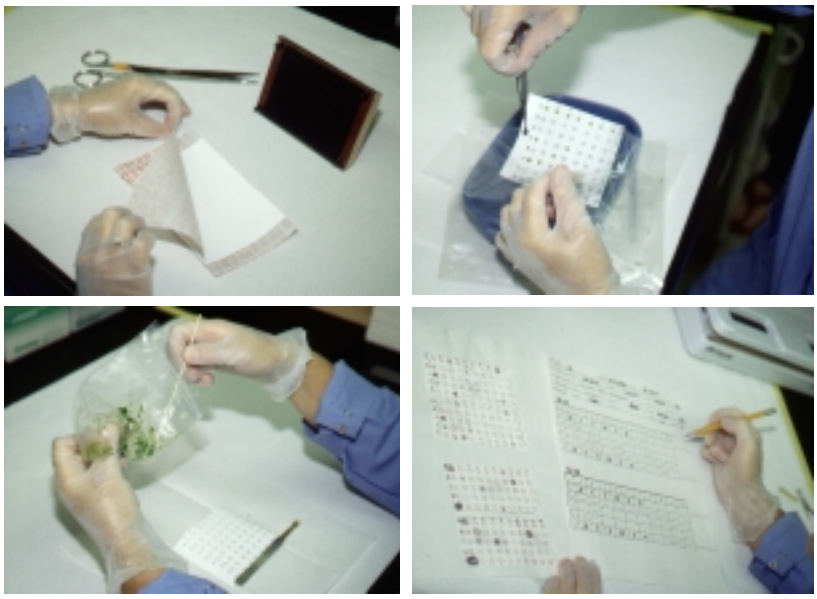
Agriculture Course Materials
ADAP 92-2

Illustrated Workbook for the Detection of Plant Viruses Infecting Cucurbits:

Demonstration of the Use of Direct Immunoblotting Assays



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Illustrated Workbook for the Detection of Plant Viruses Infecting Cucurbits: Demonstration of the Use of Direct Immunoblotting Assays

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Introduction

Insect transmitted plant viruses cause serious crop losses worldwide. It is especially serious in tropical areas where cropping is continuous and viruses and insect vectors are present year round. There are at least 5 viruses of cucurbitaceous plants (squash, melons, cucumber, etc.) that frequently cause severe to complete economic loss for cucurbit producers in the tropics. These viruses are: zucchini yellow mosaic virus (ZYMV), papaya ringspot virus watermelon isolate (PRSV-W, formerly watermelon mosaic virus 1 or WMV I), watermelon mosaic virus 2 (WMV 2), cucumber mosaic virus (CMV) and squash mosaic virus (SqMV). All five of these viruses are transmitted by a large number of aphid species in a nonpersistent fashion. This means that aphids can acquire or pick up the virus in seconds to minutes of probing an infected plant and immediately transmit or inoculate the virus to a healthy plant. The process of inoculation, like acquisition, occurs during a few seconds to minutes of probing a noninfected plant. Once the aphid acquires the virus, it does not retain it for very long. The length of virus retention may be from minutes to 24 hours, varying with the aphids behavior. For example, if an aphid probes plants frequently, they will quickly lose the virus. If they are dispersing and not probing plants, they may retain the virus for up to 24 hours. These characteristics of the virus-vector relationship make control of nonpersistently transmitted viruses extremely difficult.

Pesticides aimed at reducing aphid vector populations seldom help manage virus spread for several reasons. First, aphids can transmit the viruses so quickly that the damage is done before the pesticide can kill the insect. Second, many of the most important vector species do not colonize or live on the crop. Instead, the transient aphids that land briefly and probe crop plants while searching for an appropriate host are the most serious vectors. Thus, the most important insect species won't be targeted by spraying a pesticide on the crop. Finally, many pesticides cause aphid activity to increase. Any treatment that increases aphid activity also increases movement between plants. Frequently, when the aphids move between plants they also move the virus. Hence, pesticide treatment may result in increased virus spread. The greatest successes in managing nonpersistently transmitted viruses have involved plant resistance to the virus. Host plant resistance has been achieved through:

1) Traditional plant breeding in which plants with resistance to a virus or viruses are selected and bred with horticulturally acceptable varieties until a new commercial cultivar with resistance is achieved;

2) Classical mild strain cross protection in which a mild strain of a virus is either developed or isolated from nature and used to inoculate seedlings. Inoculated seedlings are then protected from infection by severe isolates of the same virus; and,

3) Bioengineered cross protection in which a piece of the genome or genetic material of the virus is inserted into a plant. Such plants are called transgenic plants and they will be well protected from infection by the virus from which the genetic material came. In some cases, the coat protein gene from a single virus, such as ZYMV or WMV 2 may protect the plant from several viruses in the same virus group. For example, recent information developed in the laboratory of Dr. Dennis Gonsalves at Cornell University shows that plants transformed with the coat protein gene of ZYMV and WMV 2 are protected from at least 6 different potyviruses.

To effectively take advantage of any of these methods of virus management, you must know which viruses are causing the epidemics present in crops in your region. To accurately diagnose virus infections you must use a detection technique. Many techniques are available, including host plant range analysis and serological and molecular analysis. The purpose of our workshop and this illustrated workbook is to demonstrate the use of a serological technique called direct immunoblotting. The advantage of this technique is that it requires very little special equipment, the assay is relatively easy to perform and the assay has some characteristics that make it ideal for diagnosing problems associated with surveying island communities. For all the other detection techniques, live infected tissue is required by the diagnostician. Movement of infected samples between islands is unwise because of the risk of moving insects and disease agents into areas where they did not previously exist. For this reason, movement of live tissues between islands is prohibited by quarantine regulations. The direct immunoblotting assay allows an individual in a community to blot samples onto an immunoblotting kit, dry and package the paper and mail it to a central location for testing. Hence, this method will permit wide sampling of the Pacific for plant viruses.

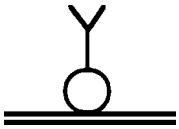
It is our hope in providing you with the skills to collect samples and prepare and process blots you will develop a network of skilled individuals who can collaborate in surveying the Pacific Basin communities and ultimately directing appropriate management strategies. Developing cucumber crops as a commodity for your island community to use locally or to export may then lead to a sustainable commercial industry and increased self- sufficiency. In this workbook, you will find a step by step illustrated protocol for conducting the immunoblotting technique, all the recipes for the solutions needed, instructions on dilutions and solution preparation, an equipment checklist and a chemical list with purchasing information.

Principles of Serology

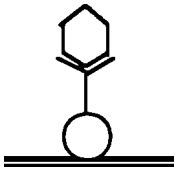
What is serology? Serology is the study of serums, especially their reactions and properties. In the technique you will be learning, detection of plant viruses with direct immunoblotting, we will be using properties of a particular type of serum called *antiserum* (a serum containing an ***antibody)*** to detect plant viruses in unknown samples. ***Antiserums*** are generally produced by injecting an ***antigen*** into a mammal, such as a rabbit or goat. An ***antigen*** is any protein or substance that the animal's immune system perceives as foreign. For purposes that simply involve broad spectrum detection of a particular virus, we can use polyclonal antibodies. These antibodies are produced by purifying a particular plant virus and injecting the purified virus (the ***antigen)*** into an animal, usually a rabbit. The animal's immune system will then build antibodies to the proteins that make up the structure of the virus. After several injections, we remove the blood of the animal (whole serum) and purify the whole serum to acquire ***antiserum***. This is the fraction containing antibodies that are specific to the viral ***antigen*** you injected. This ***antigen/antibody*** specificity lies at the heart of all detection assays because it allows the antibody to stick to the virus when it is present, but not sticking to any other proteins that may be present. Because it is the property of the antiserum and its reactions and properties that form the basis of detection, all the techniques for detection of viruses that use antisera are called ***serological techniques***.

How are antisera used in virus detection assays? In essence, what occurs in a serological test is that samples containing potential antigens (proteins making up virus particles) are allowed to stick to some solid medium (in our case a nitrocellulose membrane), See Figure 1. Then an antibody attached to a marker, like an enzyme, is allowed to react with the samples. If the antigen is present in a sample, the antibody will stick to it because of the specificity we just discussed. If the antigen is not present, the antibody should not stick. Then the substrate for the marker enzyme is added to the assay. When the enzyme and the substrate react with one another, a color change occurs. Since the antibody attached to the marker enzyme should only be present where the antigen was present, a color change tells you that the antigen was present in that particular sample. The more antigen that is present, the more the antibody will stick. Thus, more antigen will also equal a more intense color change.

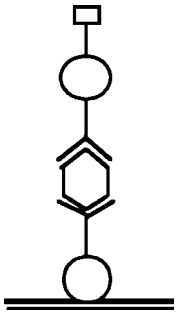
Figure 1



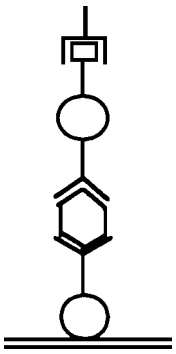
1. Coat nitrocellulose membrane with primary antibody



2. Add samples with potential antigen (the hexagon here).



3. Add antibody conjugated to an enzyme.



4. Add the substrate for the enzyme. Watch for a color change.

There are many serological assays that take advantage of the specificity that occurs between antigens and antibodies. All serological assays are based on similar principles so understanding the interactions occurring in the direct immunoblotting assay should help you to ultimately use other serological techniques. It is very important to understand the principles underlying serological assays because you must be able to evaluate your results and troubleshoot when things just don't work out as they should.

Are there problems that can occur to confuse the results of serological assays? YES! It is critical that you be aware of a few principles so you can avoid confusion in evaluation of your results. ***The first principle to understand*** is that while there is specificity between antigens and antibodies, antibodies may react with other proteins to varying degrees or nonspecifically attach to surfaces. Therefore we take great care to remove nonspecific attachment sites from our test medium, in our case the nitrocellulose membrane (NCM).

There are two important ways we do this. First, we have a blocking step in our assay in which we coat the NCM with nonfat powdered milk. The protein in the milk will coat the paper and occupy nonspecific sites. The antibodies we are using do not stick to this milk protein and hence are prevented from attaching nonspecifically to the NCM. ***Secondly***, we take extra care to remove any antibodies to healthy plant proteins that might be present in our antisera. We do this by preabsorbing our conjugated antibody (antisera attached to a marker enzyme, in our case alkaline phosphatase) with a mixture of healthy plant tissue ground in buffer.

The second principle to understand is that all serological tests must be evaluated relative to known controls. Every assay you do must include a known healthy sample, a known infected sample and buffer controls. Buffer controls are those in which you just use the buffers involved in the test without the antibodies or samples in them. ***The known healthy control*** tells you whether your antibodies reacted to any healthy plant proteins nonspecifically. They show you what a reaction to a noninfected plant should look like. If you are testing many different plant species you need to test healthy controls from as many of the species as possible. Plants vary in their protein profiles and you must be sure you are not detecting nontarget proteins. ***The known infected control*** tells you that your assay worked and your antibody detected the antigen of interest. If you are testing for several different antigens, you should include known infected samples for each one. This will tell you whether your antibody reacted nonspecifically to one of the other antigens. This is called a **cross reaction**. One must always be aware of the possibility that cross reactions can occur and try to control against misevaluations due to cross reaction. ***The buffer control*** should always be negative. If it is positive, then you know you have contamination of some type in your buffers and all the results of your test are in question.

Take Home Concepts:

1. Antigens are proteins to which antibodies are made when the antigen is injected into a mammal.

2. Antibodies are substances made by a mammals immune system in response to a foreign substance called an antigen.

3. Antiserum is the fraction purified from whole serum (blood of the mammal mentioned in 1 & 2) that contains antibodies.

4. Antigens and antibodies have a specific affinity for one another that can be used to develop detection assays of many kinds.

5. Antibodies may react nonspecifically to surfaces, healthy plant proteins and other antigens making controls essential to evaluation of serological assays.

DILUTIONS AND SOLUTIONS

I. Molarity:

Each substance has a molecular weight (M.W.) (the sum of atomic weights).

A 1 molar solution (1 M) = # grams listed as M.W. made up to 1 liter (1L).

For example: M.W.=40.2 grams (g). Weigh 40.2 g, place in a flask and bring up to 1L. This will equal a 1 M solution.

Problem:

If you have a substance with a M.W. of 50 g, how would you make a 1 M solution? a 10 mM solution? a 1 mM solution?

Hint: *0.001 M = 1 mM*
0.010 = 10 mM

II. Equivalency and Dilution Factors

a. Equivalents:

grams and liters
milligrams and milliliters
micrograms and microliters

b. Preparing solutions from stock preparations:

Dilution Factors:

What you have / what you want = the dilution factor. The dilution factor can then be used to determine what to draw out of your stock solution.

For example:

i) You have a stock solution of 1 M Tris. You need to make 50 ml of 0.05 M Tris.

1 M Tris/ 0.05 M Tris = dilution factor of 20

You want to make a final solution of 50 ml. So 50/20=2.5
Therefore, you take 2.5 ml of 1M Tris and bring it up to 50 ml to make a 0.05 M solution.

ii) You have fractionated IgG at a concentration of 1.2 mg/ml and you need to make 10 ml of a 1/1000 solution (1mg/ml).

1.2 mg (1200 mg)/1 mg = a dilution factor of 1200

You want a final solution of 10 ml (10,000 ml). So 10,000/1200 = 8.3 ml
When you remove 8.3 ml from your stock solution and bring it up to 10 ml you will have a 1/1000 solution.

Practice Problems:

a) Make a 50 ml solution of:

0.05 M Tris-HCl
0.01 EDTA
0. 15 M NaCl
1% SDS

Your stock solutions are:

1 M Tris-HCl
0.5 M EDTA
1 M NaCl
20% SDS

b) If you have a tube of IgG and the protein concentration is 1 mg/ml, how would you make a 1/1000 dilution?

Immunoblotting Kits

Producing Immunoblotting Kits

Preparing Your Nitrocellulose Membrane (NCM)

Proper handling of NCM. Nitrocellulose membranes are light weight and easy to ship between locations; however, **they are fragile,** pressure sensitive and will react in areas on which you press too hard. You must **Always wear gloves and use a forceps** to handle your NCM. **Never** handle NCM with ungloved hands and avoid touching with fingers even when you have gloves on. A flat forceps should be used for all manipulations. Designate one comer as the handling area and always use the same spot when you use the forceps to move the NCM. Nitrocellulose may be purchased in sheets, large rolls or individual, precut pieces. If you work with sheets or rolls, your first task is to cut pieces of the appropriate size with a sharp scissors as shown in Figure 1. Rolls come with the NCM between two special sheets of protective paper because the membrane itself is both light and pressure sensitive. Cut them along with the membrane and then use them to protect your NCM as shown in Figure 1. Precut pieces are very convenient, but more costly than rolls or sheets.

Keeping track of your samples. It is very important that you be able to keep track of where you place each sample on the NCM used in the immunoblotting assay. The best way to do this is with a grid and a numbering system. A self-inking stamp has been provided to you that is numbered from 1 to 10 across the top and from A to I along the left edge. Small circles are provided to help in blotting samples. Thus, you can keep track of your samples using a template (see Appendix 3). Use the self-inking stamp to place this grid on each NCM as shown in Figures 2 & 3. If you want to create your own grid or labelling, you can do so by gently writing on the NCM with an indelible ink pen or pencil. Then store the stamped NCM between the sheets of paper in which it came until you are ready for blotting (Figure 3). Again, the NCM are light and pressure sensitive, so store them in containers in a dry, dark place until you are ready to use them.

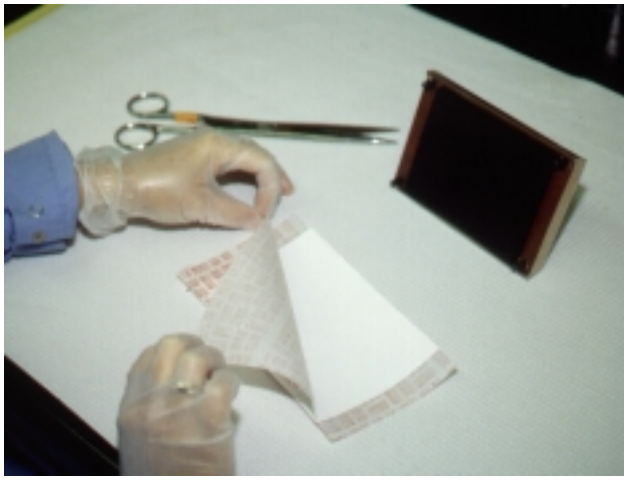


Figure 1. Cut NCM to the appropriate size keeping protective paper liners intact. Remember to use gloves whenever handling the NCM.

Figure 2. Use the self-inking stamp to create a template on your NCM. This will allow you to keep track of where you blot your sample on the membrane.

Figure 3. After using the stamp to place a template on the NCM, replace the protective paper liner and store the NCM in a container, in a dry, dark place until you are ready to proceed to the next step.

Coat NCM with Fractionated Antibody

***What is a fractionated antibody and why do we need to coat the* NCM?** When antibodies are made, immunoglobulins (IgG) are purified from the crude antiserum and fractionated across a special type of column. Each fraction contains a particular amount of IgG which should be written in milligrams (mg)/ milliliter (ml) on the vial containing the IgG. These fractions from the purification column are called fractionated antibodies. In this protocol we coat the NCM with a fractionated antibody that is specific to the virus for which we are testing, for example, zucchini yellow mosaic virus (ZYMV). This coating helps the proteins from ZYMV to adhere to the NCM and prevents cross reactions with other viruses.

Remember: Antibodies are fragile and expensive and should be handled with care and be kept refrigerated when not in use. The antibodies we will be using in this workshop are very specific and sensitive so we can dilute them considerably.

***The following describes the steps you must follow to dilute the antibody and coat the NCM*.**

Step 1. Dilute your fractionated antibody to 1 mg/ml in coating buffer (see Recipes in Appendix 1). To make this dilution: 1) look at tube of antibody to see how many mg of IgG/ml are present. If your antibody has 1 mg of IgG/ml, then 1 ul in 1 ml coating buffer = 1 mg/ml. For equivalent measurements and tips on how to make dilutions, see the section of this handbook called “How To Make Dilutions”.

Step 2. Wet the NCM with distilled water by slow immersion in a container of water as shown in Figure 4. This is best done by holding the NCM at a 45 degree angle and allowing the lower edge to enter the water. As the membrane absorbs the water continue to slowly immerse the lower edge until the entire NCM is immersed without bubbles forming underneath it. Make sure the entire NCM is wet (it will turn grey as it soaks up water). This method is optional, but recommended as it will prevent air bubbles from forming inside the NCM.

Step 3. Then place the wet NCM in the diluted antibody solution you mixed in Step l and incubate overnight at 4 degrees C (39 degrees F) or for 4-6 hrs at room temperature. This may be done in any kind of container in which NCM can be fully immersed. In this laboratory we will use seal-a-meal bags as shown in Figure 5, because they are convenient, inexpensive and provide good immersion of the NCM using the least amount of antibody. To use the seal-a-meal bags, first open the bag as wide as possible and gently place the NCM inside without bending or folding the NCM (Figure 5).

After the NCM is positioned inside the seal-a-meal bag, add your antibody solution to the bag, gently remove air bubbles and seal the open end of the bag (Figures 6 & 7). It is important to remove the air bubbles because they can prevent contact between the NCM and the antibody. If you find air bubbles after you have sealed the open end of the bag, then cut a small hole in one comer, lay the bag on the slanted

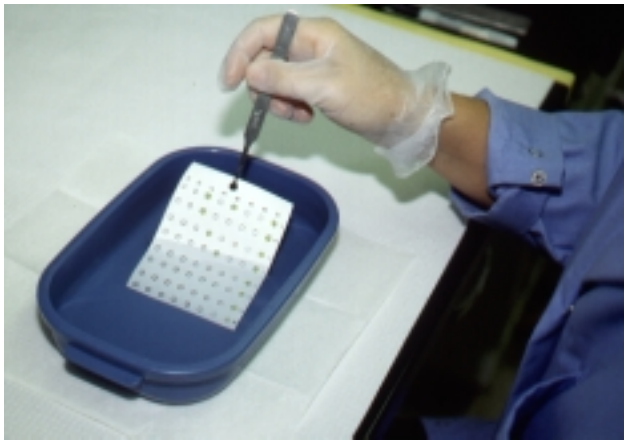


Figure 4. Wet the NCM by slowly immersing it in a container of distilled water. Be careful to avoid air bubbles under or on the NCM. Hold the NCM by a designated spot on the edge of the membrane with flat tipped forceps. Try to use the same area each time you handle the NCM.

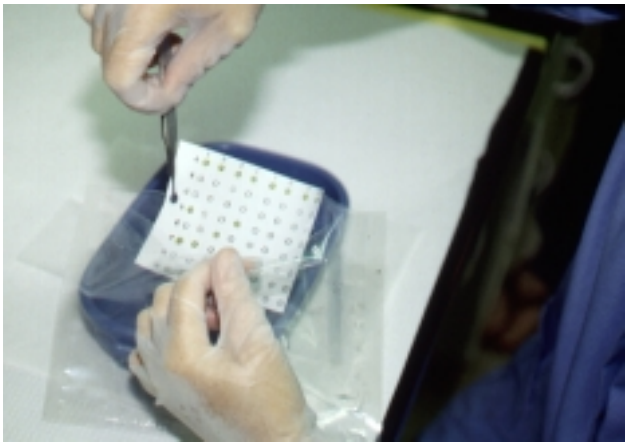


Figure 5. Incubate the NCM with the appropriate antibody in a seal-a-meal bag by first placing the NCM in the bag as shown in this illustration

Figure 6. After adding antibody to the seal-a-meal bag carefully smooth any air bubbles out of the bag fore sealing.

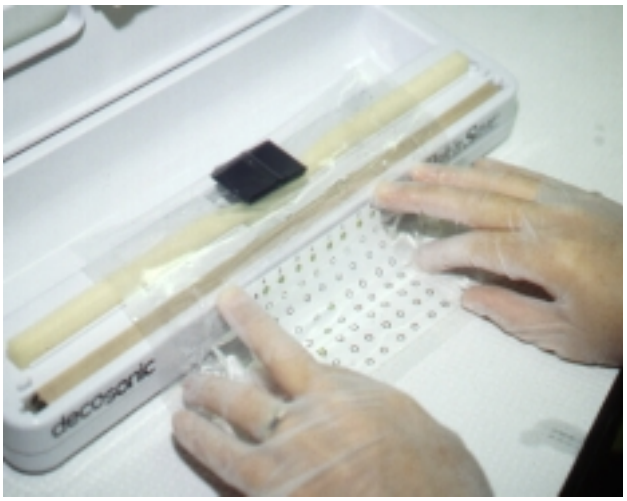


Figure 7. When the seal-a-meal bag is free of air bubbles seal the open end and begin the incubation period.

side of the sealer on top of a paper towel and force the bubble out gently, then seal the hole with tape.

If you need to process many samples, you can stack up to 4 NCM separated by plastic or nylon mesh in a seal-a-meal bag with 20-25 ml of diluted antibody. Nylon mesh sheets must be placed between each NCM to ensure that the membranes are well separated and in contact with the antibody. Results are most consistent if this incubation step is done on a shaker.

4. Following incubation, wash NCM with PBST (see Recipes in Appendix 1). First hold up each NCM with forceps and wash both sides using a wash bottle containing PBST. Then fully immerse in a clean container with PBST (100 ml/NCM) and wash for 10 minutes by placing container on a shaker. Empty wash liquid and replace with fresh PBST. Repeat this process for a total of 3 washes.

Blocking your NCM

What is blocking and why do we need to use a blocker? There are many sites on your NCM to which proteins may adhere nonspecifically. We want to remove as many of these as possible to avoid nonspecific reactions that may confuse evaluation of our samples. To overcome this problem we block these nonspecific sites with a protein that will not react with any of our antibodies. In this assay we use low fat powdered milk dissolved in PBST.

Step 5. Incubate in a solution of 5% low fat powdered milk in PBST (wt:vol) for 1.5-2 hours in a covered container at 32 degrees C. If NCM are placed in separate containers, agitation is unnecessary. If NCMs are layered, the container should be placed on a shaker. Incubation may be done at room temperature (24-27 degrees C, 75 - 81 degrees F) if an incubator is unavailable; however, blocking may not be as satisfactory.

Step 6. Pick up each NCM paper separately (don't forget to use your forceps and wear your gloves to do this) and wash with distilled water using a wash bottle.

Step 7. Place gently on clean, dry paper towels and allow to dry for at least 15 minutes prior to applying potential antigens (from your sample). Once dry, NCM that have been coated and blocked can be held in sealed plastic containers over a desiccant for up to one month (or longer) at room temperature.

Preparing Samples and Blotting Antigen

Collecting, Storing and Blotting Samples

Step 8. ***Collect the right plant tissues for blotting.*** Samples may be collected in zip lock bags or plastic sample bags. Best results will be achieved if you collect the youngest leaves showing symptoms. Figure 8 shows a zucchini plant infected with zucchini yellow mosaic virus (ZYMV). Symptoms of papaya ringspot virus the watermelon isolate (formerly watermelon mosaic virus 1) will be similar to this. The other viruses in cucurbits generally cause a mild mosaic difficult to illustrate here. Figure 9 shows how zucchini from a ZYMV infected plant can be expected to look. Samples may be stored as intact leaves in the refrigerator for 7-10 days. Once you grind or crush leaves they must be blotted IMMEDIATELY. Crushing can be done by removing air from zip lock bags and crushing the bag in your hand until the leaf is macerated releasing sap into the bag (Figure 10). Dilution in buffer or water is unnecessary when using this method although a small amount of distilled water could be added to facilitate crushing. Alternatively, samples may be ground in extraction buffer with a mortar and pestle as shown in Figure 11. You can then dip the cotton end of a Q-Tip into the sap and blot it gently onto the appropriate place on the immunoblot (Figure 12).

Figure 8.Typical foliar symptoms of zucchini yellow mosaic virus (ZYMV) infecting zucchini squash.

Figure 9.Typical symptoms of ZYMV on zucchini squash. The squash on the far right is healthy, while the four other squash show the varying levels of severity you may encounter in the field.

Figure 10. Samples may be prepared for blotting by crushing in a zip lock bag as shown here.

Figure 11. Samples may also be prepared for blotting by grinding in extraction buffer with a mortar and pestle.

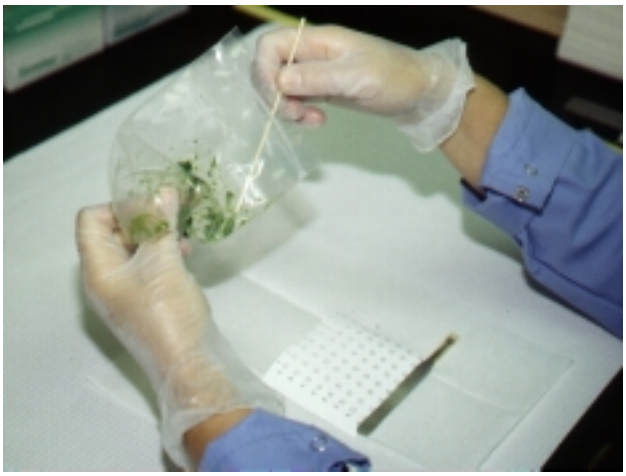


Figure 12. Dip the cotton end of a Q-Tip into the sap and blot it gently onto the appropriate place on the NCM. Remember the NCM is pressure sensitive, so do not rub the sample onto the membrane.

Step 9. ***Controls are critical to evaluation!*** Be sure to put positive (known infected), negative (known healthy), and buffer controls on every NCM. Without these controls, you will not be able to **confidently evaluate the outcome of the procedure (**see **evaluation of results** ahead**).**

Step 10. ***Record keeping is important!*** KEEP A LIST OF WHERE YOU PLACED YOUR SAMPLE ON THE NCM FOR FUTURE REFERENCE. We suggest the use of a template (see Appendix 3) in conjunction with sample numbers that allow you to maintain detailed records about each sample. You should have records of when and where you collected the sample, the plant species and the status of the plant from which you collected the sample.

Step 11. After blotting samples onto the NCM, allow the NCM to air dry. When dry, you may process immediately or store in a sealed tupperware container over desiccant. Samples may be stored this way for 2-3 weeks or even a month. It is important to check the dessicant periodically and replace it if it has absorbed too much moisture (it will change color, usually from blue to pink). If the NCM is not kept dry it may be subject to mold and this will influence the results you achieve when you process it.

Processing Immunoblots

Incubation in Conjugated Antibody

1. ***What is conjugated antibody?*** In this assay we will be using antibodies specific to the viruses for which we are testing that have been conjugated or linked to an enzyme called alkaline phosphatase. Later in our procedure, we will react our immunoblots with a substrate for this enzyme. Wherever the antibody adhered to the NCM (hopefully only where the appropriate virus has adhered as well), the enzyme will be present. A chemical reaction occurs between the enzyme and the substrate causing a color change. This color change is the signal that the virus was present in a sample.

2. ***Preparing your conjugate.*** The conjugated antibody is usually very sensitive and specific. We must dilute it to a concentration that will give us the desired reaction, but not be so strong that it causes nonspecific reactions. In addition, to ensure we have removed possible reactions to healthy plant proteins, we preabsorb the antibody with a preparation of healthy plant tissue.

Step 12. ***Preabsorbing your conjugate.*** Preabsorbing your conjugate is advised to reduce possible nonspecific reactions. First, prepare preabsorbent (see Recipes in Appendix 1). Take a 2 ml aliquot of preabsorbent and to this add the number of ml of conjugated antibody required for your final dilution. Mix well and allow to rest at room temperature for 15 minutes. Then bring the solution up to the final volume required with conjugate buffer. Be sure to mix well. Volume will depend on how many NCM you are processing and dilution will depend upon the antibody. One NCM in a seal-a-meal bag requires 5 ml of diluted conjugated antibody, while up to four NCM in one seal-a-meal bag requires 20 ml of diluted conjugated antibody.

Step 13. ***Rehydrating your NCM.*** Rehydrate NCM by immersing it in distilled water as described earlier. (Or if you wish, wash 3 times as previously described with PBST. We do not find this option to be worth the additional time it requires.)

Step 14. ***Incubation.*** Place the NCM and preabsorbed, diluted conjugated antibody solution in a seal-a-meal bag, remove air and seal (or put in a covered container). If processing many samples, up to 4 NCM

may be stacked with a piece of nylon mesh between each. Place on shaker at room temperature and incubate over night or for 3-6 hours at 32 degrees C (90 degrees F).

Step 15. ***Washing the NCM.*** After incubation, rinse NCM with PBST using a wash bottle, then wash in 3 changes of PBST for 10 minutes each. Washes should be done on a shaker.

Reacting the NCM with the Enzyme Substrate

Step 16. During the last wash (step d in previous section), mix NBT substrate solution (see Recipes in Appendix 3). This solution is light sensitive and deteriorates after 15 minutes, so mix and use immediately.

Step 17. Place NCM in NBT substrate solution. Incubate in a covered container on shaker for approximately 1 hour at room temperature. This time is dependent on the rate at which the reaction occurs. Where the enzyme is present (on samples containing your antigen) you will see a blue to purple color change. Monitor the reaction and stop it when you consider it complete.

Step 18. When reaction is complete, remove NCM and rinse with distilled water.

Step 19. Soak in 2% hypochlorite (vol:vol in water) (hypochlorite is the active ingredient in household bleach, if you use bleach, make a 50% solution in water) for 10 minutes in a covered container on shaker at room temperature. This step removes the green coloration from chlorophyll in the plant tissues. Monitor the progress of this step and stop it before 10 minutes if the chlorox is removing the blue color from the enzyme-substrate reaction.

Step 20. Rinse with distilled water. Place in 3 changes of distilled water, 10 minutes per change. Use a covered container placed on shaker. Be sure to wash adequately. If you do not the chlorox will continue to bleach the NCM and all the color will eventually fade from the NCM. If you wash well and store your NCM in a dry, dark place, the colors should remain for many months.

Step 21. Remove NCM and allow to air dry. You are ready to evaluate the status of your samples.

Evaluation of Results

Like all serological assays, you evaluate the results of immunoblotting relative to the controls you placed on the NCM. Look at your known infected control to see what kind of reaction is present and compare this reaction to that present for your healthy and buffer controls. If your procedure was successful, the infected control, known to be infected with the virus for which you are testing, will turn blue. The more virus present, the darker the color. Optimally, the blotted areas for healthy and buffer controls should be white having had no reaction. Sometimes a slight green or yellow color will be present from the plant material if you did not adequately wash with the chlorox. This is fine as long as you can distinguish between these colors and the blue of a true positive reaction.

Now evaluate each of your samples relative to your controls. Sometimes, if a sample is weakly positive, just the edge of the blotted area turns blue. It is also advisable to check both sides of your NCM when evaluating samples. The antigen sometimes soaks through the paper and the reaction is best seen on the backside of the NCM. Unlike ELISA, sample reactions can't easily be quantified with a reader (unless you have access to a densitometer which makes analysis of the intensity of the color changes possible). Thus, you must make your own system for recording the intensity of the color changes for each sample. We generally use the following system:

a sample with no reaction =	-
a sample with a very slight reaction =	+/-
a sample with a weak, but definite reaction =	+
a sample with a strong, definite reaction =	++

If you prepare NCM and ship them elsewhere for samples to be collected and blotted, we recommend that you place presample and postsample controls on the NCM. This will allow you to evaluate any degradation the NCM may have undergone during shipping. See Figure 13 for an example of a completed NCM survey. You may have results which are ambiguous at times. It is critical that you reexamine these plants if possible or sample again from that particular area.

Figure 13. Final evaluation of samples. Processed NCM are on the left and the sample template to be used for recording sample placement and results is on the right.

Appendix 1 Recipes for Immunoblotting Buffers

PBS	8.0 g NaCl 0.2 g KH_2PO_4 1.15 g Na_2HPO_4 0.2 KCl made up to 1L in distilled water adjust to pH 7.4 with HCl or NaOH
PBS	0.5 ml Tween 20 + 1L PBS (readjust pH to 7.4)
PBST + 5% milk	50 g powdered non-fat milk + 1L PBST
Coating buffer	1.59 g Na_2CO_3 2.93 g $NaHCO_3$ made up to 1L in distilled water adjust pH 9.6 with HCL or NaOH
Extraction buffer	20 g PVP-40 + 1L PBST (readjust to pH 7.4)
Pre-absorbant	1g healthy plant tissue + 5 ml conjugate buffer. Grind, filter (store and freeze in 1 ml aliquots for use as needed)
Conjugate buffer	2% PVP-40 0.2% Ovalbumin 0.02% NaN_3 (optional for storage) made up to 1L in PBST adjust to pH 7.4 with HCL or NaOH Be sure to stir well so PVP-40 is fully dissolved.
Tris Buffer 0.2M + Mg Cl_2 4mM	24.2 g tris 0.819 $MgCl_2$-$6H_2O$ made up to 1L in distilled water adjust to pH 9.5 with NaOH
BCIP	10.0 ml CH_3OH 5.0 ml Acetone 60.0 mg BCIP (or 3 tablets) store aliquoted at -20°C for months

NBT	5 mg NBT (use tablet form if possible) 0.75 ml BCIP sol to 50 ml Tris buffer MAKE FRESH — DO NOT STORE!

Alternative Process for Substrate (Expensive, for Small Volumes Only)

NBT/BCIP	1 tablet + 10 ml of dH_2O
Substrate tablets	dissolve completely (make fresh)
Sigma #B5655	if not available, substitute with Tris buffer + $MgCl_2$, BCIP, NBT

Appendix 2 Equipment and Supplies Needed for Immunoblotting

The following list will give you an idea what equipment and supplies you need to conduct immunoblotting assays at your home institution. This list does not include common laboratory supplies such as glassware, shaker, scissors, etc. We have provided you the names of the companies from which we normally purchase our supplies. You may identify other companies that also carry these items. To help you when you initially order supplies we have also supplied current catalogue numbers (current as of December 1992). Naturally, companies often change these numbers, so you need to obtain catalogues and make sure you are ordering the correct items.

EQUIPMENT:

1. Refrigerator
2. Shaker or gyrator
3. Incubator
4. Stir plate and stir bars
5. pH Meter
6. Sealer

Supplies:

To order in US:

1. Fisher Scientific,
   711 Forbes Ave.,
   Pittsburgh, PA 15219-4785.
   PH: (412) 562-8300
   FAX: 1-800-926-1166
   Web page: www.fisher1.com.sg/

To order in Asia and Pacific:

Fisher Scientific,
No. 101 Thomson Road,
16-05 United Square,
Singapore 1130
PH: 65-250-9766
FAX: 65-253-2286

MSI Nitrocellulose Transfer Membrane (30cm x 3m roll) ..... Cat #EO4HYOOO1O
TRIS (THAM) Buffer (500gm) ..... CAT #T370-500
Seal-a-meal Bags (6 1/2 x 8") 2mils ..... CAT #01-812-16
Eppendorf Pipetter(1-10ml) ..... CAT #21-381-200
Pipette tips (1-250ml) ..... CAT #21-381-10A
Pipette tips (0.5-10ml) ..... CAT #21-197-2E
Gloves (large) ..... CAT #11-394-36C
Gloves (medium) ..... CAT #11-394-36B
Gloves (small) ..... CAT #11-394-36A
Membrane Forceps (41/2") ..... CAT #09-753-50
Black Permanent ink pens (fine) ..... CAT #13-382-50
Black Permanent ink pens (broad) ..... CAT #13-382-51
PBS-NaCl ..... CAT #S271-500
- KH_2PO_4 ..... CAT #P382-500
- Na_2HP0_4 anhydrous ..... CAT #S374-500
- KCl ..... CAT #P217-500

PBST-Tween-20 ..... CAT #BP337-100
Coating Buffer-Na_2C0_3 ..... CAT #S263-500
-$NaHCO_3$ ..... CAT #S233-500
MgC12 ..... CAT #M33-500
Acetone (IL) ..... CAT #A18-500
Methanol (IL) ..... CAT #A412-500

2. Sigma Chemical CO.,
P.O. Box 14508
ST. Louis, MO 63178-9916.
PH: (314) 771-5750 call collect
FAX: (314) 771-5757.
Web page: www.sigma-aldrich.com

NBT Tablets (25) ..... CAT #N-5514
BCIP Tablets (25) ..... CAT #B-0274
Ovalbumin (10gm) ..... CAT #A-5378
PVP-40 (500gm) ..... CAT #PVP-40
Substrate Tablets ..... CAT #B5655

3. Schwaab Inc.,
11415 W. Burleigh Street
P.O. Box 26069,
Milwaukee, Wisconsin 53226-0069.
PH: (414) 771-4150
FAX: (414) 771-7165.
Web page: www.schwaab.com

Black pre-inked stamp.

4. Rainin Instrument Co.,
5400 Hollis Street.,
Emeryville, CA 94608
PH: (510) 654-9142
FAX: (510) 652- 8876
Web page: www.rainin.com

Pipetman (20-200ul)

Appendix 3 Sample Record Template

Test No.: _____Date: _____
Coating--Ab--ID: _____ Concn.: _____ Time: _____ Temp:_____
Antigen Prep: _____ Time: _____ Temp:_____
Ab Conjugate 10: _____ Concn.: _____ Time: _____ Temp:_____
Substrate: Type: _____ Concn.: _____ Time: _____ Temp:_____

	1	2	3	4	5	6	7	8	9	10
A	Z	H	P	B						
B										
C										
D										
E										
F										
G										
H										
I	Z	H	P	B						

	1	2	3	4	5	6	7	8	9	10
A	Z	H	P	B						
B										
C										
D										
E										
F										
G										
H										
I	Z	H	P	B						