Diagnostic Tools and Definitions

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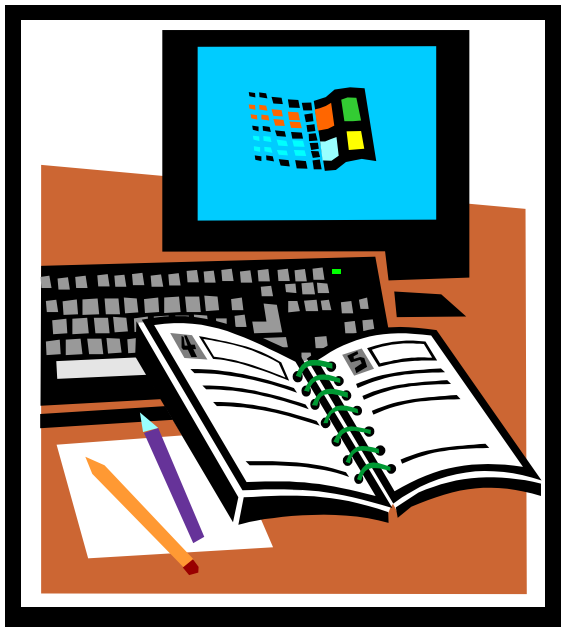
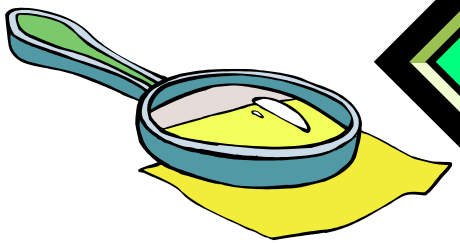
Beltsville Laboratory

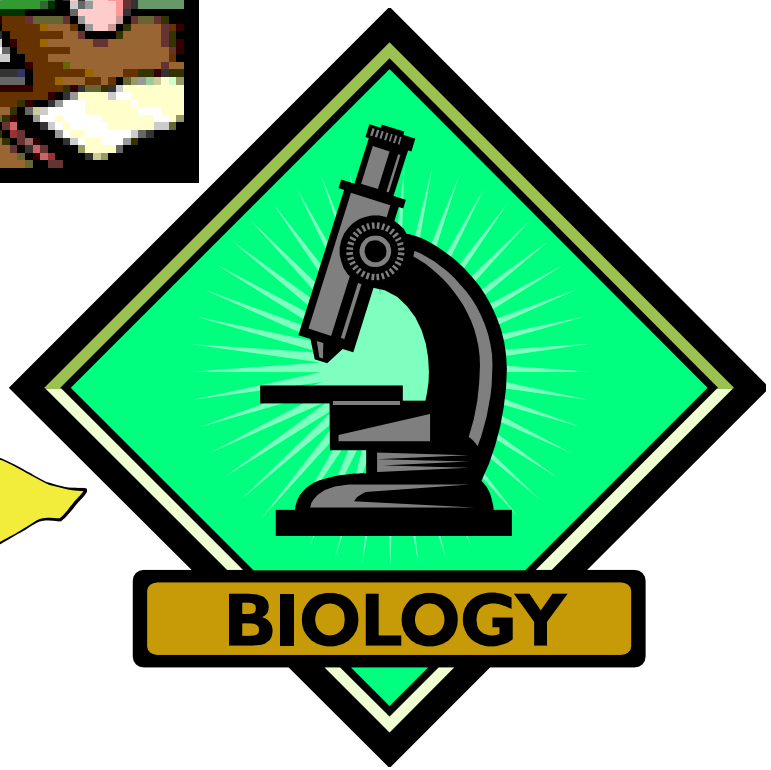
- **Federal Confirmatory Operational Diagnostics**
- **Development and Validation of Diagnostic Methods for Regulatory Pathogens**
- **Proficiency testing**
- **Evaluating new diagnostic technologies**
- **Provide Training on Diagnostics**
- **Scientific Solutions for PPQ**

DIAGNOSIS

- Symptoms
- Signs

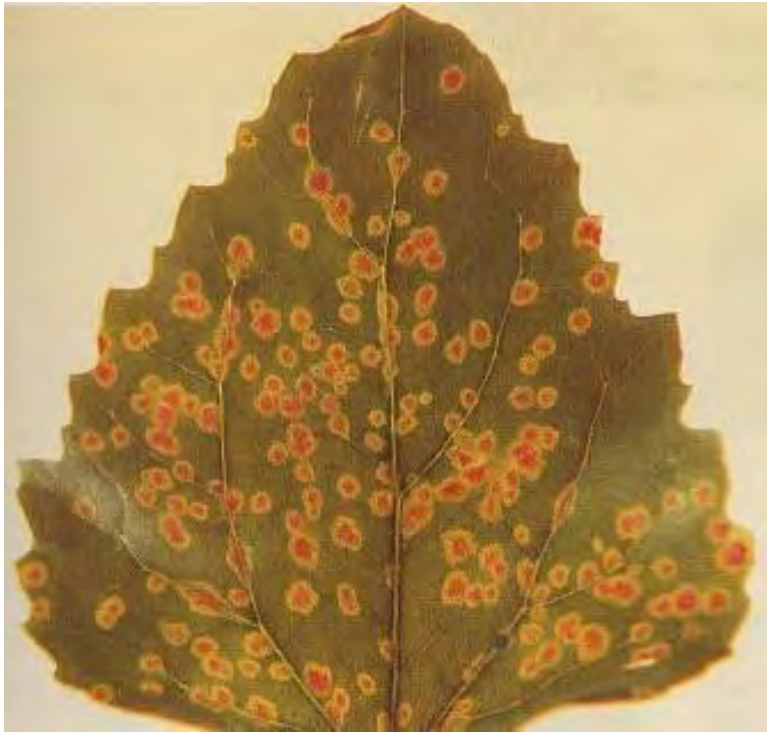






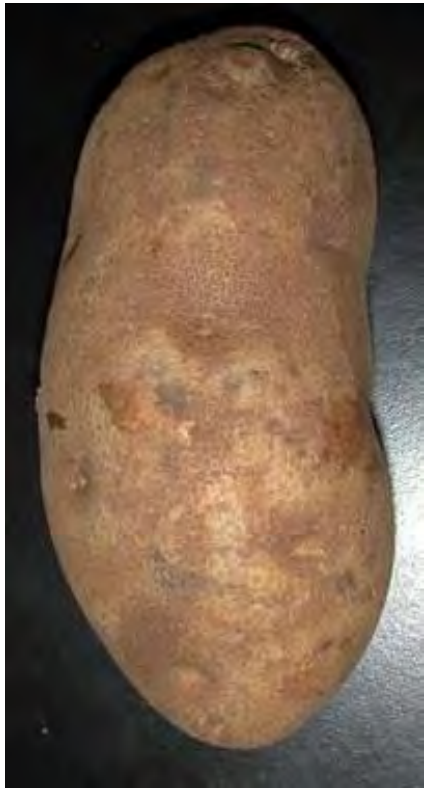
Symptoms

- Host-Pathogen Interaction
- Indicator plants/ biological Indexing



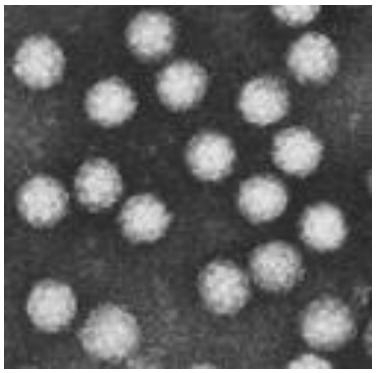
What if we can not see symptoms?

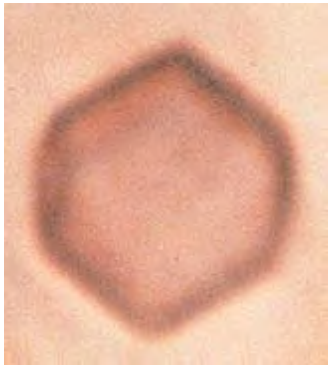
- Potato tuber (no symptoms)
- Bean seed (no symptoms)
- Tree seedling (dormant)

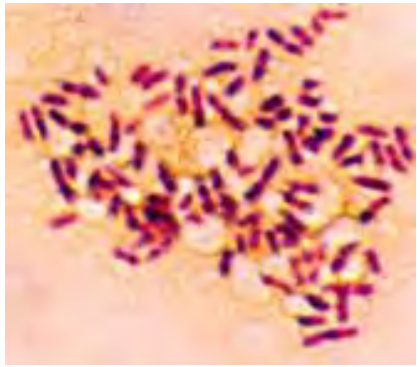


Magnification: The Power of the lens

- Signs are too small to be seen
  - Microscopy
    - Fungal structures
    - Bacteria
    - Inclusion bodies
  - Electron Microscope
    - Viruses

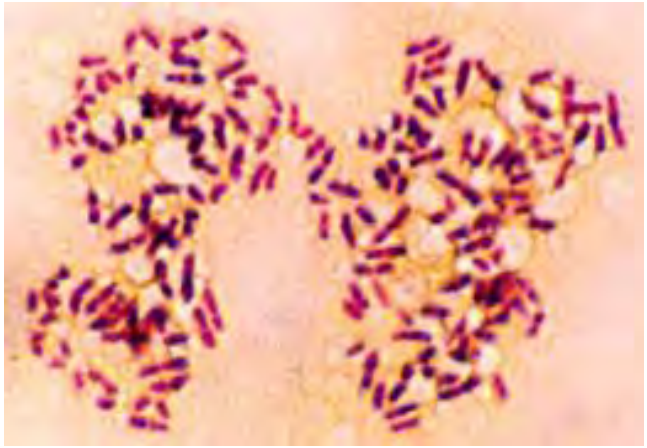






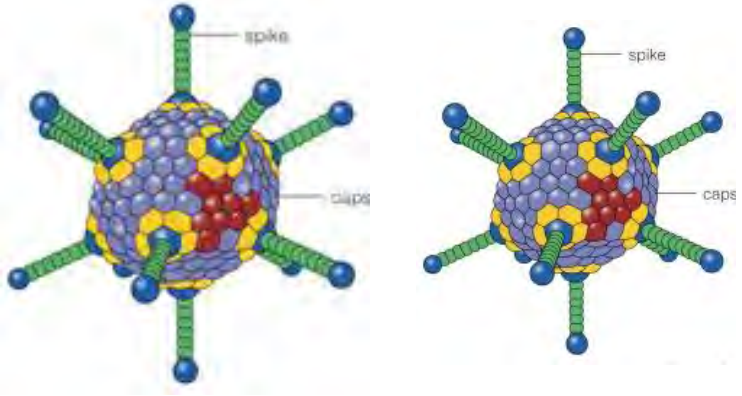
What if we can not differentiate signs?

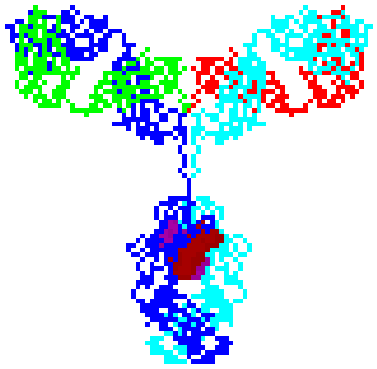
- Hyphae but no spores!
- Spores but which species or strain?
- Bacteria???
- Virus???



Serological Detection

- Specific binding between antigen and antibody
- Antigen
- Immunization
- Antibodies
- Polyclonal
- Monoclonal

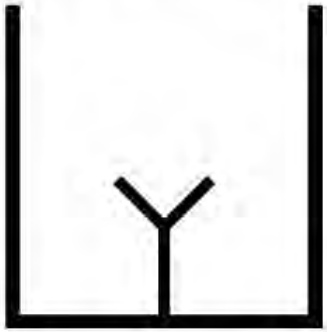




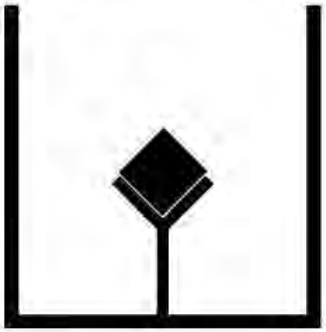
Enzyme-Linked Immunosorbent Assay

- ELISA

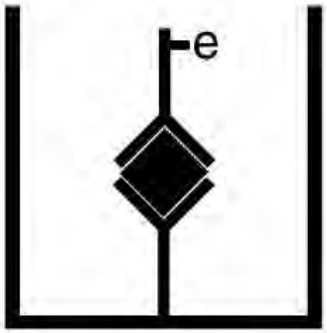
Step 1
Antigen-specific antibody is attached to a solid-phase surface



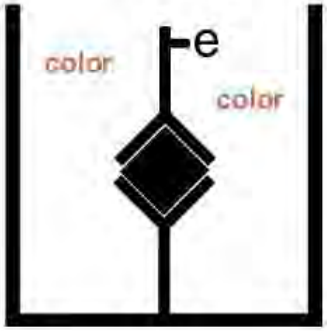
Step 2
Test specimen is added, which may or may not contain the antigen



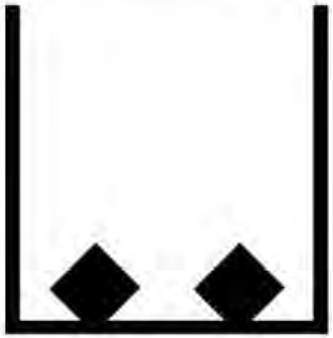
Step 3
An enzyme-labeled antibody specific to the antigen is added (conjugate)



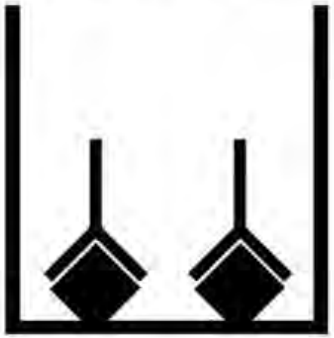
Step 4
Chromogenic substrate is added, which in the presence of the enzyme, changes color.



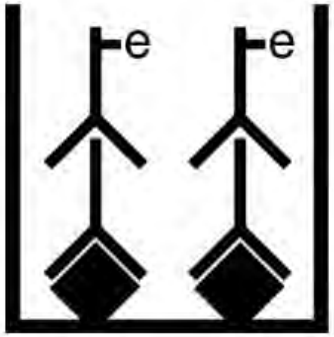
Step 1
Specific antigen is attached to a solid-phase surface



Step 2
Test specimen is added, which may or may not contain the antibody



Step 3
An enzyme-labeled antibody specific to the test antibody is added (conjugate)



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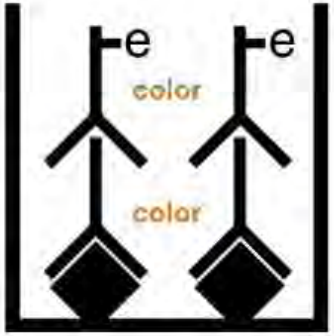
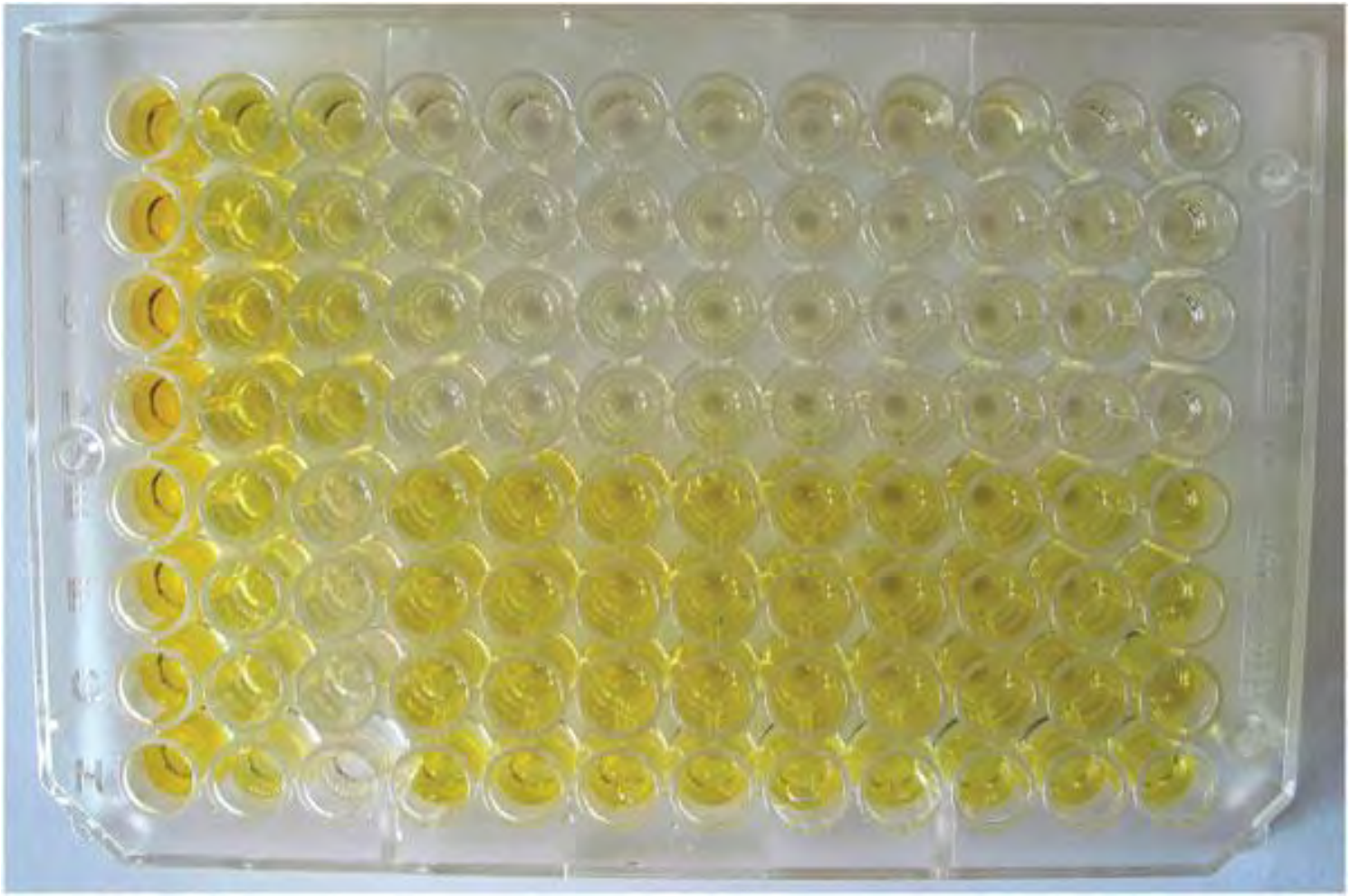
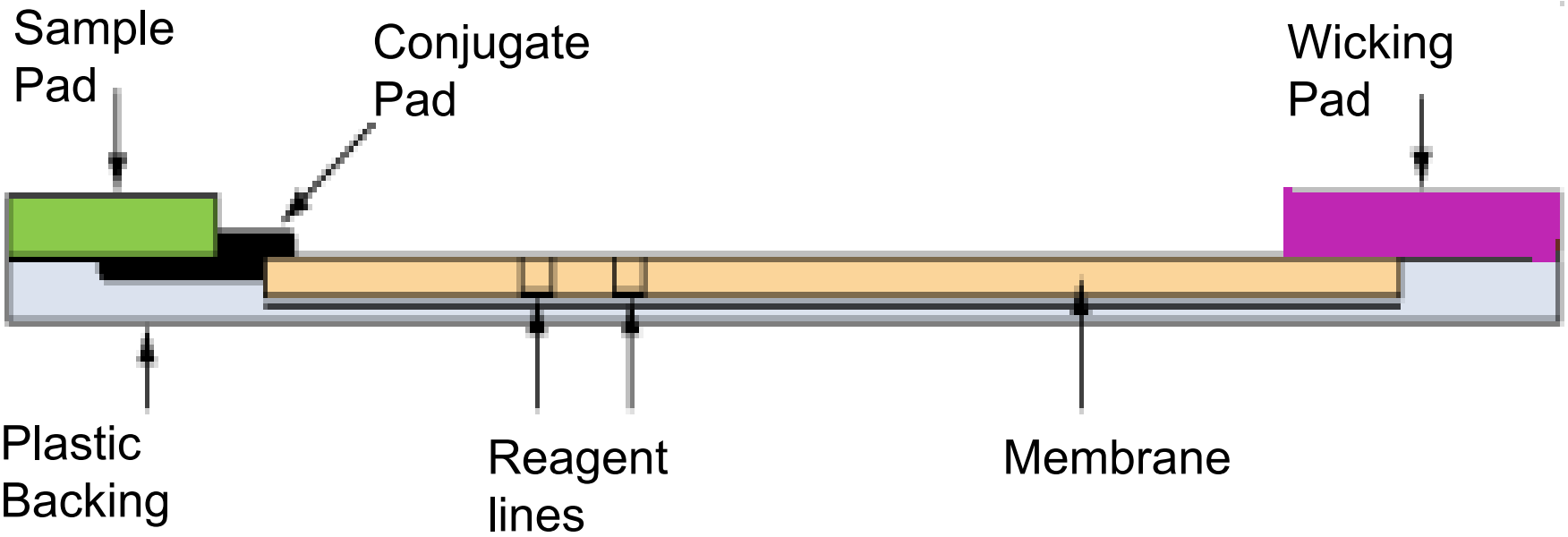


Enzyme-Linked Immunosorbent Assay

- ELISA



ImmunoStrips



Nucleic Acid Detection

- Overcomes many of the problems associated with serological methods
  - Some pathogens (viroids) do not have proteins
  - Difficult to make antibodies for some pathogens
  - Low titer of antigen
  - Cross-reaction of antibodies with heterologous antigens
  - Nucleic acid sequence is not affected by growth conditions (Developmental or environmental)
  - No experimental animals are required for primer or probe production

Nucleic Acid Detection

- Polymerase chain reaction (PCR)
  - (DNA viruses)
  - Reverse transcriptase PCR (RT-PCR)
    - (RNA viruses and viroids)
- Hybridization:
  - Specific probes
  - General probe
- Sequencing

Polymerase Chain Reaction (PCR)
Mullis (1985)

- Powerful technique with widespread application
- Amplify a specific NA fragment that lies between two regions of known sequence

```
5′ ATTGCATCGAATAGxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxGCTACGGCAGCT 3′
3′ TAACGTAGCTTATCxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxCGATGCCGTCGA
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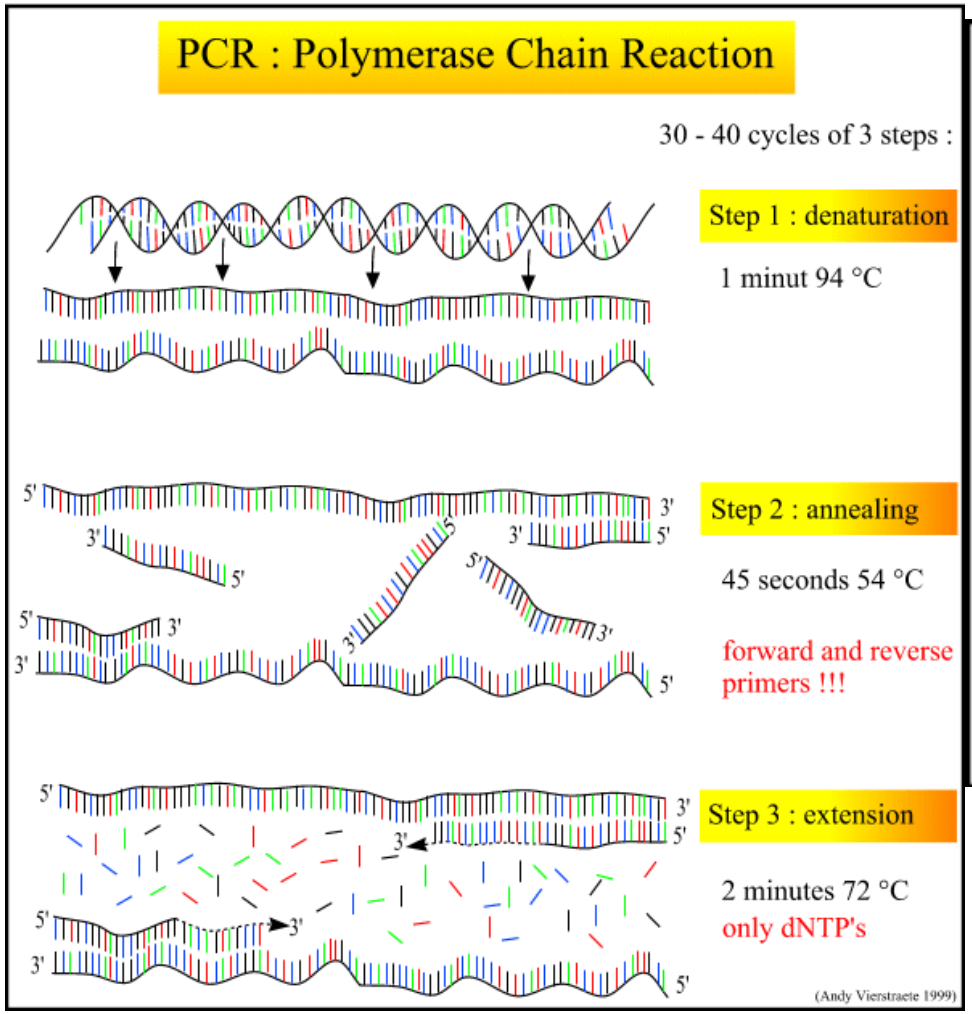
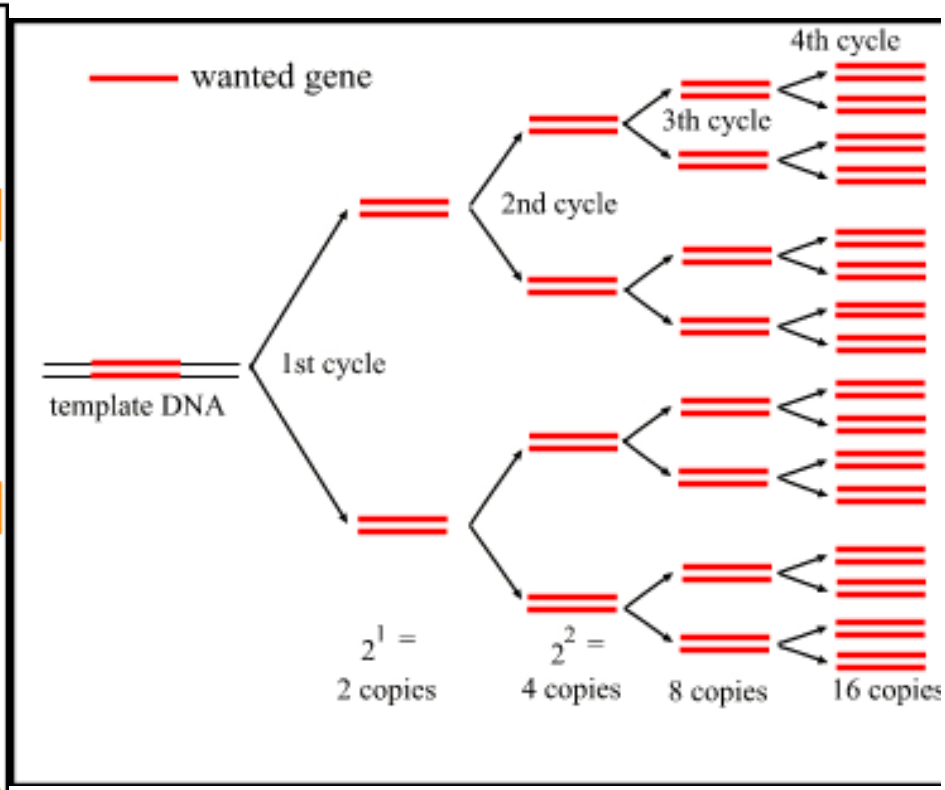
- Very sensitive, very small amount of target is needed (fresh, dried, partially degraded)
- High specificity based on NA sequence
- Make a huge number of copies of DNA
  - (like a copy machine for genes)





35 cycles:
2^{35} = 24 billion copies

Which Method I Should Use?

- Specificity
  - No false positive
  - Accuracy
  - Precision
- Sensitivity
  - No false negative
  - Limit of Detection
  - Limit of Quantification
- Robustness/ Ruggedness
- System Suitability

Method Validation

The Specificity of a method defines the ability of the method to measure the pathogen of interest to the exclusion of other relevant components.

The Limit of Detection (LOD) is the lowest concentration of pathogen that can be distinguished from background.

The limits of quantitation (LOQ) are the lowest and the highest concentrations of pathogen in a sample or specimen that can be measured with an acceptable level of accuracy and precision.

Method Validation

- Accuracy is the measure of exactness of an analytical method
  - Requires a “gold standard”

The Precision of a method is the degree of agreement among individual test results.

- Within-day variability (repeatability)
- Between-day variability (reproducibility)

Method Validation

- The Robustness of a procedure is a measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability in normal usage.

- Ruggedness is the of reproducibility of the test results obtained for identical samples under normal (but variable) test conditions.

Controls

- Aliquots of the pathogen that have been made in the sample matrix separately from the standard curve and which are run in every assay
- Should be at the low, medium and high end of the standard curve.

Which Method I Should Use?

Method	Specificity	Sensitivity	Time
Biological Indexing	++	++	+++++
EM	+	+	++
Serology	+++	+++	+
PCR	+++++	+++++	+

Other Things to Consider

- Approved Work Instructions
- Laboratory Accreditation
- Diagnostician Certification
  - (Proficiency Testing)

Document Control Number **WI-B-T-1-32**	**WORK INSTRUCTION** **USDA, APHIS, PPQ, CPHST,** **Beltsville Laboratory, Bldg 580, BARC-East**	Revision Number **Original**
Effective Date: **10/20/2011**	**Detection of *Citrus Leprosis Virus*-Cytoplasmic Type (CiLV-C) using a Multiplex One-Step Reverse Transcription (RT) Conventional PCR (K565 and K568)**	

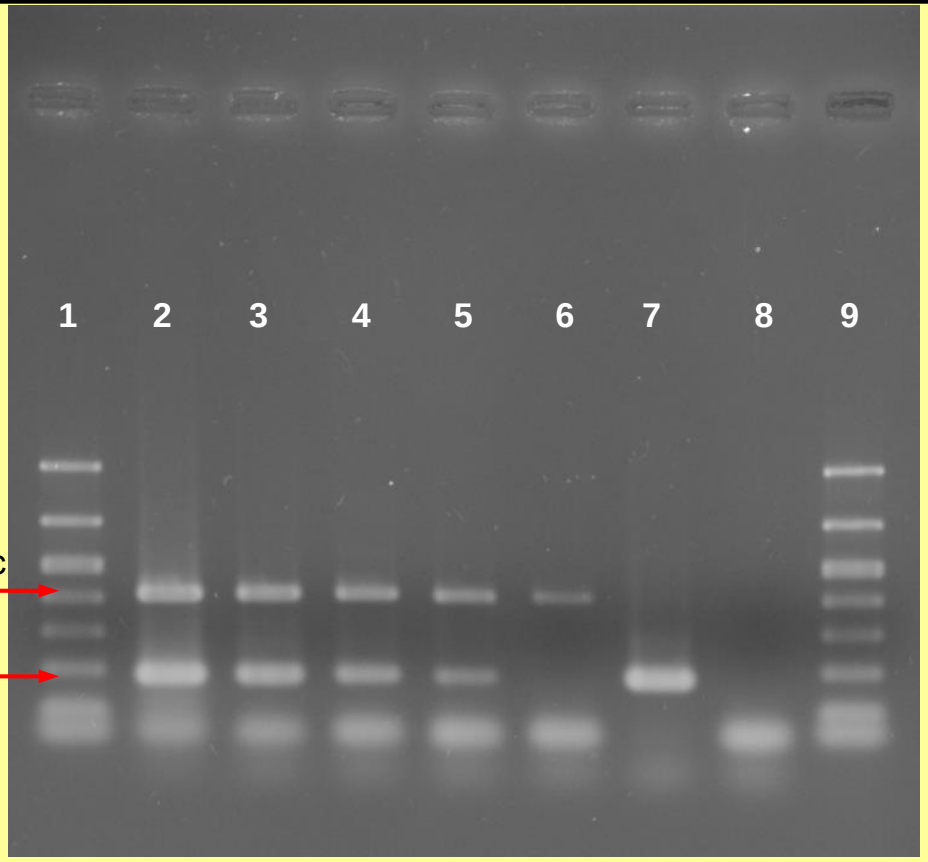


Fig. 1 Lanes:

1) **Biomarker Low**
2) **Undiluted (117ng/µl) CiLV-C positive control**
3) **1:1,000 diluted (117pg/µl) CiLV-C positive control**
4) **1:10,000 diluted (11.7pg/µl) CiLV-C positive control**
5) **1:100,000 diluted (1.17pg/µl) CiLV-C positive control**
6) **1:1,000,000 diluted (117fg/µl) CiLV-C positive control**
7) **Healthy Control**
8) **PCR NTC**
9) **Biomarker Low (1000, 700, 500/525, 400, 300, 200, 100, 50 bp)**

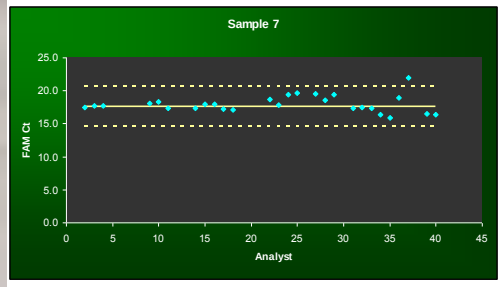
National Plant Protection Laboratory Accreditation Program (NPPLAP)

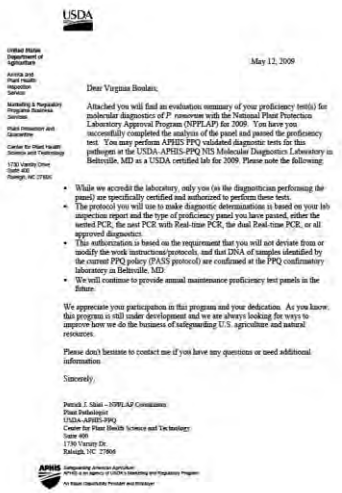
Enhance PPQ ability to respond to and manage intentional and unintentional introductions of plant diseases and pests by increasing the reliability and speed of diagnostic tests.

- **Accredit laboratories in the National Plant Diagnostic Network (NPDN), State Depts. of Ag., federal and private or commercial sectors**
- **Carry out diagnostic tests on plant pathogens or pests *of regulatory concern***
- **Defined standards for facilities, equipment, personnel training, sample tracking, and methods.**

Proficiency Testing (PT)

- A method to verify that the performance of each network laboratory diagnostician is in line with other lab/s diagnosticians performing the same analysis.
- Provides external and independent assessment of accuracy of the results generated by each participant.




USDA

May 12, 2009

Dear Virginia Boulais,

Attached you will find an evaluation summary of your proficiency test(s) for molecular diagnostics of *P. ramorum* with the National Plant Protection Laboratory Approval Program (NPPLAP) for 2009. You have you successfully completed the analysis of the panel and passed the proficiency test. You may perform APHIS PPQ validated diagnostic tests for this pathogen at the USDA-APHIS-PPQ NIS Molecular Diagnostics Laboratory in Beltsville, MD as a USDA certified lab for 2009. Please note the following:

- While we accredit the laboratory, only you (as the diagnostician performing the panel) are specifically certified and authorized to perform these tests.
- The protocol you will use to make diagnostic determinations is based on your lab inspection report and the type of proficiency panel you have passed, either the nested PCR, the nest PCR with Real-time PCR, the dual Real-time PCR, or all approved diagnostics.
- This authorization is based on the requirement that you will not deviate from or modify the work instructions/protocols, and that DNA of samples identified by the current PPQ policy (PASS protocol) are confirmed at the PPQ confirmatory laboratory in Beltsville, MD
- We will continue to provide annual maintenance proficiency test panels in the future.

We appreciate your participation in this program and your dedication. As you know, this program is still under development and we are always looking for ways to improve how we do the business of safeguarding U.S. agriculture and natural resources.

Please don't hesitate to contact me if you have any questions or need additional information.

Sincerely,

Patrick J. Shiel - NPPLAP Coordinator
Plant Pathologist
USDA-APHIS-PPQ
Center for Plant Health Science and Technology
Suite 400
1730 Varsity Dr.
Raleigh, NC 27606

APHIS

Thank You