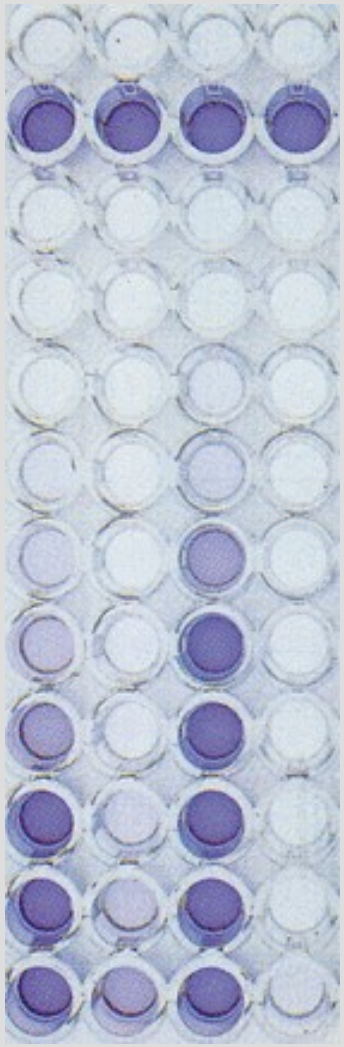


Enzyme Linked ImmunoSorbent Assay

DOUBLE ANTIBODY SANDWICH

DAS-ELISA

ELISA:

Used for the detection of proteins

Sensitive: 1-10 ng to 300 pg virus/ml can be detected

Requires small amounts of antibodies

Suitable for large scale testing of samples

Highly portable, easily stored: can be made into mass-marketed kits

Can be standardized using kits

Both qualitative and quantitative measurements are possible

Commercial Sources For Immunoreagents and Kits for Detection/Diagnosis of Plant Viruses

Neogen Europe Ltd ADGEN Phytodiagnostics
http://plant.neogeneurope.com/default.asp

NEOGEN EUROPE Ltd - ADGEN Phytodiagnostics

AgDia
http://www.agdia.com/

agdia®

BIOREBA AG
http://www.bioreba.ch/

BIOREBA

BASIC STRUCTURE of ELISA:

- **Solid support (plastic)**
- **Antibody made against the antigen (one or more)**
- **Antigen (virus)**
- **Antibody-conjugated to an enzyme**
- **A soluble enzyme substrate**

Reagents can be used in different orders

Good animation of different ELISA formats:

http://www.sumanasinc.com/webcontent/animations/content/ELISA.html

BASICS Of ELISA FOR PLANT VIRUS DETECTION:

Required:

- An antiserum with specificity for the test antigens (virus)
- Sample(s) to be assayed
- A positive control consisting of a reactive source of the virus antigen used in preparing the antiserum
- A negative control from samples from non-infected plants of the same species (cultivar)

Negative (and positive) controls allow you to interpret results, without them you can easily make mistakes in interpreting the results!

SELECTION OF PLANT TISSUE FOR TESTING:

- The plant host (age, species, growth conditions) can influence:

  1) the concentration of the antigen in the sample

  2) distribution of viral antigen within the plant
     ("not all samples are created equal")

Enzyme-linked immunosorbent assay (ELISA)

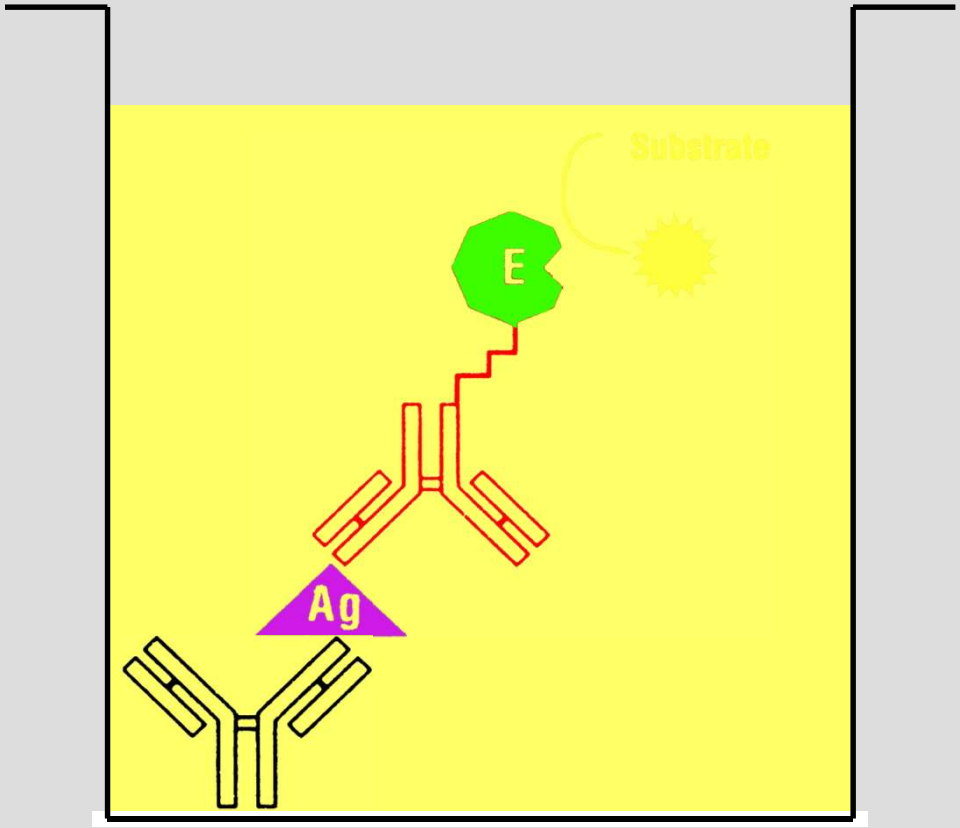
Most widely used test for the routine detection of plant viruses.
The results can easily be quantified (spectrophotometer) and the whole testing procedure can be automated.

Different Strategies:

Direct or Indirect

Antigen labeled or Antibody Labeled

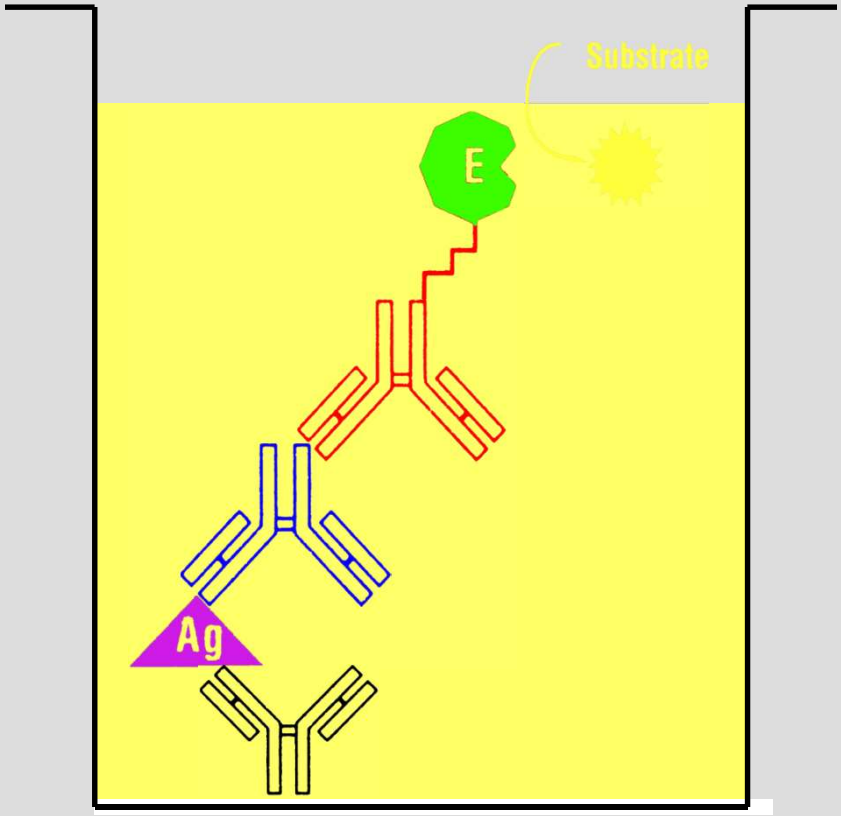
Double Antibody Sandwich ELISA



Direct,
Antibody trapping

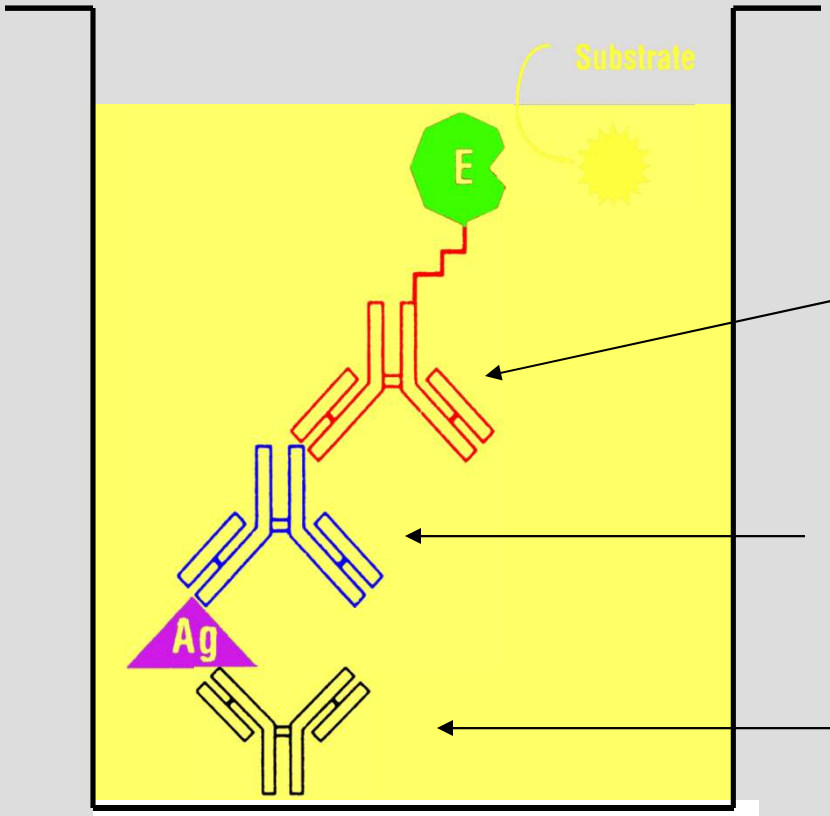
(the assay you will execute this week)

Indirect antibody-labeled ELISA
“Triple” Antibody Sandwich ELISA



ELISA

Use of monoclonal and polyclonal antibodies (Antigen trapping triple antibody ELISA)



3° Antibody monoclonal or polyclonal anti-mouse), "Goat anti-mouse"

2° Antibody (monoclonal made in mouse) "Mouse anti-virus"

1° antibody or Trapping Antibody (Polyclonal made in rabbit) "Rabbit anti-virus"

Overview of Laboratory Procedure:

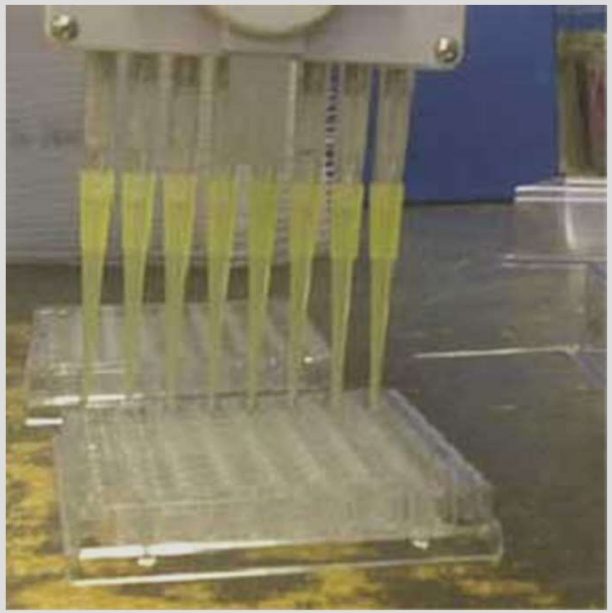
Monday:

- Plates have been pre-coated with virus-specific antibodies to allow this lab to be completed within two labs
- You will add the antigen (plants from your host range study)
- Plates will be incubated at 4^{o} C

Wednesday:

- Wash plates
- Add conjugate/secondary antibody
- Incubate for 1 hr at 37 C
- Wash plates
- Add substrate and record results with spectrophotometer
- Calculate results and discuss

Coating of the Antibody to the Plate



- The first step is to add an antibody to a specific virus to the ELISA plates. Commercial ELISA test kits with antibodies for all major viruses are available.

- The pH and ionic strength of the coating buffer have been specifically formulated to create an optimal coating environment which maximizes the adsorption efficiency of most antibodies and protein antigens onto polystyrene plate surfaces.

*** To save time, this step was done by Heather to allow us to complete the lab in 2 sessions**

Coating buffer:
15 mM Na_2CO_3,
35 mM $NaHCO_3$
pH 9.6

Adding the Antigen

Grind the plant tissue with extraction buffer (1g tissue / 10ml extraction buffer)

The extraction buffer contains:

- 20 mM Tris-HCl
- 150 mM NaCl
- 2.7 mM KCl
- 2% PVP
- pH 7.4

This homogenate is loaded into the coated plates using a P100 or P200 micropipette.

Add 100 ul per well

Use in Diagnostics:

A positive control and a negative control must be included in each ELISA plate.

The positive control is sap obtained from plants known to be infected with the target virus purchased from AgDia.

Sap from a healthy plant of the same species as the test plant is used as a negative control. Plant proteins also are antigenic and they can react with antisera that contain antibodies to the host proteins

After addition of the antigen, the plates are incubated overnight at

4 ^{o}C OR

4h at room temperature OR

37 ^{o}C for 1 hr

Washing to Remove Unbound Antigen

In this step we will remove any protein or antigen that did not bind to the coated antibody

Empty the wells of plant debris and extraction buffer by quick flip of the wrist into the bin provided

Wash plates with PBS-T 4 times; one minute each:

* Fill wells by immersion in PBS-T
* Let sit for one minute
* Empty the PBS-T into the waste bin
* Repeat 3 times
* Gently tap plate upside down on dry paper towel to remove excess buffer

PBS-T Buffer:

10 mM phosphate buffer pH 7.4,
150 mM NaCl,
0.05% Tween 20

Adding Conjugated Antisera

The anti-virus conjugate will bind to the virus captured by the coated antibody

Add the labeled second antibody (anti-virus-IgG conjugated to alkaline phosphates).

The antibody dilution to be added has been determined in preliminary experiments.

Wash to Remove Excess Conjugated Antisera

remove all the conjugated antibody that did not bind to the trapped virus

Empty the contents of the wells into the wash bin by quick flip of the wrist

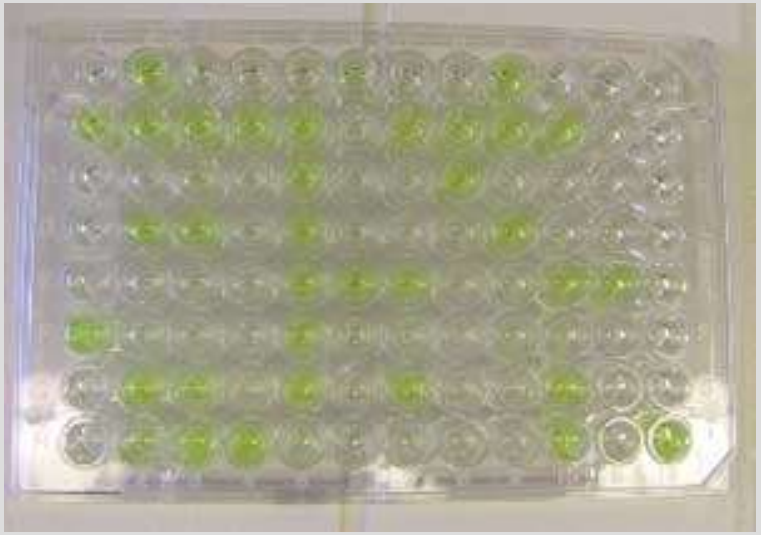
Wash plates with PBS-T 4-6 times; 1 minute each
*Immerse in PBS-T as before
*Let sit for one minute
*Empty the PBS-T
*Repeat 4-6 times
*Gently tap plate upside down on dry paper towel to remove moisture

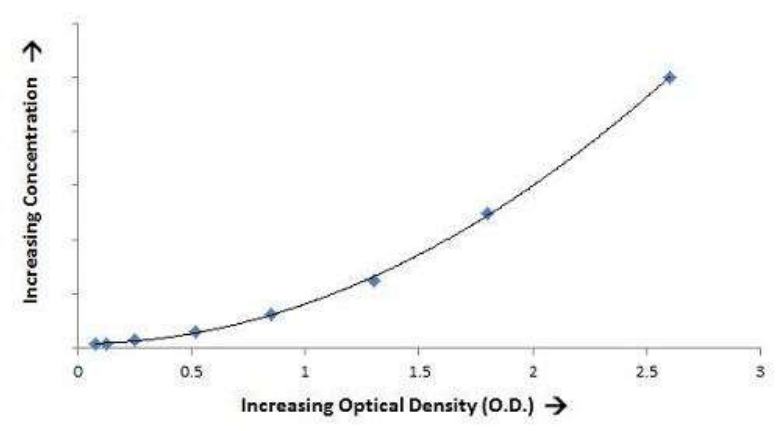
Addition of Enzyme Substrate

An alkaline phosphate substrate is added that will react with the enzyme attached to antibody. The resulting color will give an indication if virus was present in the plant sap.

Prepare pNPP substrate solution (p-nitrophenyl phosphate)
Dispense 100 µl of pNPP substrate per well (entire plate)
Incubate plates 5-15 minutes at room temperature in the dark
Stop color development by adding one drop of 1M NaOH

pNPP substrate:
1.0 mg/ml pNPP ((p-nitrophenyl phosphate)
0.2 M Tris-HCl pH 9.8 or 10% Diethanolamine
5 mM $MgCl_2$





While not strictly necessary for diagnostics, a microplate reader is used to measure the absorbance of UV light at a selected wavelength as it is passed through each well of the plate. More color = more absorbance (useful for weak positives)

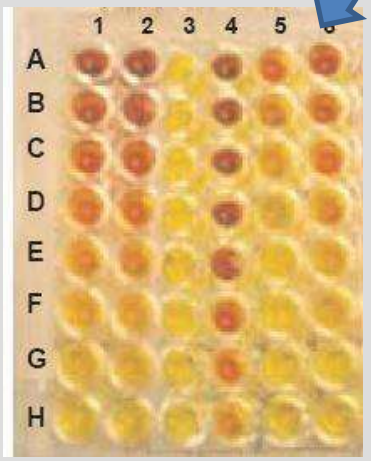
The greater the color change = the greater the amount of enzyme = the greater the amount of antigen.

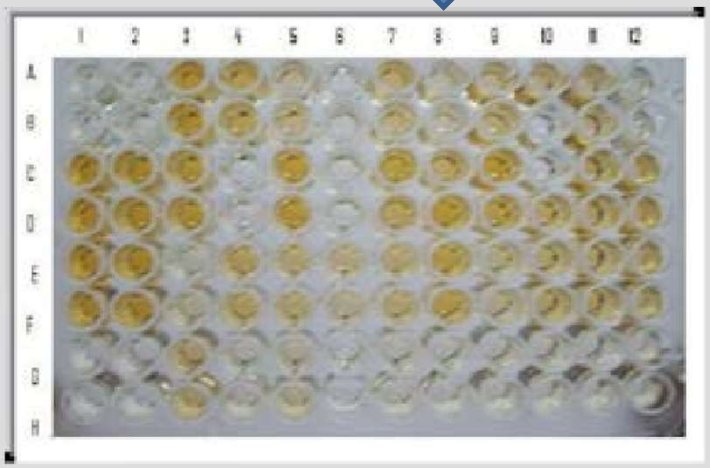
So the absorbance values are used to roughly quantify the amount of antigen a sample relative to another sample.

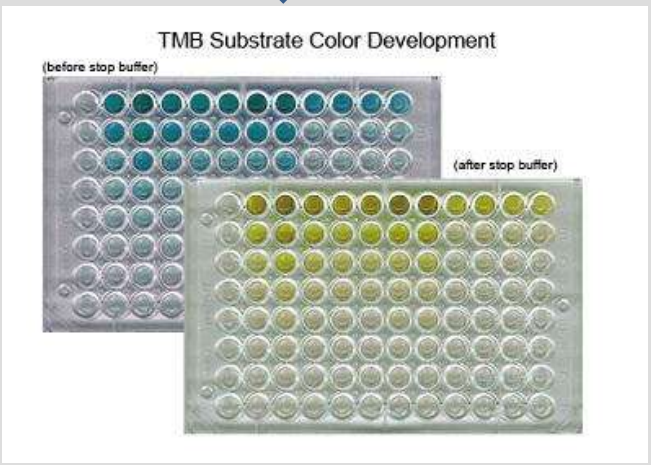
How could you set up an ELISA to quantify the amount of coat protein in a sample?

Other Enzymes and Substrates are Available

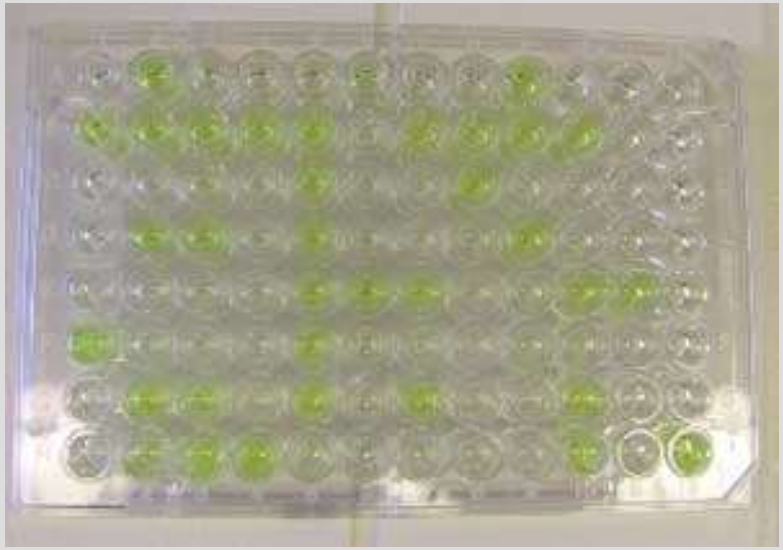
Reagent			
Enzyme	**Gal** Beta galactosidase	**HRP** Horseradish peroxidase	**HRP** Horseradish peroxidase
Substrate	**CPRG** (Chlorophenol red-B-D glactopyrano)	**OPD** (o-phenylenediamine dihydrochloride)	**TMB** (3,3',5,5'-tetramethylbenzidine)
UV wavelength (nm)	570-595	492	370 + 652 (before acid) 450 (after acid)

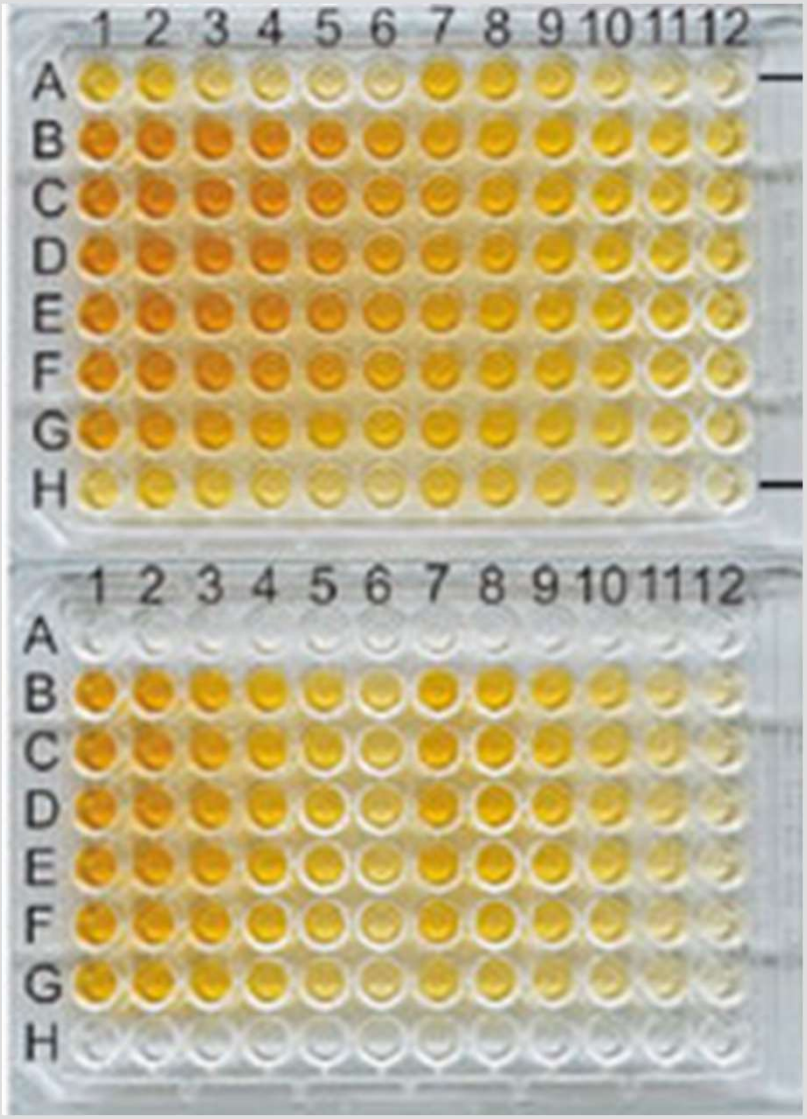






Determining whether a reaction is positive or negative





Determining which reactions are positive:

Various methods have been used:

Table 1. Results of 2-year (1984 and 1985) survey of four journals[a] for methods used to set ELISA threshold values

Method	Number of papers
Not stated[b]	49
Visual	7
$2\bar{x}$ Negative mean	10
$3\bar{x}$ Negative mean	5
Negative mean + $2s$	2
Negative mean + $3s$	5
Negative mean + $4s$	1
Other	2
Total	81

[a] PLANT DISEASE, *Plant Pathology*, *Annals of Applied Biology*, and *Journal of General Virology*.
[b] Some articles cited another article for materials and methods.

Table 5. Summary of false-negative and false-positive results[a] of ELISA for potato leafroll virus infection in 297 potato sprouts, employing commonly used statistical methods vs. discriminate analysis for determining the positive-negative threshold

Threshold absorbance value	Method for setting threshold	Percentage of false negatives	Percentage of false positives
0.040	$2\bar{x}$	0.00	9.09
0.050	$\bar{x} + 3s$	0.34	8.42
0.060	$3\bar{x}$	1.01	7.07
0.065	$\bar{x} + 3s$	1.01	6.73
0.080	$\bar{x} + 4s$	2.02	4.71
0.118	CORROD[b]	9.09	2.36
0.131	LnCORROD[b]	11.11	2.04

[a] Determined by visual plant symptoms.
[b] Discriminate function.

Increasing the threshold (cut-off):
% false negatives rises and % of false positives falls

Mean of the healthy controls plus 3x standard deviation

Sutula et al 1986 Plant Dis. 8:722-726

Objectives of this laboratory:

1. Improve understanding of how a protein-based assay works
2. Learn the basics of how to perform an ELISA
3. Successfully detect the presence of ZYMV coat protein in the different leaf tissues of ZYMV-infected squash plants.
4. Use ELISA to compare the relative amounts of coat protein in:
    A) Green and chlorotic leaf tissues
    B) Older vs newer leaves on the same plant.

This exercise should provide an improved understanding of the following:

- Distribution of a virus in a plant
- Importance of sampling with respect to assay results