

**NPDN Training – May 14, 2007**

**Legume Virus IPM-PIPE  
Laboratory Assay Training**

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# Legume Virus IPM-PIPE

## ▶ Purpose

- To test for selected viruses in common bean and soybean
- To increase preparedness of NPDN labs for virus assays by using a high throughput, economical method

## ▶ Outputs

- Useful and relevant information for RMA – what viruses pose the most significant risks
- A linkage of virus incidence with vector emergence
- A new application of a method for wide-scale survey and detection of viruses

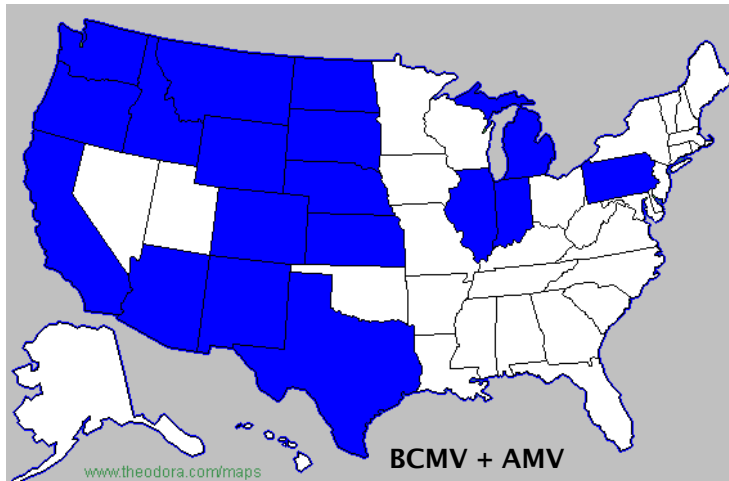
# The Assay Method

- ▶ The tissue blot immunoassay (TBIA) system was selected to be developed as a high-throughput, membrane-based, virus kit-based assay for the Legume IPM- PIPE
- ▶ Development and scale-up of TBIA was funded by a CSREES Critical Issues grant to Sue Tolin (Virginia Tech) and Chet Sutula (Agdia, Inc.)
  - TBIA for 2007 is solely for the Legume Virus IPM-PIPE, and is not for commercial sale
  - A Pilot Project for 2007 – feedback and comments are welcome

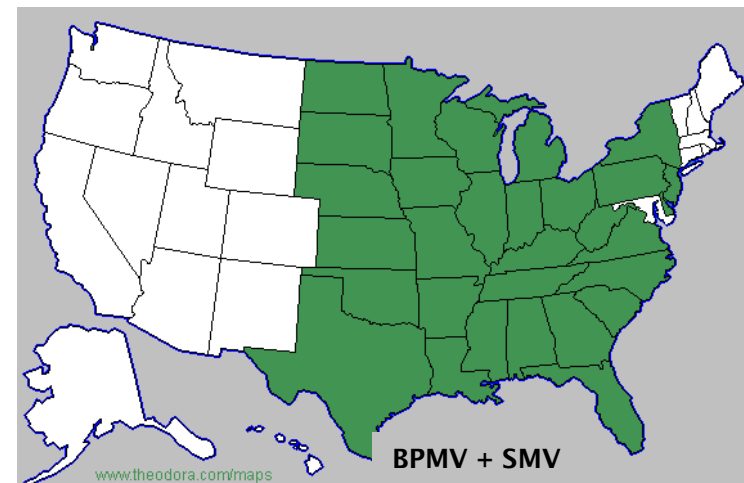
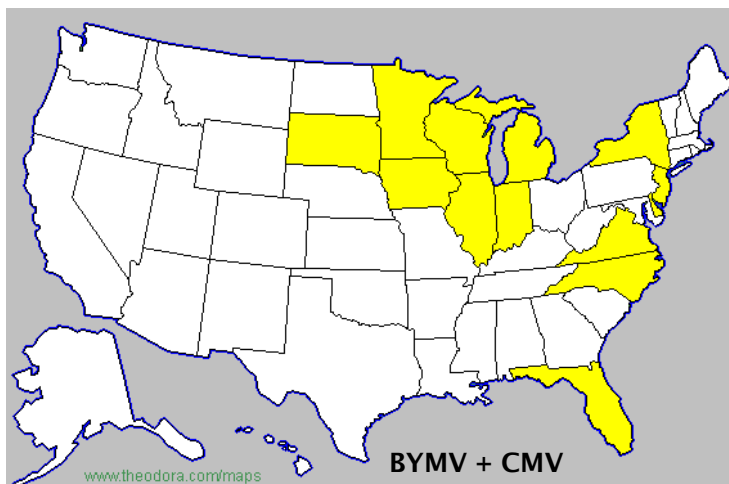
# TBIA for Specific Virus Detection

- ▶ Virologists assessed known viruses in bean and soybean and selected priority viruses
- ▶ TBIA Test Cards prepared for the IPM-PIPE Pilot Project for 2007 are:
  - BCMV + AMV (for bean and other food legumes)  
[*Bean common mosaic virus* and *Alfalfa mosaic virus*]
  - BYMV + CMV (for bean and other food legumes)  
[*Bean yellow mosaic virus* and *Cucumber mosaic virus*]  
[*Clover yellow vein virus* may also be detected by BYMV Ab]
  - BPMV + SMV (for soybean)  
[*Bean pod mottle virus* and *Soybean mosaic virus*]
- ▶ Participating NPDN labs receive a Soybean card and a Bean card for each plot for each testing time.

# Virus Testing in Sentinel Plot Sites



Legume plots vary by state in number and type. States chose one card; some purchased a second card.



2 Soybean Rust Sites per State

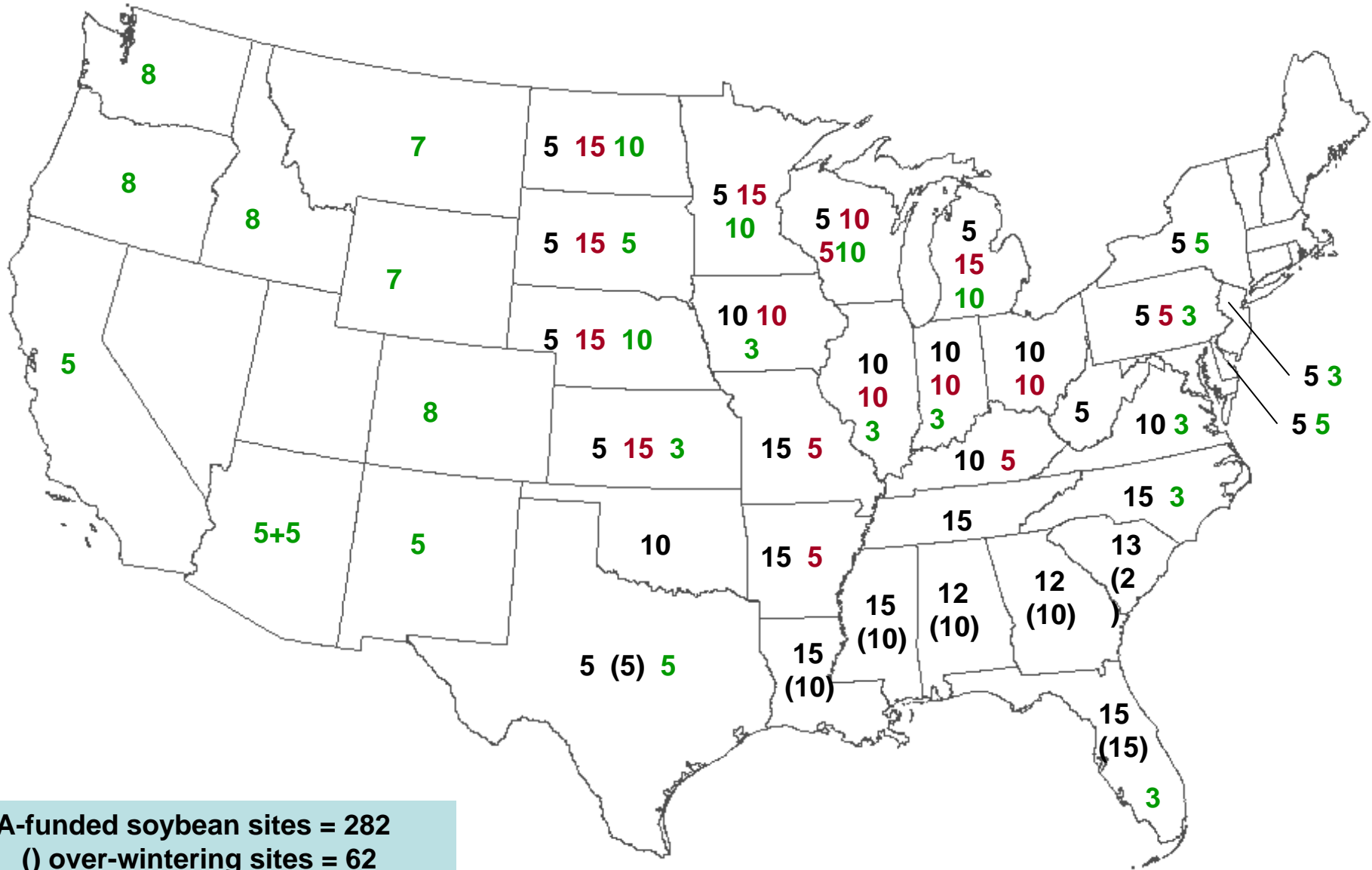
# Nucleic Acid-Based Assay for BCTV

- *Beet curly top virus* (BCTV)
  - Genus *Curtovirus*; Family *Geminiviridae*
- ▶ BCTV is prevalent in bean in the west but cannot be detected by a serological assay.
- ▶ Western states are currently developing a protocol for the PIPE for BCTV detection by NPDN labs.
  - That protocol is not included in this training session
- ▶ BCTV DNA detection will be from a leaf pressed onto nylon membranes, using the same format as for TBIA.
- ▶ Participating states will receive one BCTV card per site and await further instructions.

# Sampling Protocol for Viruses

- ▶ Sentinel Plots or commercial legume fields  
(Dry bean, snap bean, other legume, soybean)
  - Two to ten bean/legume plots in relation to acreage of RMA-insured bean plots in each state
  - Two soybean plots per state in the sentinel plot system
- Two sampling times per season
  - Late vegetative to early flowering stage
    - 4-6 weeks post planting
  - Early to mid-pod growth stage
    - 8-10 weeks post planting

# 2007 SENTINEL SITE NETWORK



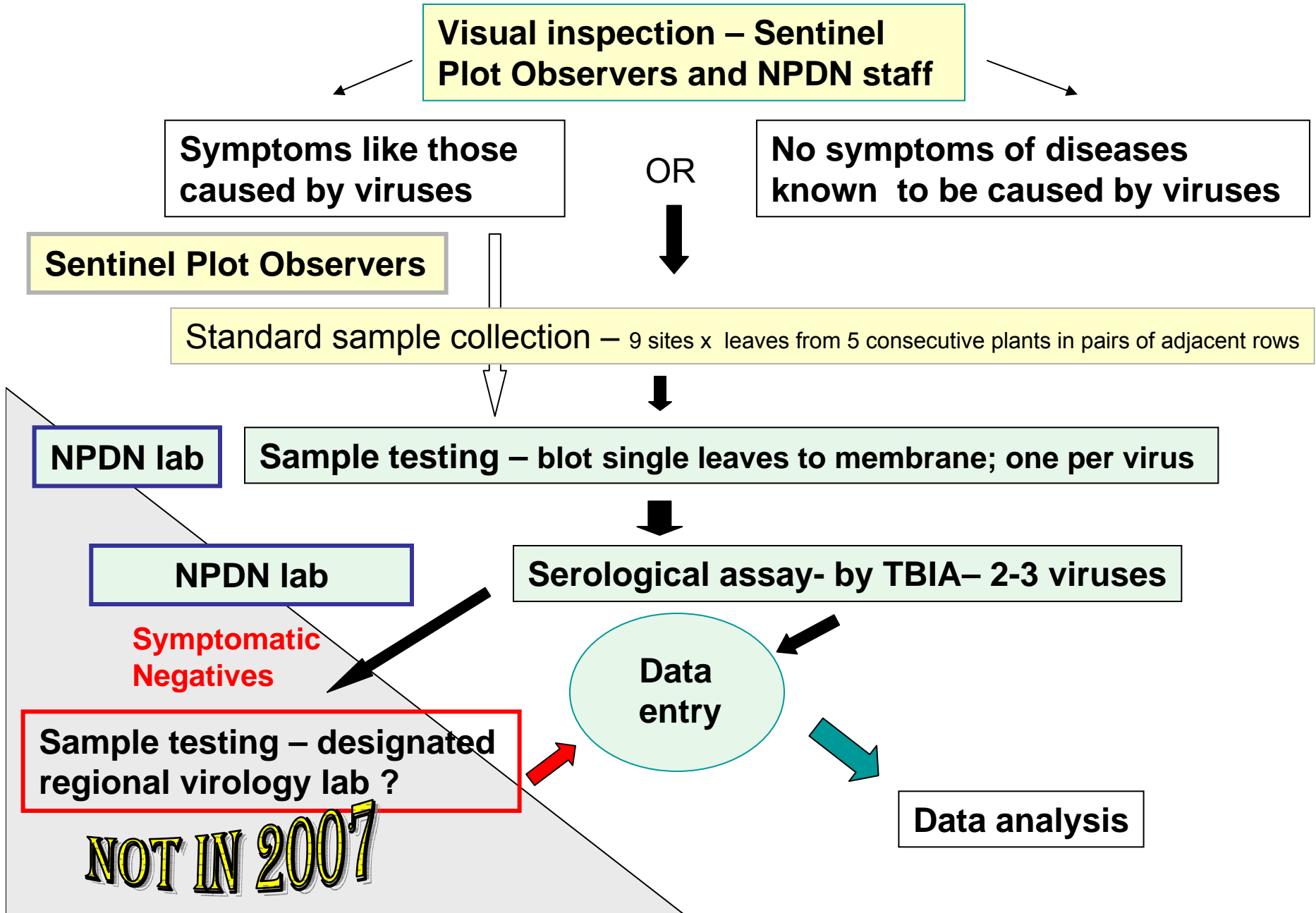
RMA-funded soybean sites = 282  
 ( ) over-wintering sites = 62  
 Checkoff-funded soybean sites= 160  
 RMA- funded legumes sites=161

2 soybean sentinel sites in each state will be used in the legume PIPE program and sampled twice and assayed for BPMV and SMV

# Sampling Protocol for Viruses

- ▶ Standard Sentinel Plot or commercial legume field
  - Walk between rows; stop at 5-10 paces; collect leaves from 5 adjacent plants; put in single bag. Repeat at 9 sites per plot between 2-3 paired rows
    - Collect from upper canopy; include a fully expanded leaf
  - Transport 45-plant sample (9 bags of 5 plants each) to NPDPN lab for TBIA assay for viruses
- ▶ Optional: collect plants with virus-like symptoms
- ▶ Optional: collect aphids that may be potential vectors

# Sample management/movement protocols



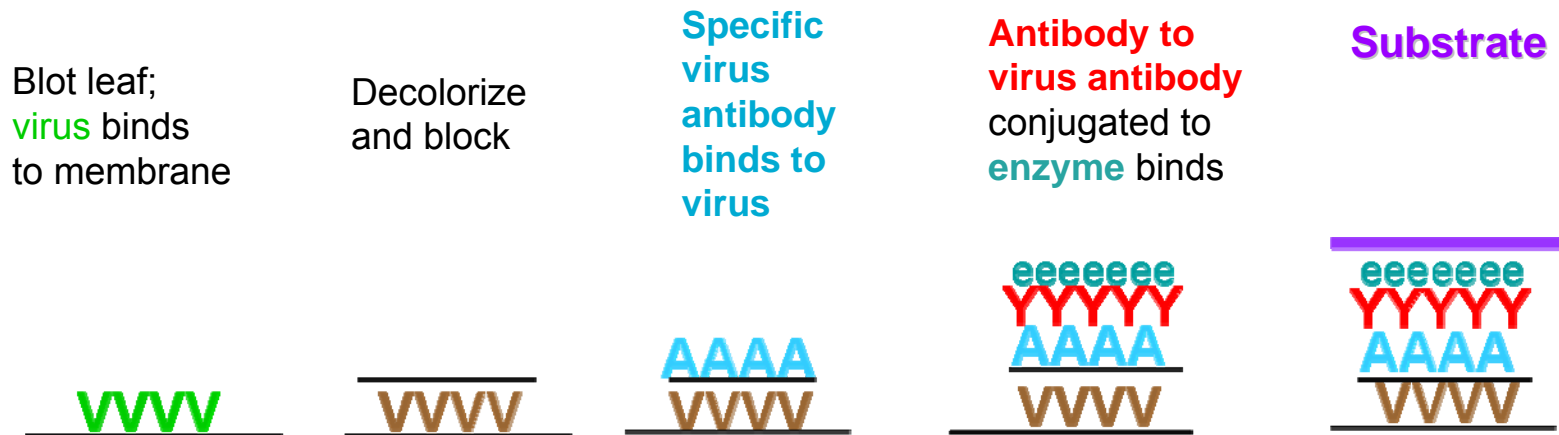
# Optional Research Protocol

- ▶ Record additional information about samples
  - Note symptoms
  - Report if symptomatic leaves test negative
- ▶ Attempt to identify virus
- ▶ Populate an Excel spreadsheet with data
  - May lead to publication on results of virus assays
- ▶ Sign up with Legume IPM-PIPE coordinator
  - Eastern - Marie.Langham@SDSTATE.EDU
  - Western - Howard.Schwartz@ColoState.EDU

# The TBIA Assay

An indirect ELISA in which virus is trapped directly from plant tissue onto a nitrocellulose membrane.

Detection is by enzyme-conjugated antibodies bound to specific virus antibodies, and NBT/BCIP substrate, similar to a Western blot.



# Virus Detection by TBIA

**Enzyme:** alkaline phosphatase conjugated secondary antibody (EC)

**Substrate:** BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) and NBT (NitroBlue Tetrazolium). Phosphate groups on BCIP are hydrolyzed and NBT is reduced to form an insoluble, **purple precipitate** of NBT formazan

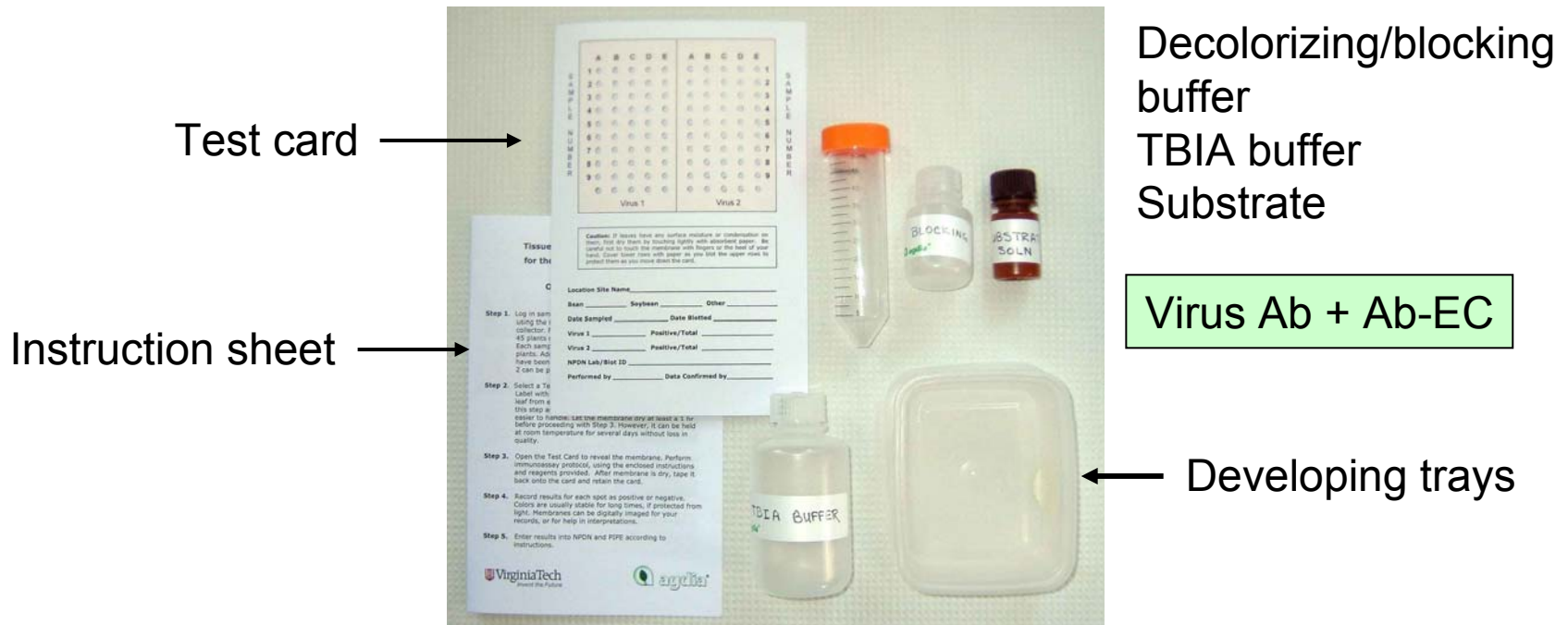
- ▶ A Qualitative Test, scored as positive or negative for virus
- ▶ Sensitivity is comparable to DAS-ELISA
  - Precipitate can be seen more clearly if viewed in bright through a good hand lens or a stereoscope.

# Top 10 Advantages of TBIA

1. No need to grind or extract sap from leaves
2. Can blot many spots from a leaf, and test for many viruses
3. Blotted membrane can be held for better time management and scheduling of assay completion
4. Several membranes of 50 samples can be processed together
5. Minimal equipment requirements - shaker, timer, forceps
6. Short developing time of approximately 3 hours
7. Purple precipitate is permanent; scoring can be deferred or revisited
8. Alpha-numeric format for easy sample tracking and data recording
9. Economical, easy to use kit with all reagents supplied
10. Ready for certain viruses in 2007

# Contents of the TBIA Kit

- ▶ TBIA assays will be conducted by using a kit assembled by Agdia®, Inc. specifically for NPDN and the IPM-PIPE.
- ▶ Contents of the kit are below:



# TBIA Kit Instruction Sheet

Front Page: Outline of Instructions

Step 1: Receive samples and log in

Step 2: Blotting leaves to membrane in Test Card

Detailed instructions on page 2

Step 3: Immunoassay processing and development

Detailed instructions on page 3

Step 4: Recording results

Step 5: Data entry

Back Page: Acknowledgements, who to contact for help.

# Additional Materials for TBIA

Graduated cylinder  
Marking pen  
Soft-leaded pencil  
Forceps, flat-tipped  
Office transparent tape  
Timer  
Razor blade or scalpel  
50 ml Centrifuge tube  
Plastic rinse container

## Not Pictured

Shaker (recommended)  
Gloves, latex or vinyl  
Paper towels  
Distilled or de-ionized water  
Refrigerator for sample and reagent storage



# Sample Handling

- ▶ Log in samples of bean, soybean, or selected legumes, using the same Location Site Name used by the collector.
- ▶ Leaves should be blotted to membranes as soon as possible. Fresh leaves are easier to handle and give better results.
- ▶ Store leaf samples in plastic bags in the cold until they can be blotted onto membranes. Do not freeze the leaves. Leaves should be turgid with no surface moisture.
- ▶ Leaves can be discarded according to standard lab protocols, as endemic pathogens are expected.

# Preparing to Blot Leaves

- ▶ Select an Agdia® Test Card for each assay.
- ▶ Label Test Card with Location Site Name, plant type, and viruses to be tested.
- ▶ Assign a unique number to the card for tracking purposes

From field collectors →

Assigned by NPDN lab performing assay →

Location Site Name VAmontgomeryST01

Bean \_\_\_\_\_ Soybean X Other \_\_\_\_\_

Date Sampled 4/30/07 Date Blotted \_\_\_\_\_

Virus 1 BPMV Positive/Total \_\_\_\_\_

Virus 2 SMV Positive/Total \_\_\_\_\_

NPDN Lab/Blot ID xxx/597

Performed by \_\_\_\_\_ Data Confirmed by \_\_\_\_\_

# Sample Sorting and Handling

- ▶ For each Location Site and sampling time, leaf samples will be collected in a standardized 9-sample transect pattern, with leaf sub-samples taken from 5 consecutive plants for a total of 45 plants sampled.
  - If bags are not labeled individually, label them 1-9.
  - If leaves are combined, process in groups of 5.
- ▶ Additional plants with virus-like symptoms that may have been collected can be designated sample 10 and blotted in row 10 of the membrane.

# Example of Soybean Leaf Samples



Group of upper trifoliate leaves from 5 plants. Select one leaflet from each plant and blot to row 1, columns A-E.



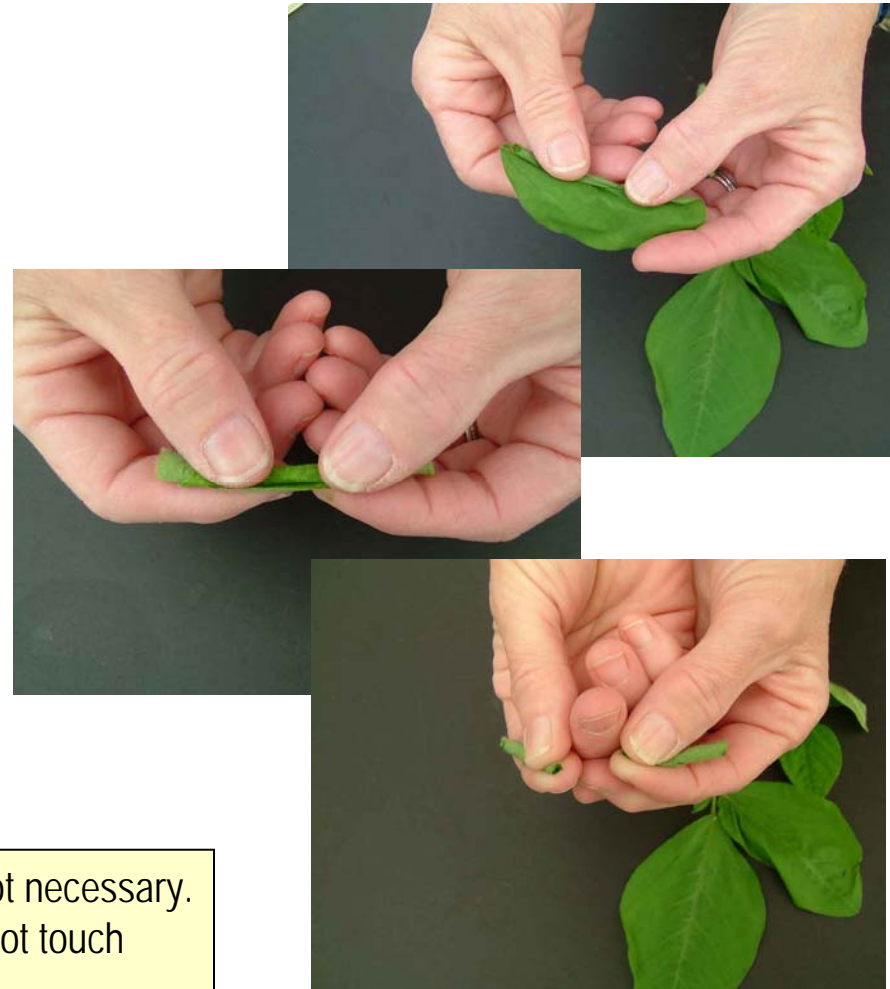
Label a remaining leaflet from each plant as A - E, to enable trace-back to single plants, if desired.

# Preparing to Blot Leaves to Membranes

- ▶ Separate the 5 plants from Sample Transect 1
- ▶ Follow instructions on the cover sheet included with the Test Card and outlined in the S.O.P.
- ▶ If participating in the optional research project:
  - Note whether virus-like symptoms are evident on any of the leaves, and record information in spreadsheet
  - Slide sets and symptom information are under development for posting on PIPE and NPDPN-associated websites

# Blotting Leaves to Membranes

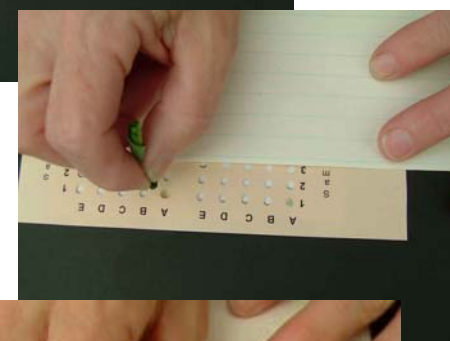
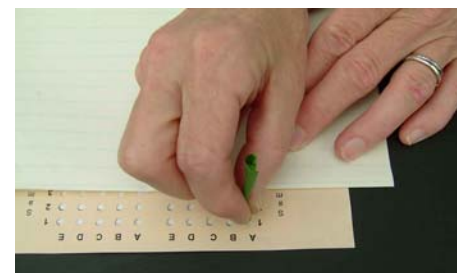
1. Select a leaflet from plant A. Starting from one side, tightly roll the leaf into a tight coil.
2. Hold the coiled leaf between thumb and forefinger of both hands, thumbs nearly touching.
3. Pinch and give a quick jerk, breaking the leaf into two pieces.



NOTE: Gloves are not necessary. Your fingers should not touch the torn leaf edge.

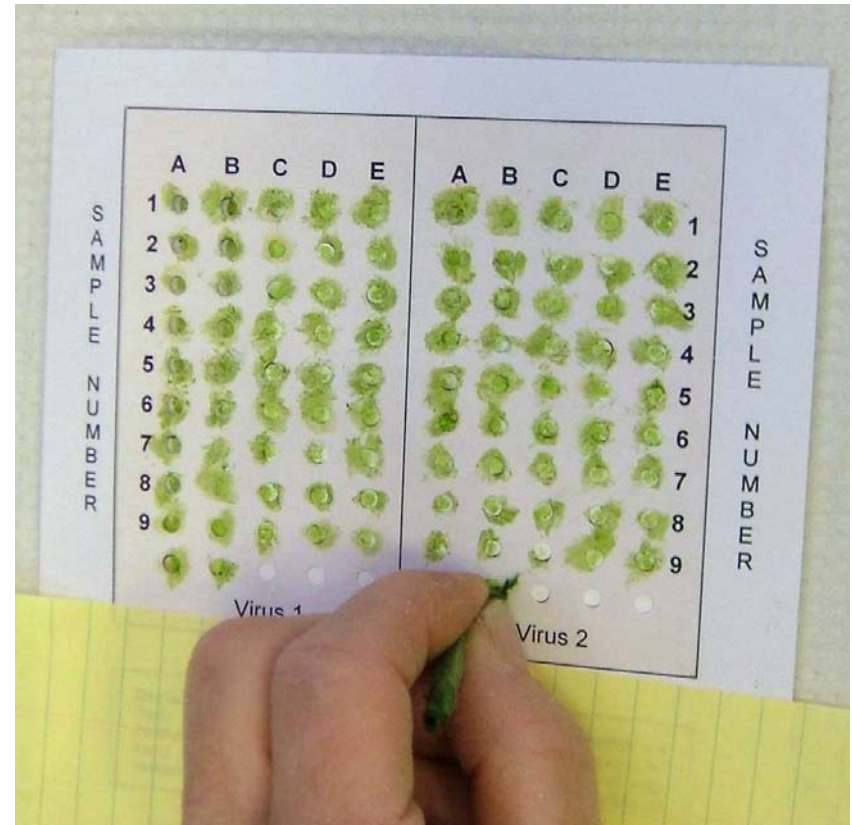
# Blotting Leaves to Membranes

1. While still holding the coil, lightly press torn edge several times (1-2 sec each) onto the membrane in the first spot (1A), until the spot is uniformly green.
2. Use a series of pressing motions over the hole in the template to soften the torn leaf edges until sap from the leaf sticks to the membrane and green color is obvious. Do not press too hard, as it damages the membrane.
3. Repeat with the same leaf in the corresponding 1A spot on the other side of the card.
4. Repeat the process for the rest of the leaves. Use row 10 for additional samples or to correct errors. Make note of these samples.



# Finished Blotted Test Card

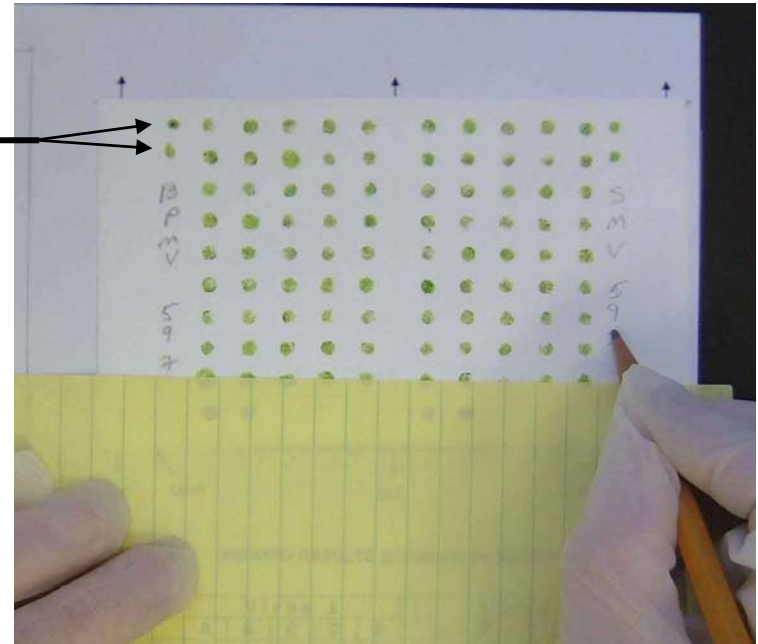
- ▶ The covering mask should be green around edges of the holes, but there should be no cross-contamination.
- ▶ Let membrane dry for at least 1 hour before developing.
- ▶ Once blotted, the membranes can be held at room temperature in a protected place for days until they can be developed.



# Labeling the Membrane

1. Open the test card to expose the membrane. Spots hidden by the mask are positive and negative controls.
2. Use a soft leaded pencil to label the left side and right sides with the name of the virus being tested on this membrane and a unique ID number (597).
3. It helps in later orientation and scoring to number rows 1, 5, and 10, and tick mark other rows on either the center and sides. Letter or tick mark the top and bottom of the columns.

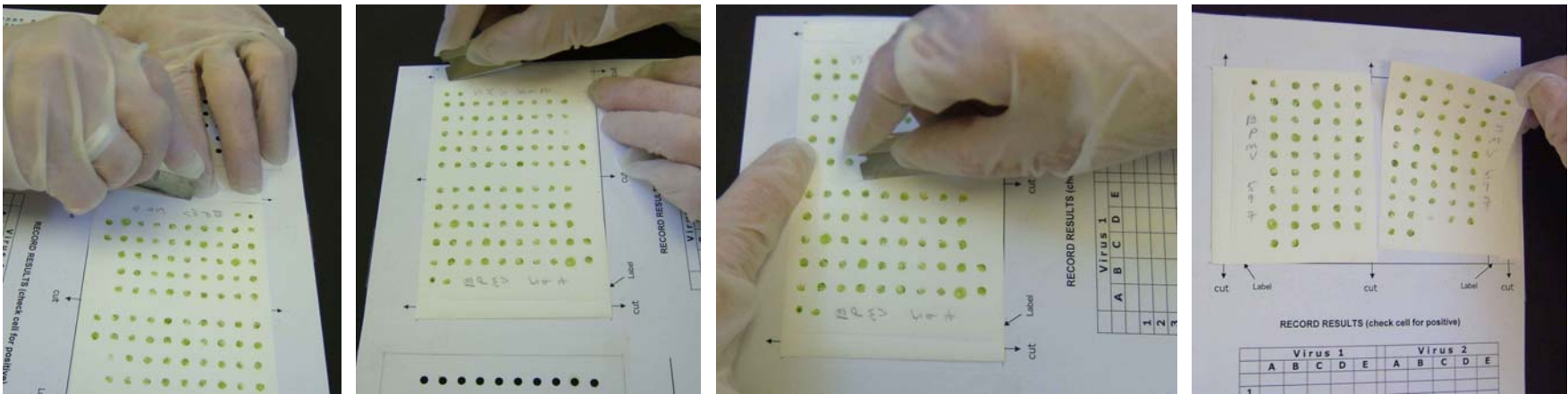
Controls



NOTE: Wear gloves to protect the membrane and prevent finger oils from contacting the nitrocellulose, which block binding of antibody to virus.

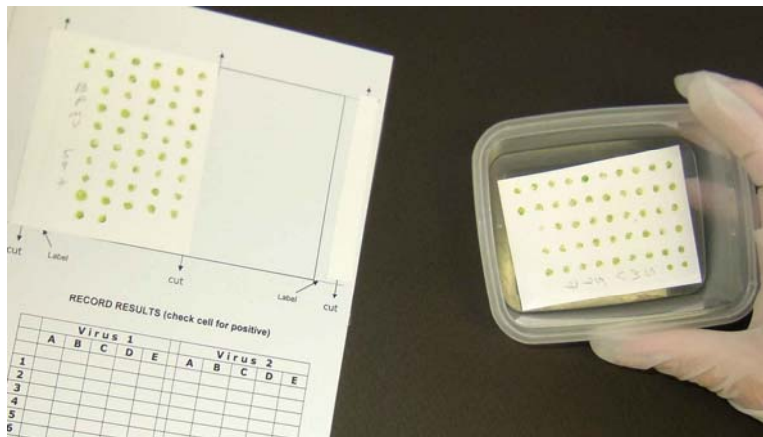
# Freeing and Dividing the Membrane

1. With a razor blade or scalpel, carefully cut the membranes from the card on each side, following **Cut** lines on the card.
2. Cut the two blocks apart with the blade or scissors.
3. Lift membrane carefully from the card.



# TBIA Protocol: # 1 – Decolorize/Block

1. Place both membranes into one developing tray.
2. Pour contents of bottle labeled “Decolor/Blocking” into one of the trays provided in the Agdia® kit.
3. Incubate for 60 min, with gentle shaking (recommended).
4. Green spots should be nearly colorless after 30 minutes. If not, touch membranes lightly with a folded Kimwipe to brush off adhering plant tissue. Continue incubation.



# Record the Development Process

## Inside of card:

Date Processed \_\_\_\_\_ Date Scored \_\_\_\_\_

Virus #1 - Left Side \_\_\_\_\_

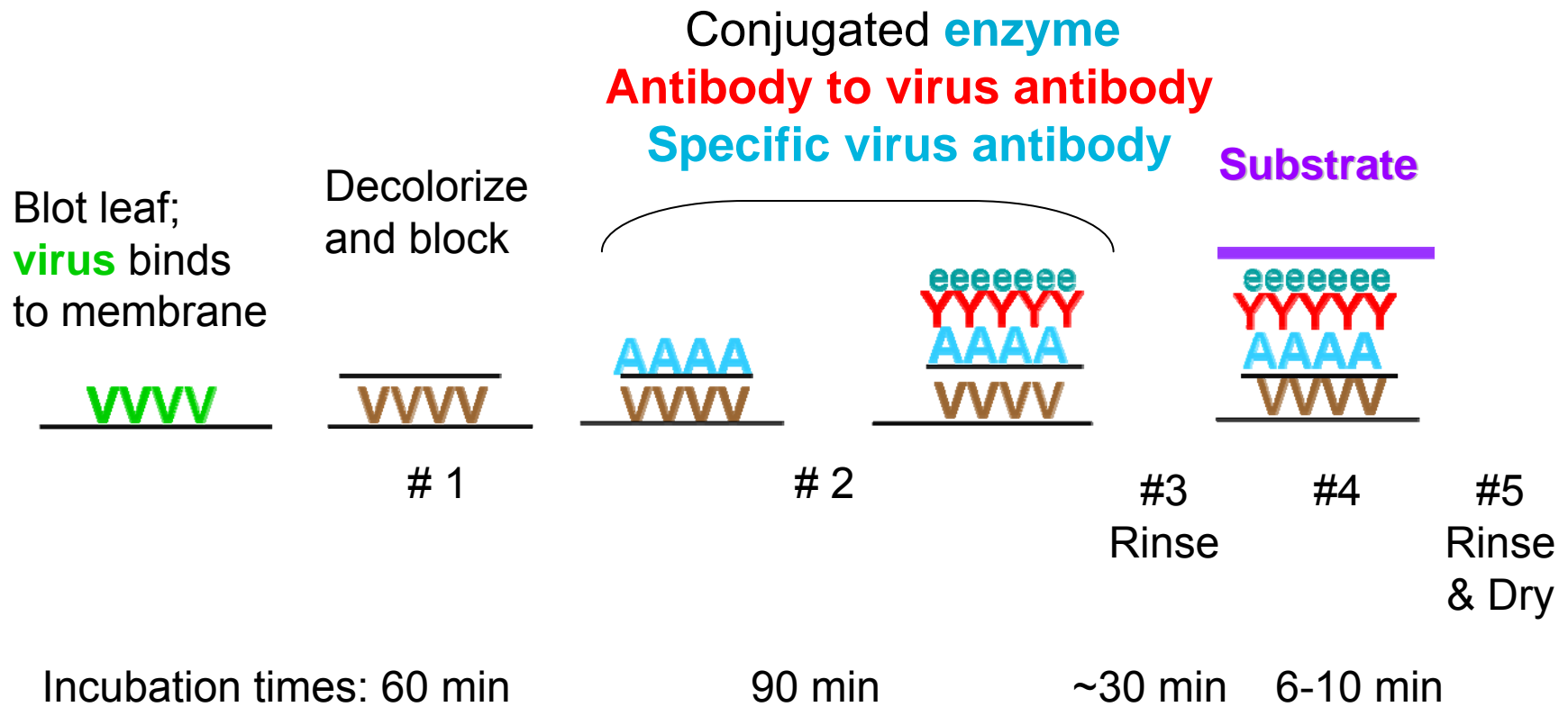
#1 Decolorize/Block	Start at: _____	End at: _____
#2 Antibody	Start at: _____	End at: _____
#3 Rinse	Start at: _____	End at: _____
#4 Substrate	Start at: _____	End at: _____

Virus #2 - Right Side \_\_\_\_\_

#1 Decolorize/Block	Start at: _____	End at: _____
#2 Antibody	Start at: _____	End at: _____
#3 Rinse	Start at: _____	End at: _____
#4 Substrate	Start at: _____	End at: _____

Notes: Any additional information, such as symptomatic plants

# Incubation Times for Development



# TBIA Protocol: Prepare for Step # 2

Near the end of blocking step, prepare antibody

1. Measure 20 ml of TBIA buffer into the second tray.
2. Add contents of the green-capped vial containing antibody to Virus 1, then add contents of one clear-capped vial of enzyme-conjugated anti-animal antibody. Label the tray Virus 1.
3. Prepare 20 ml of antibody for Virus 2 in a 50 ml Falcon-type centrifuge tube or similar container in the same way, using second vial of EC-Ab.



+  + EC/AB

NOTE: Follow specific instructions included in each the Agdia kit for antibody preparation. Details may vary for different tests.

# TBIA Protocol: # 2 Antibody Incubation

1. With forceps, lift the Virus 1 membrane from the decolor/block solution, drain, place it in Virus 1 antibody-labeled tray.
2. Pour blocking solution out of tray, holding membrane in with a different pair of forceps (touch only edges)
3. Add Virus 2 antibody solution and label tray with that virus.
4. Incubate both membranes for 90 minutes



NOTE: Using the same forceps without thorough rinsing may cause cross-contamination of antibody and false positives.



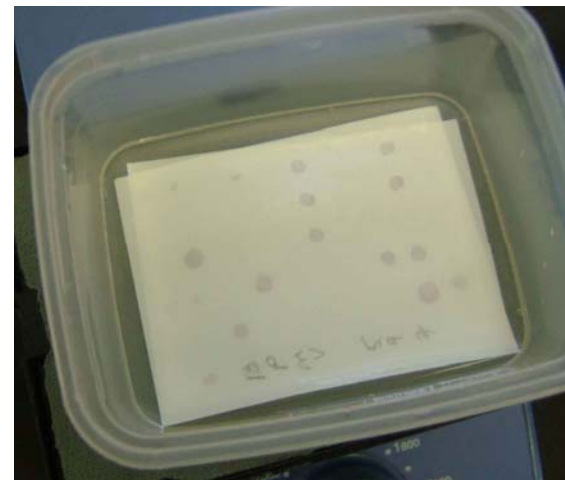
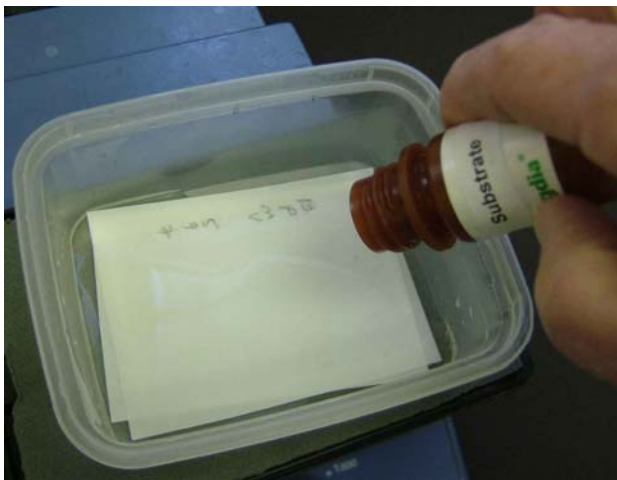
# TBIA Protocol: # 3 Rinse

1. Pour out antibody solution, one virus at a time.
2. Add 20 ml TBIA buffer; gently agitate for 10 min. Pour out.
3. Repeat twice for 5 min each. Leave membrane in final rinse until substrate is ready.
4. During the rinse, remove substrate from storage in the cold and allow to come to room temperature.



# TBIA Protocol: #4 Substrate

1. Transfer both membranes to one new container along with the contents of the bottle labeled "Substrate".
2. Agitate gently with shaker or by hand, 6-10 minutes, until purple color is evident over positive controls, and membrane and negative controls are clear.



NOTE: Membranes incubated in different antibodies can be combined in a single container at this step.

# TBIA Protocol: # 5 Final Rinse

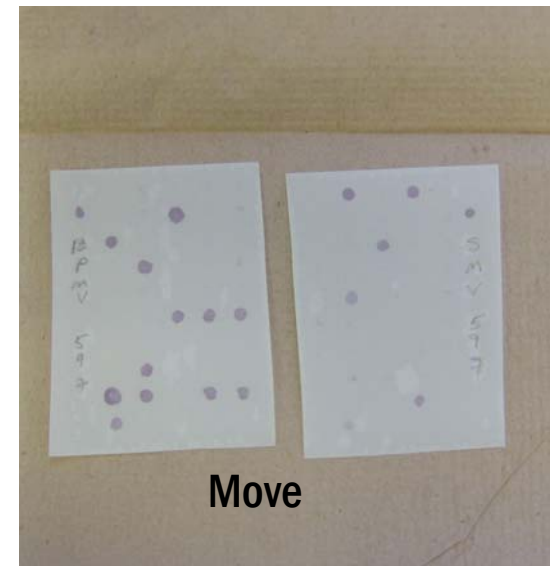
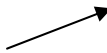
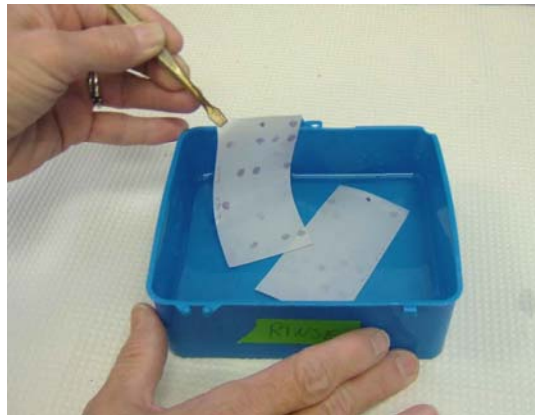
1. Move membranes to a larger container containing a generous amount of distilled water (~50 ml or more).
2. Leave for 10-20 seconds – swirl the container or the membrane for thorough rinsing.
3. Pour water out, and repeat with two additional water rinses to stop color development.



**NOTE: Incompletely rinsed membranes may turn purple over time in areas without spots.**

# TBIA Protocol: # 5 Drying

4. Lift membranes out and place on paper towels to drain.
5. Pat gently with another paper towel to remove surface water.
6. Move membranes between dry towels to dry ~ 1 hr to overnight.



# TBIA : The Developed Membrane

1. Re-tape membrane to original card, applying tape only at the edges of the membrane and not over test spots.
2. Record results in the table below the membrane.

Caution: Wear gloves to handle membrane before processing.

Date Processed 5-1-2007 Date Scored 5-2-07

Virus #1 - Left Side BPMV

#1 Decolorize/Block	Start at: <u>12:00</u>	End at: <u>1:00</u>
#2 Antibody	Start at: <u>1:00</u>	End at: <u>2:30</u>
#3 Rinse	Start at: <u>2:30</u>	End at: <u>2:32</u>
#4 Substrate	Start at: <u>2:32</u>	End at: <u>2:59</u>

10/5/5

Virus #2 - Right Side SMV

#1 Decolorize/Block	Start at: <u>12:01</u>	End at: <u>1:01</u>
#2 Antibody	Start at: <u>1:01</u>	End at: <u>2:31</u>
#3 Rinse	Start at: <u>2:31</u>	End at: <u>2:33</u>
#4 Substrate	Start at: <u>2:33</u>	End at: <u>2:58</u>

10/5/5

Notes:  
 10A - Mottled Leaves  
 10B - Leaf curl distortion

RECORD RESULTS (check cell for positive)

	Virus 1					Virus 2				
	A	B	C	D	E	A	B	C	D	E
1							+			+
2			+							
3	+								+	
4		+								
5							+			
6			+	+	+					
7										
8		+								
9	+	+		+	+					+
+										

# Counting Positives

- ▶ For each, membrane count the number of positive samples (purple spots) and the total number tested.
  - Example: Virus 1 (BPMV):
    - 12 positives (11 in rows 1-9) + (1 in row 10)
  - Total number tested:
    - 45 standard samples + 2 symptomatic (row 10)
  - Reporting:
    - 12 positive
    - 47 total tested

# Recording Results on Test Card

1. Enter the viruses tested and results
2. Retain the cards and membranes, as colors remain stable for long periods of time.

From field collectors

Assigned by NPDN lab performing assay

Location Site Name VAmontgomeryST01

Bean \_\_\_\_\_ Soybean X Other \_\_\_\_\_

Date Sampled 4/30/07 Date Blotted 5/1/07

Virus 1 BPMV Positive/Total 12/47

Virus 2 SMV Positive/Total 5/47

NPDN Lab/Blot ID xxx/597

Performed by MB Data Confirmed by ST

# Getting Started

- ▶ Receive the Test Kits
  - Open and remove the instruction sheet and test card; store at room temperature.
  - Refrigerate the remaining kit containing buffer, antibodies, and substrate.
- ▶ Coordinate with Sentinel Plot Samplers
  - Schedule times to expect to receive leaf samples based on planting dates of plots to be sampled.
- ▶ Clarify systems you will use to enter data.
- ▶ Ask questions and seek assistance, as needed.