely described the physical appearance of plants and were usually associated with catastrophic crop failure. By 1870 fungal and bacterial diseases of plants were diagnosed, but it was not until after 1900 that virus diseases were identified. The science of plant pathology in its true sense came into being when it became possible to treat plants to control pathogens or to use husbandry to avoid pathogen problems. Physical symptoms on plants can be diagnostic, but very often they can be caused by several pathogens and a more scientific approach is required. Diagnostic methods mainly developed for the medical and veterinary sciences have now been applied to diseases of plants and we now have a bewildering assortment of methods at our disposal. Plant diseases still account for heavy losses in many parts of the world where total crop failure due to disease can lead to human misery.

In the western world, much emphasis is now placed on effective disease control by the use of clean seed and appropriate chemical intervention, but both rely on good diagnostics to establish disease status prior to action being taken. The development of quick and cheap methods for disease detection ensures that crop plants remain free of pathogens.

Plant pathology techniques fall into three categories: traditional, serological and nucleic acid, although some span more than one discipline. Traditional methods include the use of indicator plants to produce visual symptoms of disease on susceptible hosts and the use of synthetic media to encourage the growth of microorganisms which can then be identified by colony morphology. These methods can be coupled with more advanced techniques where additional information is required for diagnosis.

Serological methods are all based on the unique ability of animal antibodies to bind to small target areas known as epitopes on the proteins that elicited their synthesis. Antibodies to plant pathogens can be raised in the serum of animals quite easily by immunising them with preparations of microorganisms and then collecting the serum by live donation. Antibodies can be recovered from serum by simple chemistry or chromatography and reagents developed by adding markers such as chromogenic chemicals or fluorescent dyes. Monoclonal antibodies can be made in cell lines, using tissue culture obviating the use of animals for serum production. Such methods have long been established in the medical world and now provide rapid robust tests for pathogens in plant material.

Nucleic acid methods are the newest of the technologies but provide the most exciting possibilities. Methods are all based on the fact that small areas of nucleic acid exist within the genome that are unique to an organism and can be used to identify its presence. Once the sequence of these regions is known, synthetic fragments can

be made which will bind to the specific areas on the pathogen DNA. These areas can then be made to replicate to produce multiple copies that can then be visualised either by electrophoresis or by fluorescent-based methods. The polymerase chain reaction (PCR) is fundamental to the nucleic acid technologies as it provides a way to amplify selected specific regions of DNA so that they can be visualised and thus provide a measurable signal indicating the presence of the target DNA.

This first edition of *Plant Pathology Techniques and Protocols* seeks to provide workers with both basic methods and more advanced techniques for the diagnosis of plant pathogens. Those with limited experience will find easy to use protocols and those with more experience will find methods that they may wish to use as alternatives to those already in place. Methods cover pathogens which cause major problems in crop plants globally. Issues of crop identity and authenticity have become more important in recent years and two chapters have been included which will allow workers to genotype samples from two major food crops. Authors are all active researchers and the methods are those currently being used in their laboratories.

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Robert Burns

Chapter 2

Development of Enzyme Linked, Tissue Blot and Dot Blot Immunoassays for Plant Virus Detection

Hei-ti Hsu

Summary

Immunoassays are among the most powerful and useful techniques for analysis of biological materials. There are numerous variations in which immunoassays can be performed. Coupled with enzyme, using chromogenic substrates, the enzyme immunoassay technique is used to trace the target antigen in tissues. The technique also is used to measure the concentration of antigen in tissue extracts. This chapter provides fundamental information that is needed to carry out the routinely used procedures in plant virus research.

Key words: ELISA, Dot blot, Tissue blot, $F(ab')_2$, Protein A, Enzyme-labeling, Biotinylation.

2.1. Introduction

Enzyme Immunossay (EIA) is a simple, effective and powerful tool for identification and analysis of biological materials. The method is based on the principle of specificity of antibody–antigen interaction. Coupled with enzyme using chromogenic substrates, the technique is used to trace target antigen in tissue sections or tissue blots. Upon hydrolysis of the substrate, the presence of antigen is visualized by an insoluble color precipitate. The technique also is used in quantitative measurement of antibodies in body fluids (*see* **Note 1**) or antigens when extracted from tissues. In this procedure, a color soluble substance is produced by the enzyme. The concentration of the antigen present in the extract is determined spectrophotometrically.

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Before getting into the details of basic EIA, a couple of terms commonly used in serological procedures need to be clarified. Direct EIA utilizes enzyme molecules that are covalently linked to antibodies specific to the target antigen in question. In indirect EIA, an unlabeled target-specific antibody is first reacted with antigens. These antibodies are called primary antibodies. In second step, this globulin–antigen complex is reacted with enzyme-labeled anti-globulin conjugate (secondary antibody). If the enzyme activity is detected following the addition of enzyme substrate, we have indirect evidence of the presence of the specific antigen–antibody complex formed in the first step.

Indirect EIAs are versatile. The extension of the indirect procedure is the use of Protein A- or protein G-labeled antibody conjugate and biotinylated antibody. The ability of protein A molecules to bind specifically to the carbohydrate moiety of many immunoglobulins makes enzyme-labeled protein A or protein G conjugate a useful detection reagent *(1, 2)*. In procedures where biotinylated primary or secondary antibodies are used, avidin–enzyme conjugate is employed as a detection reagent *(3)*. Both enzyme-labeled protein A and protein G and biotinylated species specific anti-globulin antibody conjugates are readily available from a number of reliable commercial sources. Its cost effectiveness and consistency in quality make the modified procedures very attractive to investigators.

Direct detection is the simplest, most reliable and highly specific of all EIA procedures but is somewhat a little less sensitive than the indirect method. One drawback of the direct method is that each virus specific antibody reagent must be labeled with the enzyme.

The merit of the indirect method lies on the amplification of primary and secondary antibodies. Maximal amount of the target specific antibody reacts with the antigen followed by the excess amount of labeled secondary antibody reacting with the primary antibody yielding stronger signal than the direct method. With the increase of reacting reagents, the specificity of detection is slightly compromised.

This chapter intends to provide the fundamental information needed to execute the commonly used technology in biological sciences, and is written for plant pathologists having little or no previous exposure with EIA. It is almost impossible that the chapter be exhaustive and extensive to cover every possible variation and modification because of versatility and applicability of the EIA to almost every discipline in biological sciences. I shall focus on a few EIAs routinely used in plant virus laboratories. These shall include basic Enzyme Linked Immunosorbent Assay (ELISA) *(4)*, Tissue Blot Immunoassay (TBIA) *(5)* and Dot Blot Immunoassay (DBIA) *(6)*. Useful variations and modifications will also be discussed. Step-by-step basic procedures shall be

presented. It is hoped that the technique will extend its application to various fields in plant pathology.

2.2. Materials

2.2.1. Preparation of Enzyme–Antibody Conjugates

1. Alkaline phosphatase, Type VII (Sigma).
2. 0.01 M Phosphate buffered saline solution (PBS) pH 7.5.
3. 10% Glutaraldehyde, reagent grade (freshly opened vial).
4. 1% Bovine serum albumin (BSA) in PBS.
5. 10% Sodium azide (*see* **Note 2**).
6. Centrifuge.

2.2.2. Preparation of F(ab′)$_2$ Antibody Fragments

1. Protein-A Sepharose.
2. 0.5 M Sodium acetate, pH 4.5.
3. Pepsin solution: 1 mg pepsin in 1 mL warm 0.1 M sodium acetate buffer, pH 4.5.
4. Tris–HCl salt.
5. 0.1 M Glycine, pH 3.2.
6. Saturated ammonium sulfate, pH 7.8.
7. Normal saline solution: 0.85% sodium chloride.

2.2.3. Preparation of Biotinylated Antibody

1. 0.2 M Borate buffer,\ pH 8.5.
2. *N*-Hydroxy succinimidobiotin.
3. Dimethylsulfoxide (DMSO).
4. 0.01 M Phosphate buffered saline (PBS), pH 7.5.

2.2.4. ELISA Procedure

1. ELISA plates.
2. Coating-buffer: (0.05 M carbonate, pH 9.6). Dissolve 1.59 g sodium carbonate, and 2.93 g sodium bicarbonate in 1,000 mL distilled water.
3. 0.01 M Phosphate buffered saline (PBS), pH 7.5.
4. PBS-Tween: 0.05% (v/v) Tween-20 in PBS.
5. Blocking solution: 1% nonfat dry milk, 0.5% BSA in PBS-Tween.
6. Alkaline phosphatase–antibody conjugates (either virus-specific antibody or species-specific anti-globulin antibody) in PBS-Tween.
7. Substrate buffer: 97 mL diethanolamine, 0.1 g $MgCl_2 \cdot 6H_2O$, pH 9.8. Add distilled water to 1,000 mL.

8. Enzyme substrate: Dissolve 25 mg *p*-nitrophenyl phosphate in 25 mL substrate buffer.
9. Shaker.
10. ELISA Reader.

2.2.5. TBIA and DBIA Procedures

1. 0.2- or 0.45-μm nitrocellulose membrane.
2. Razor blades.
3. Gloves or forceps.
4. Small (about 10 × 12 cm) glass tray.
5. Phosphate suffered saline solution (PBS): pH 7.5.
6. PBS-Tween: 0.05% Tween-20 in PBS.
7. Blocking solution: 1% nonfat dry milk, 0.5% BSA in PBS-Tween.
8. Alkaline phosphatase-labeled antibody conjugate.
9. Substrate buffer: Solution A: 35.5 mL distilled water, 2.0 mL 2.5 M Tris–HCl, pH 9.5, 2.0 mL 11% sodium chloride, 0.2 mL 1 M magnesium chloride; Solution B: 14 mg nitro tetrazolium in 300 μL methanol; Solution C: 7 mg 5-bromo-4-chloro-3-indolyl *p*-toluidine salt in 50 μL dimethylsulfoxide. Prior to use, add B to A, mix well. Add C dropwise with shaking, mix completely. Stop solution. 0.01 M Tris-HCl, 1 mM EDTA (pH 7.5).
10. Shaker.

2.3. Methods

2.3.1. Preparation of Enzyme–Antibody Conjugates

During early years when the ELISA technique was developed, scientists had to prepare enzyme–antibody conjugates for their own research *(7)*. In recent years with the exception of a few viral pathogens and previously unreported new viral causal agents, enzyme–antibody conjugates to many viruses are now readily available from a number of commercial suppliers (*see* **Note 3**). The great benefits of obtaining conjugates from commercial sources are that they come in either alkaline phosphatase form or horseradish peroxidase form and that they are consistent in quality. However, the following illustrates the procedures used to prepare reagents for use in EIA.

This procedure is useful for either alkaline phosphates or horseradish peroxidase.

1. Centrifuge alkaline phosphatase, type VII, which comes in ammonium sulfate suspension. Dissolve 2 mg alkaline

phosphates in 0.5 mL of PBS containing 1 mg specific immunoglobulin. Dialyze against PBS.

2. Add gluteraldehyde to the enzyme–antibody mixture to a final concentration of 0.2%. Stir at room temperature for 3 h. Dialyze overnight against PBS.
3. Dilute the conjugate solution with PBS containing 1.0% bovine serum albumin (or egg albumin) to make a 10% solution (0.5 mL of enzyme–antibody + 4.5 mL PBS + 0.01 mL 10% azide sodium). Store at 4°C. This is stock solution.

2.3.2. Preparation of F(ab′)$_2$ Antibody Fragment

In the double antibody sandwich ELISA (DAS-ELISA) procedure, use of F(ab′)$_2$ antibody fragments in coating microtiter plates eliminates the necessity of preparing various enzyme–antibody conjugates for each of individual different viruses. Enzyme-labeled protein A is used as a universal signal generating reagent.

1. Take 5 mg antibody which has been passed through protein A column, and 0.5 M sodium acetate buffer, pH 4.5, to give 0.1 M sodium acetate, pH 4.5 (5 mg antibody in 0.4 mL saline + 0.1 mL 0.5 M acetate).
2. Prepare pepsin solution: 1 mg pepsin in 1 mL warm 0.1 M sodium acetate, pH 4.5. Add 50 µL pepsin solution (i.e. 50 µg) to antibody.
3. Incubate at 37°C for 20–24 h.
4. Neutralize by adding few Tris salt crystals to pH 8.0 to pH 8.4.
5. Run digested antibody through protein A column. The first peak is F(ab′)$_2$. Then elute column with 0.1 M glycine, pH 3.0 to remove any undigested antibody.
6. Concentrate F(ab′)$_2$ fraction by adding one-half volume saturated ammonium sulfate.
7. Dialyze against several changes of saline. Centrifuge at low speed to remove any insoluble material.

2.3.3. Preparation of Biotinylated Antibody

Avidin is an egg white protein which is a tetramer with four identical subunits. Each contains a high affinity binding site for biotin. The biotin molecule can be coupled to antibodies as illustrated.

1. Dilute antibody to 1 mg/mL in 0.2 M borate buffer, pH 8.5.
2. Prepare 1 mg/mL biotinyl-*N*-hydroxysuccinimide in DMSO.
3. Add 100 µL of biotin solution to 1 mL antibody dropwise while slowly stirring.
4. Continue stirring 5–10 min. Let sit for 4 h at room temperature.
5. Dialyze against PBS, with several changes at 4°C overnight.
6. Aliquot and store at 20°C below zero.

2.3.4. ELISA Procedure

Either antigen or antibody can be attached to the surface of microtiter plates made from polystyrene or polyvinyl. Although round bottom well plates are acceptable, flat bottom well plates are recommended if results are to be read spectrophotometrically (*see* **Note 4**). The optimal conditions for running the test are pre-determined by checker-board titration method using reference reagents. These include the concentration of reactants, time and temperature of incubation. Once determined, these conditions are to be followed in subsequent tests if reproducible results are to be anticipated.

For most viral investigation, protein (viruses, viral proteins, and antibodies) at 1–10 μg/mL in coating buffer at pH 9.6, are used for coating microtiter plates. Coating are usually carried out at room temperature (some people prefer 37°C) for 1–2 h. For convenience, incubation at 4°C overnight gives satisfactory results. Blocking is an essential step in EIA. Egg albumin, BSA and/or nonfat dry milk are used following the step of coating. Blocking or quenching is a process in which unoccupied protein binding sites on the surface of microtiter wells are saturated so that detecting antibody molecules in direct procedure (also primary and secondary antibodies in indirect methods) do not bind non-specifically resulting in background formation. Washing is the separation of bound and free reactant after an appropriate time of reaction. It is usually accomplished on a shaker for 10–20 min with washing solution.

2.3.4.1. ELISA Using Antibody-Sensitized Plates, Direct Procedure

The following assay system illustrates the most commonly used DAS-ELISA direct procedure in plant virus research. Antigen in tissue extract is specifically reacted with and bound to antibody that is already attached to microtiter wells. Unless otherwise noted, incubations are at room temperature for 1–2 h or 4°C overnight.

1. Coat plates by adding 100–150 μL antibody-coating solution to each well and incubate.
2. Discard the antibody solution and wash the plate once.
3. Add 200 μL blocking solution and incubate.
4. Invert and tap plates to remove blocking solution and wash once.
5. Add samples and incubate.
6. Discard samples and wash once.
7. Add enzyme-labeled virus-specific antibody and incubate (*see* **Note 5**).
8. Empty enzyme–antibody conjugate.
9. Wash 3–4 times, 3–5 min each! This is an important step. Be sure that no residual unbound enzyme conjugate is left behind.

10. Add enzyme–substrate solution and incubate for color to develop.
11. Read spectrophotometrically (A_{405}) or record visually.

2.3.4.2. ELISA Using Antigen-Sensitized Plates, Indirect Procedure

Antigen coating procedure in indirect ELISA offers an advantage that one single, species-specific enzyme-labeled anti-globulin antibody conjugate can be used in assays of various different viruses. It eliminates the necessity of preparing various enzyme–antibody conjugates for individual viruses.

1. Dilute infected tissue extract in coating buffer.
2. Coat plate by adding 100–150 µL antigen-coating solution to each well.
3. Discard the coating solution and wash the plate once.
4. Add 200 µL blocking solution and incubate.
5. Discard blocking solution and wash once.
6. Add 150 µL virus-specific mouse monoclonal antibody or dilute rabbit antiserum and incubate.
7. Empty plate and wash once.
8. Add 150 µL mouse specific alkaline phosphatase-labeled anti-globulin antibody (or rabbit specific anti-globulin antibody if rabbit anti-virus antibody is used), and incubate (*see* **Note 5**).
9. Discard the conjugate solution and wash the plate 3–4 times, 5–10 min each. Be sure that no residual unbound enzyme-conjugate is left behind.
10. Add enzyme-substrate solution and incubate for color development.
11. Read spectrophotometrically (A_{405}) or record visually.

2.3.5. TBIA and DBIA Procedures

Similar to coating procedure in ELISA, proteins can be immobilized on nitrocellulose membranes. The superior binding capacity of at least 80 µg of protein per square centimeter of nitrocellulose membrane makes it an excellent solid substrate in blot immunoassay of viral antigens *(8)*. Membranes 0.45 µm are suitable for TBIA or DBIA *(5, 6)*. Use gloves or forceps while handling the membrane.

Tissue blot is a process of transfer of protein antigens from a freshly cut tissue surface to nitrocellulose membrane. It is achieved simply by bringing a freshly cut tissue surface in direct contact with a dry nitrocellulose membrane. The tissue imprint is made by application of a slight pressure of the cut tissue surface while it is in contact with the nitrocellulose membrane. Do not squeeze juice out from tissues. This will smear the imprint.

In the Dot Blot procedure, the samples are applied to nitrocellulose membrane while it is attached and clamped into a

manifold. Add 0.25–100 µL tissue extract containing virus or viral protein to each well. Membranes are removed. From here on both membranes from tissue blot or dot blot are processed similarly as microtiter plates in ELISA. Either direct or indirect procedure can be used.

Detection enzymes for use in both tissue blot and blot immunoassay procedures should be carefully evaluated before the system is established. Horseradish peroxidase is commonly employed in enzyme-linked immunoassay for detection of a number of plant pathogens. It may not be suitable for use in tissue blot immunoassay on nitrocellulose membranes since endogenous peroxidase from plant tissue interferes with the assay. Alkaline phosphatase-antibody (both primary virus-specific and secondary immunoglobulin-specific) conjugates are generally available in plant pathology laboratories and are convenient to investigators. Enzyme-labeled protein A or avidin–biotin system works equally well in membrane blot assays. Substrates that produce soluble colored products are used in ELISA. With the same enzyme conjugate in TBIA, the substrates that yield insoluble colored products precipitating at the site of enzyme reaction should be the choice. When chemiluminescent substrates are utilized in tissue blot immunoassay, the presence of antigens can be recognized by the image registered on a light-sensitive X-ray film *(9)*. This is especially useful when colored pigments of plant tissue origin interfere with the results of analysis using chromogenic substrates.

2.3.5.1. TBIA Direct Procedure

1. Excise tissues (leaves, petioles, stems, flower buds, emerging shoots, bulb, etc.).
2. For thin tissue such as leaves, roll them into a tight core.
3. Hold tissue in one hand and cut with a new razor blade in steady motion with other hand to obtain a single-plane cut surface (*see* **Note 6**).Press for about a full second the newly cut surface onto a nitrocellulose membrane to obtain a tissue blot. Use a firm but gentle force (*see* **Note 7**).
4. Block tissue blots by immersing nitrocellulose membranes in the blocking solution for 30–60 min with occasional shaking at room temperature (*see* **Note 8**).
5. Wash blots once with washing solution for about 1 min with gentle shaking (about one rotation per second on a mechanical shaker).
6. Incubate tissue blots in a glass dish with alkaline phosphatase-labeled virus-specific antibodies diluted in PBS-Tween for 60 min at room temperature. Be sure that the reagent solution covers the blot (*see* **Note 9**).
7. Wash blots 4 times in washing solution for 30 min with shaking.
8. Soak blots in substrate solution 2–5 min at room temperature to detect enzyme activity.

9. Rinse blots for a few seconds in distilled water.
10. Stop reaction by immersing blots in stopping solution, two to three changes, 10 min each.
11. Dry nitrocellulose membranes on two to three layers of tissue wipes in a dust-free area.

2.3.5.2. DBIA Indirect Procedure

1. Grind 0.2 g of liquid nitrogen frozen midrib excised from phytoplasma infected plants in 3.0 mL PBS buffer in a mortar and pestle. This constitutes 1:15 dilution of antigen preparation. Transfer the extract to a glass test tub and let the extract undisturbed for 5 min at room temperature. Leaving a sample suspension undisturbed for a few minutes allows particulates, which may interfere with the sample application onto the membrane, to settle by gravity.
2. Wet nitrocellulose membrane in PBS for 1–2 min.
3. Place wet nitrocellulose onto manifold and clamp into place following manufacturer's instructions.
4. Turn on vacuum; add 50 µL antigen to well.
5. Let antigen filter through membrane completely under vacuum. Wash each well with 100 µL PBS.
6. Turn off vacuum and disassemble apparatus.
7. Block tissue blots by immersing nitrocellulose membranes in the blocking solution for 30–60 min with occasional shaking at room temperature.
8. Wash blots once with PBS-Tween for about 1 min with gentle shaking.
9. Incubate tissue blots in a glass dish with virus-specific mouse monoclonal antibodies diluted in PBS-Tween for 60 min at room temperature. Be sure that the reagent solution covers the blot.
10. Incubate tissue blots with alkaline phosphatase-labeled mouse-specific antibodies diluted in PBS-Tween for 60 min at room temperature. Be sure that the reagent solution covers the blot (*see* **Note 8**).
11. From here on, follow **step 8** described in **Subheading 2.3.5.1**.

2.4. Notes

1. Labeled antigen is routinely utilized in EIA in medical diagnosis of infectious diseases to measure the level of specific antibodies after viral infection. This format is rarely applicable in plant sciences.

2. Sodium azide inhibits the horseradish peroxide activity. Instead, use merthiolate as preservative.
3. Companies that offer services and/or antisera for plant viral disease diagnosis are: Adgen Ltd, http://www.adgen.co.uk; Agdia, Inc., http://www.agdia.com; American Type Culture Collection, http://www.atcc.com; Bactochem Ltd, Fax 972-8-9401439; Bio-Rad Laboratories, http://www.bio-rad.com; Bioreba AG., http://www.bioreba.ch; Durviz, Inc., http://www.durviz.com; German Collection of Microorganisms and Cell Cultures, http://www.dsmz.de; LCA BIOTEST, http://perso.wanadoo.fr/lcab/; LOEWE Biochemica GmbH, http://www.loewe-info.com; and Plant Research international, http://www.plant.wageningen-ru.nl/
4. Round bottom well plates are manufactured specifically for hemaglutination, complement fixation and other serological tests.
5. Horseradish peroxidase-labeled antibody conjugate is suitable for use in ELISA, but is not recommended due to that its substrate, *ortho*-phenylene diamine, is possibly carcinogenic.
6. In tissues that contain a high concentration of latex, the cut surface is first drained on tissue paper to remove excess exudate before blotting onto nitrocellulose membranes.
7. Membrane blots, after preparation, can be stored for a period of time up to 4 weeks in a dust-free environment or mailed to a processing laboratory. This allows membrane blots to be prepared in one location and sent to another location for diagnosis.
8. Blocking should only be done immediately before incubation with antibodies.
9. For economical reasons, alkaline phosphatase labeled antibody conjugate can be recovered and used repeatedly for a few times before the solution shows losses of enzyme activity.

References

1. Goding, J.W. (1978). Use of staphylococcal protein A as an immunological reagent. *J. Immunol. Methods* 20:241–253.
2. Langone, J.J. (1982). Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumococci. *Adv. Immunol.* 32:157–251.
3. Guesdon, J.L., Ternynck, T., and Avrameas, S. (1979). The use of avidin–biotin interaction of immunoenzymatic techniques. *J. Histochem. Cytochem.* 27:1131–1139.
4. Clark, M.F., and Adam, A.N. (1977). Characterization of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475–483.
5. Lin, N.S., Hsu, Y.H., and Hsu, H.T. (1990). Immunological detection of plant viruses and a mycoplasma like organism by direct tissue blotting on nitrocellulose membranes. *Phytopathlogy* 80:824–828.
6. Hsu, H.T., Lee, I.M., Davis, R.E., and Wang, Y.C. (1990). Immunization for generation of hybridoma antibodies specifically reacting with plants infected with a mycoplasma like organism (MLO) and their use in detection of MLO antigens. *Phytopathology* 80:946–950.

7. Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* 6:43–52.
8. Gershoni, J.M., and Palade, G.E. (1982). Electrophoretic transfer of proteins from sodium dodecyl sulphate–polyacrylamide gels to a positively charged membrane filter. *Anal. Biochem*. 12: 396–405.
9. Makkouk, K.M., Hsu, H.T., and Kumari, S.G. (1993). Detection of three plant viruses by dot-blot and tissue-blot immunoassays using chemiluminescent and chromogenic substrates. *J. Phytopathol*. 139:97–102.