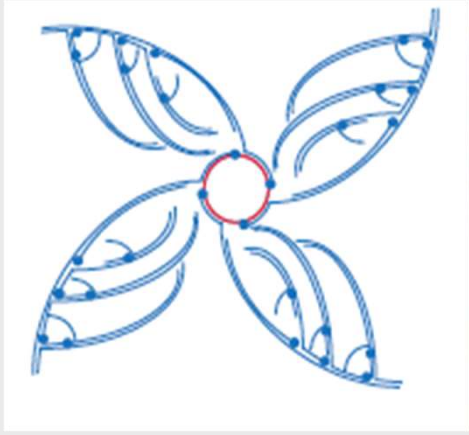
Biological and Nucleic Acid Based Assays for Plant Virus Detection



Three Broad Categories of Assays:

A. Biological Assays

B. Viral Nucleic Acid Assays

C. Viral Protein Assays

Objectives:

1. Know what and how different Biological Assays are employed for virus detection and identification based on the virus as an entity

2. Know what and how different Nucleic Acid Assays are used to detect and identify viruses based on their genomes

3. Be able to use this knowledge to interpret refereed/non-refereed papers, seminars, etc…

Summary- Available Diagnostic Assays

A. Biological Assays

- Host range
- Symptoms
- Methods of transmission

B. Viral Nucleic Acid Assays

- Inclusion body visualization
- dsRNA visualization
- Nucleic acid hybridization
- Microarray
- Amplification Assays:
  - PCR, RT-PCR,
  - RCA (rolling circle amplification)
  - RPA (recombinase polymerase amplification)
  - LAMP (loop-mediated isothermal reaction)
  - Crisper based detection assay
- Viral genome sequence (ecogenomics, deep sequencing,)

A. Assays based on Viral Biology:

Macroscopic Symptomatology

Use of characteristic disease symptoms either on field plants or on experimental indicator hosts

Host Range

Identification of the species of plants which can and cannot be infected

Experimental Transmission Properties

Determine the mode(s) of transmission (mechanical, type of vector, seed, etc…)

A. Assays based on Viral Biology Con't:

Strengths:

- Essential to fulfilling Koch's Postulates

  (virus isolated from the initial host must reproduce the disease when a healthy host is inoculated.)

- Establishes the viral nature of a disease

  Enables the distinction of virus-induced disease from non-pathogen induced symptoms such nutrient deficiency, and physiological and genetic disorders.

- Are often the best assays to distinguish viral strains.
- Very helpful to identify the causal agents when more than one virus is the cause of the disease

A. Assays based on Viral Biology Con't:

Limitations:

- Time consuming
- Requires greenhouse facilities
- Environmental factors may affect symptoms
- Several viruses may give similar symptoms on the same indicator plants
- Latent infections can be difficult to diagnosis by these assays
- Some viruses are difficult to transmit under experimental conditions.

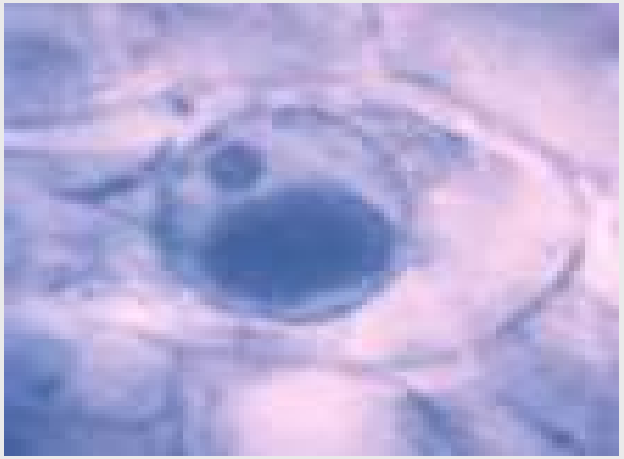
B. Assays Based on Viral Nucleic Acid

- Inclusion body visualization
- dsRNA visualization
- Nucleic acid hybridization
- Microarray
- Amplification Assays:
  - PCR, RT-PCR, Real Time PCR, Real time RT-PCR
  - RCA (rolling circle amplification)
  - RPA (recombinase polymerase amplification)
  - LAMP (loop-mediated isothermal reaction)
- Analysis of complete or partial viral genome sequences

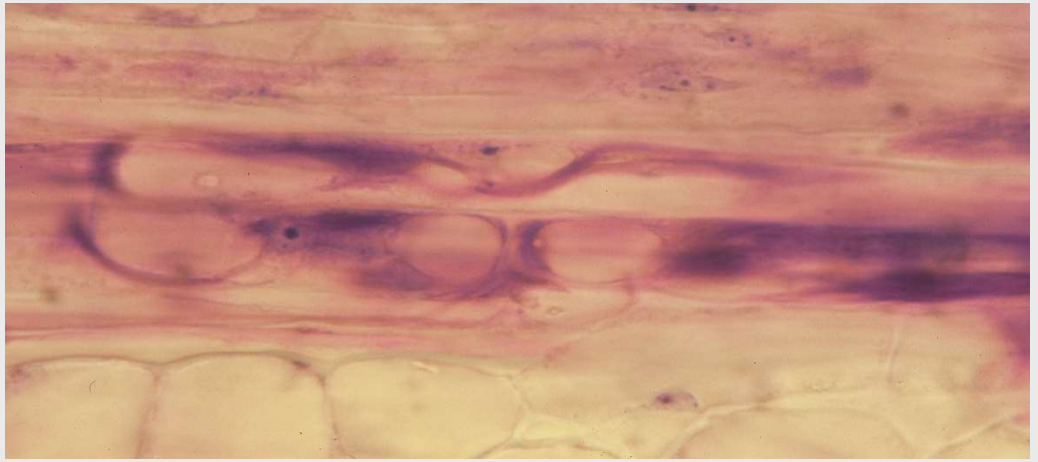
B. Assays Based on Viral Nucleic Acid

1. Inclusion Body Visualization

Choice of stain – can indicate type of nucleic acid
Inclusions stained with Azure A indicates present of RNA (pink) or DNA (blue)



DNA virus



RNA virus

B. Assays Based on Viral Nucleic Acid

2. dsRNA (double stranded RNA) Analysis

dsRNAs are produced as an intermediate product in the replication of many plant viruses. dsRNAs are not present in a healthy plant cell.

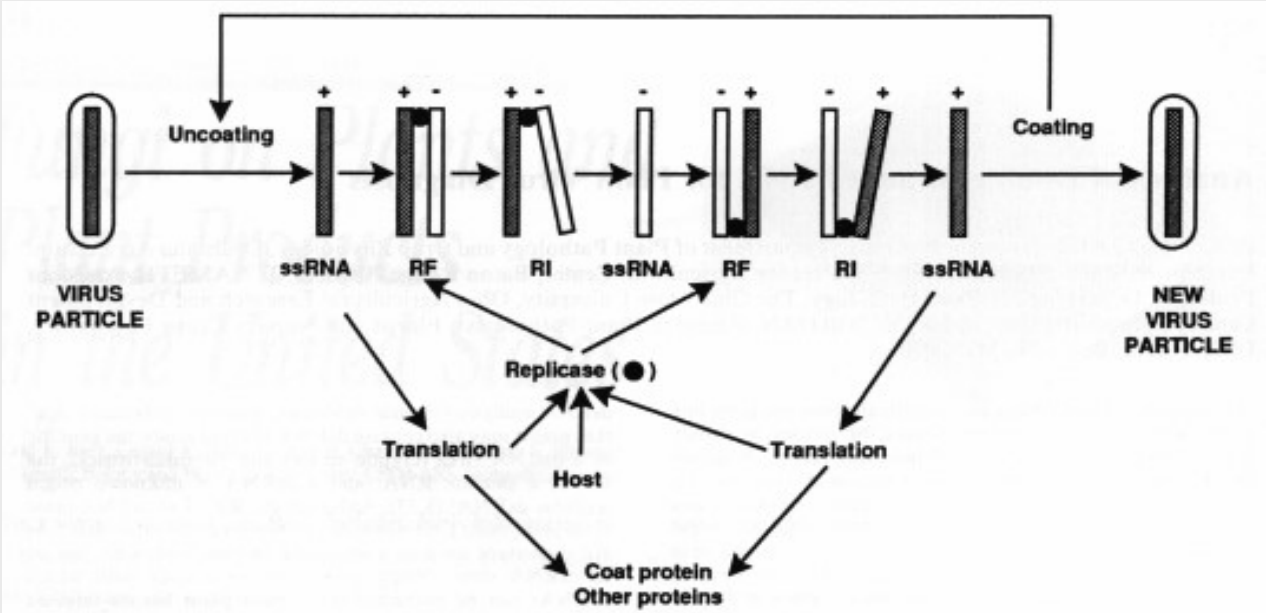


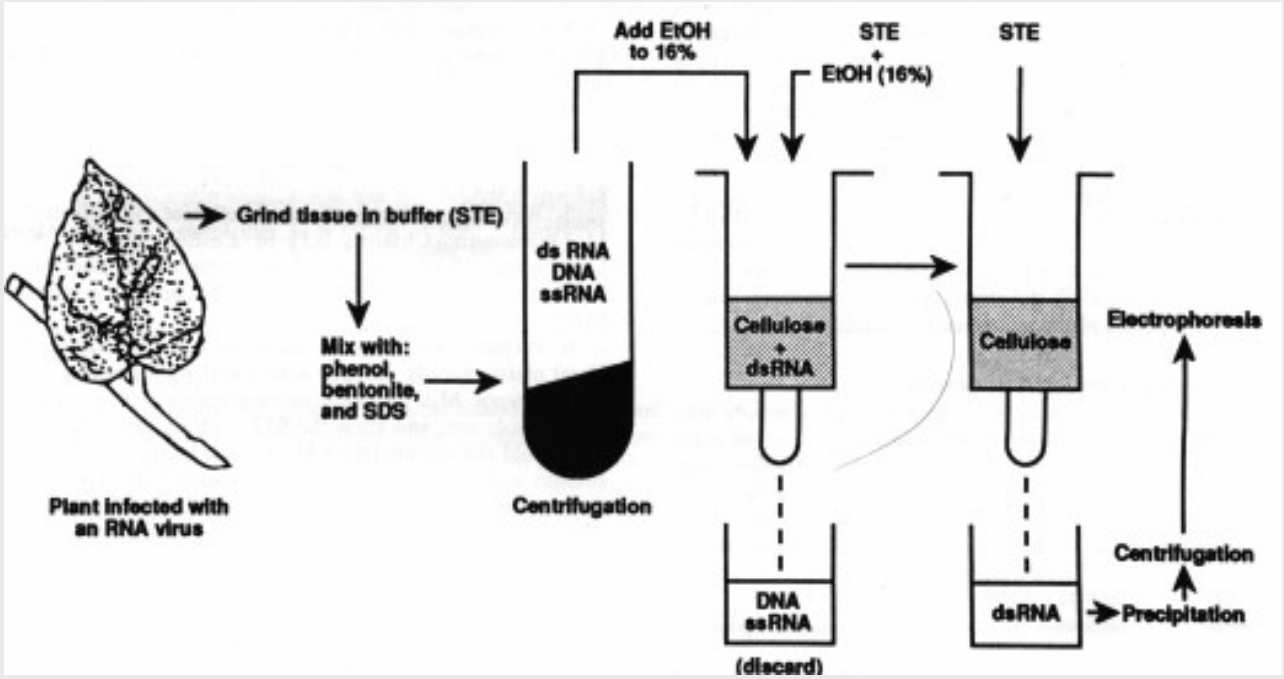
Fig. 1. Schematic representation of the proposed replication cycle of a typical single-stranded RNA (ssRNA) plant virus. Only the replication of the genomic RNA is illustrated. In some cases, however, replicative forms (RFs) of subgenomic RNAs are also produced. Double-stranded RNAs obtained from plants infected with ssRNA viruses are probably RFs. Replicative intermediate (RI) consists of dsRNA that is partially single-stranded and therefore more susceptible to RNase degradation.

Taken from Valverde, R. A. &Nameth, S. T. 1990, Plant Disease 74, 255-258

B. Assays Based on Viral Nucleic Acid

dsRNA (double stranded RNA) Analysis

The basic steps involved in the extraction of dsRNA from plant tissue:



Valverde, R. A. & Nameth, S. T. 1990, Plant Disease 74, 255-258

B. Assays Based on Viral Nucleic Acid

dsRNA Analysis

- These dsRNAs are readily isolated and characterized by polyacrylamide gel electrophoresis.
- Many viruses produce a characteristic number and size of dsRNAs

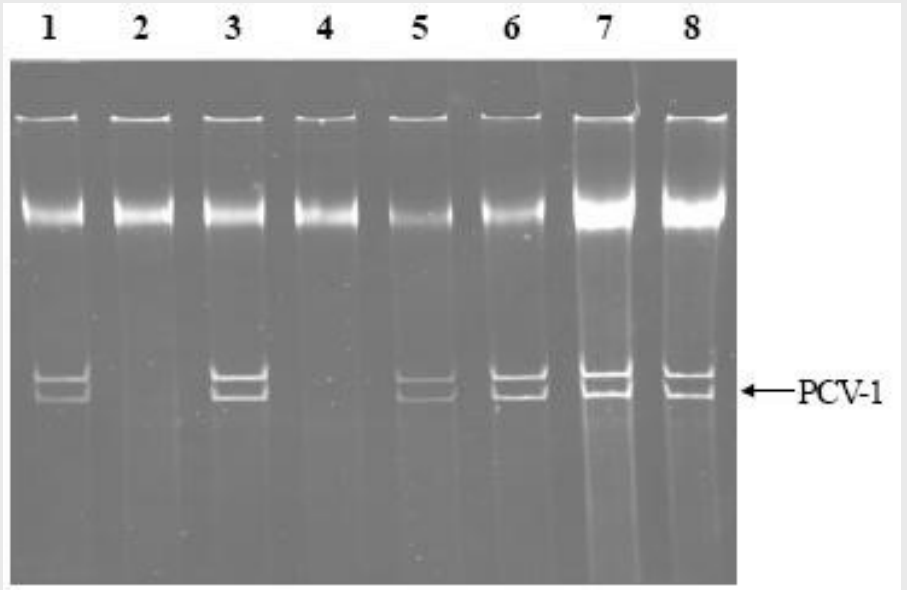
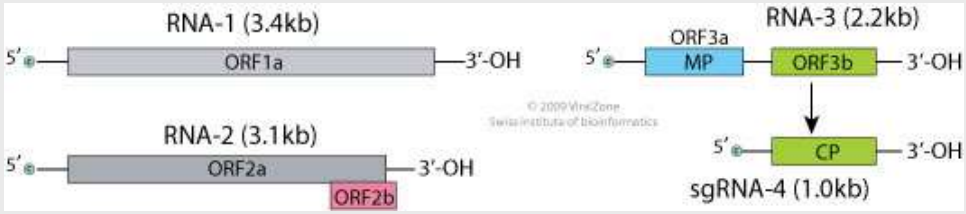


Fig. 1. Polyacrylamide gel electrophoresis of dsRNAs extracted from eight Jalapeño M pepper (*Capsicum annuum*) plants. Lanes 1, 3, 5, 6, 7, and 8 are from Jalapeño M plants infected with pepper cryptic virus -1 (PCV-1). Lanes 2 and 4 are from Jalapeño M plants free of the virus.

Pepper cryptic virus 1
(*Partitiviridae, Deltapartitivirus*)

Can be used to characterize or recognize different virus strains as well as different viruses

This example:
Pattern of dsRNAs produced by different isolates and strains of *Cucumber mosaic virus* (tripartite virus)



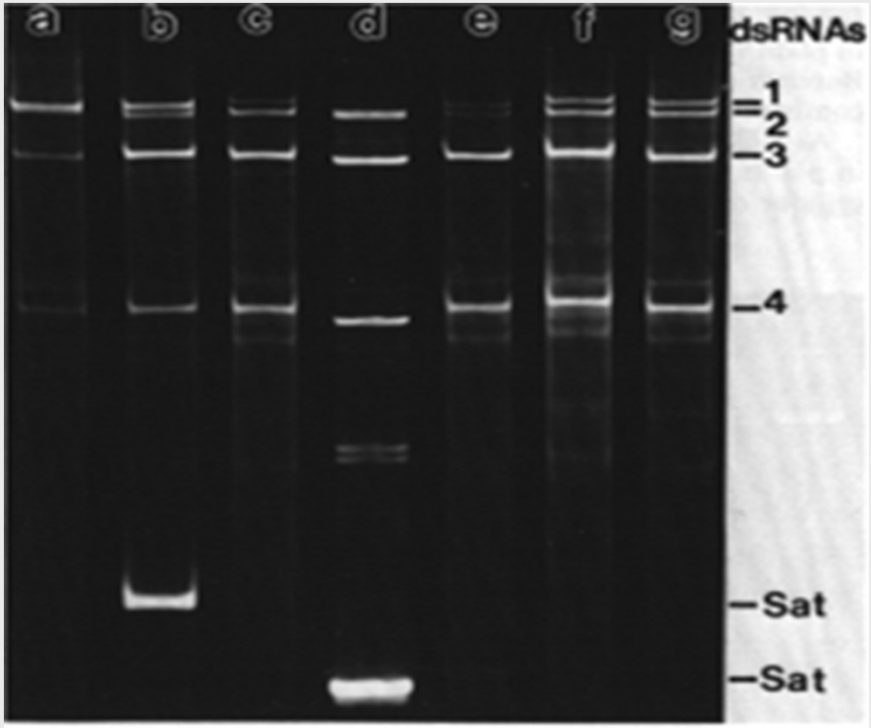
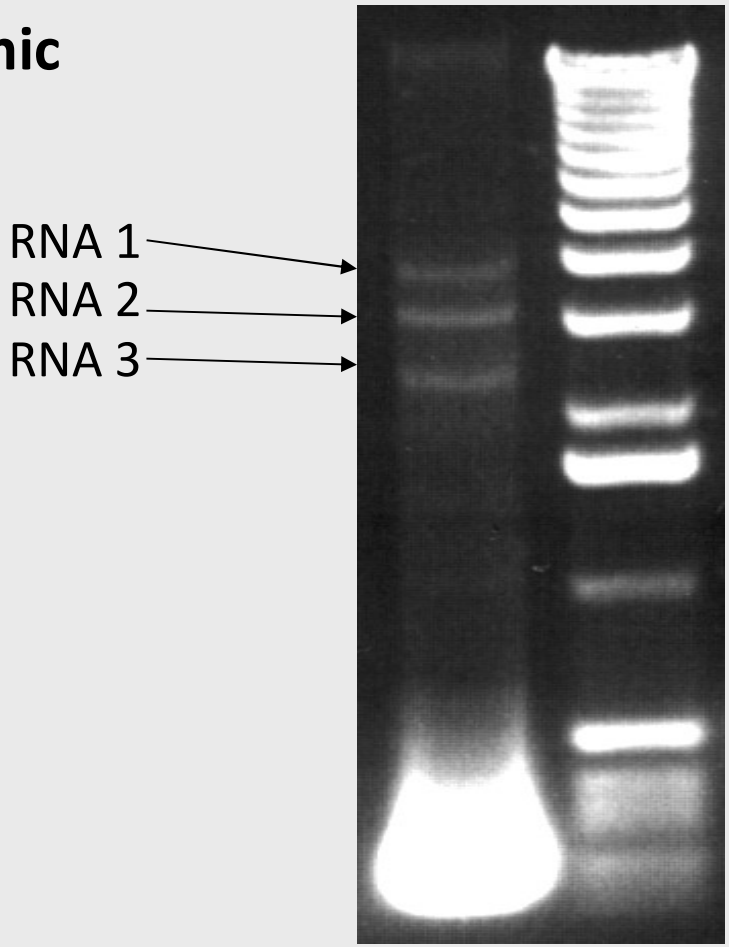


Fig. 5. Polyacrylamide gel electrophoresis of dsRNAs extracted from tobacco cv. Turkish infected with several strains and field isolates

Valverde & Nameth 1990, Plant Disease 74, 255-258

dsRNAs can be used to obtain viral genomic sequences



- Collect dsRNA from gel,
- Use Reverse transcription to produce cDNA
- Clone cDNA
- Sequence cDNA
- Compare with known sequences

Attoui et al., 2000. Strategies for the sequence determination of viral dsRNA genomes. J.of Virol. Meth. 89:147–158.

B. Assays Based on Viral Nucleic Acid

dsRNA

Strengths:

- Non-specific test
- Segmented viral genomes, the number and sizes of these segments are often diagnostic for a strain, species, genus, or family of viruses.
- Identifies subgenomic segments which represent less than full-length copies of genomic RNA.
- dsRNAs are useful as templates to produce complementary DNA (cDNA) by reverse transcription which is useful for cloning, PCR, and preparation of labeled probes for use in other assays.
- Useful in detecting mixed infections, satellite RNAs, defective interfering RNAs, and satellite viruses.

B. Assays Based on Viral Nucleic Acid

dsRNA

Limitations:

- Time consuming and expensive… best for analysis of small no.'s of samples
- Specialize skill and experience are required for preparation and evaluation of dsRNA
- Some viruses produce very small amounts of dsRNA (ex. Species of *Potyviridae*)
- The technique requires chromatographic and electrophoresis equipment
- Detects non-specific dsRNAs - the presence of multiple viruses, cryptic viruses, viroids

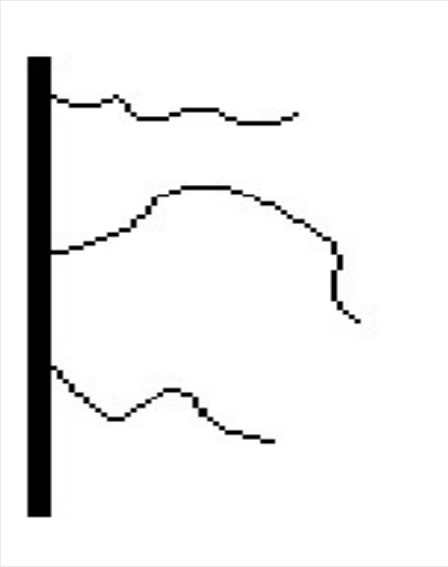
B. Assays Based on Viral Nucleic Acid

3. Nucleic Acid Hybridization

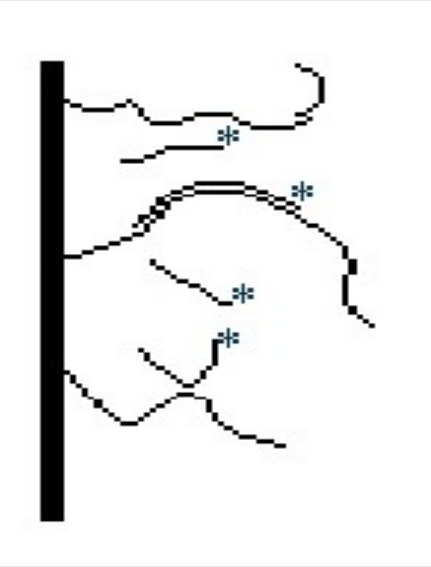
- This assay is based on the similarity of a nucleic acid probe with the target nucleic acid (ie degree of genome sequence homology)
- Target nucleic acid is immobilized onto a solid matrix
- A probe is made. Probe can be RNA or DNA - it binds to the target nucleic acids which have complimentary sequences
- The probe is nucleic acid linked to a reporter molecule which produces a signal that can be detected
- Reporter molecules can be nucleotides which are produced with P^{32},or S^{35}, which are used to synthesize the probe
- Detection at femtogram level (10^{-10})

Nucleic acid hybridization (NASHA)

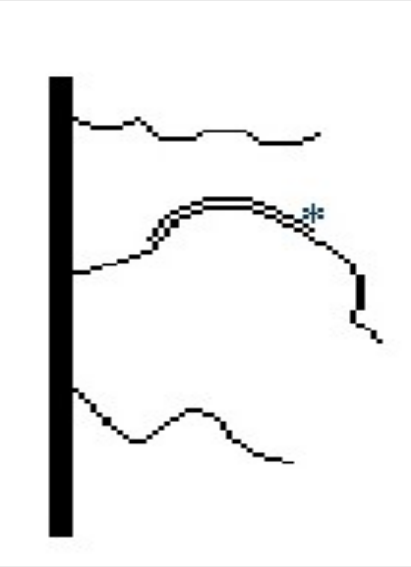
Hybridization



Add probe



Wash

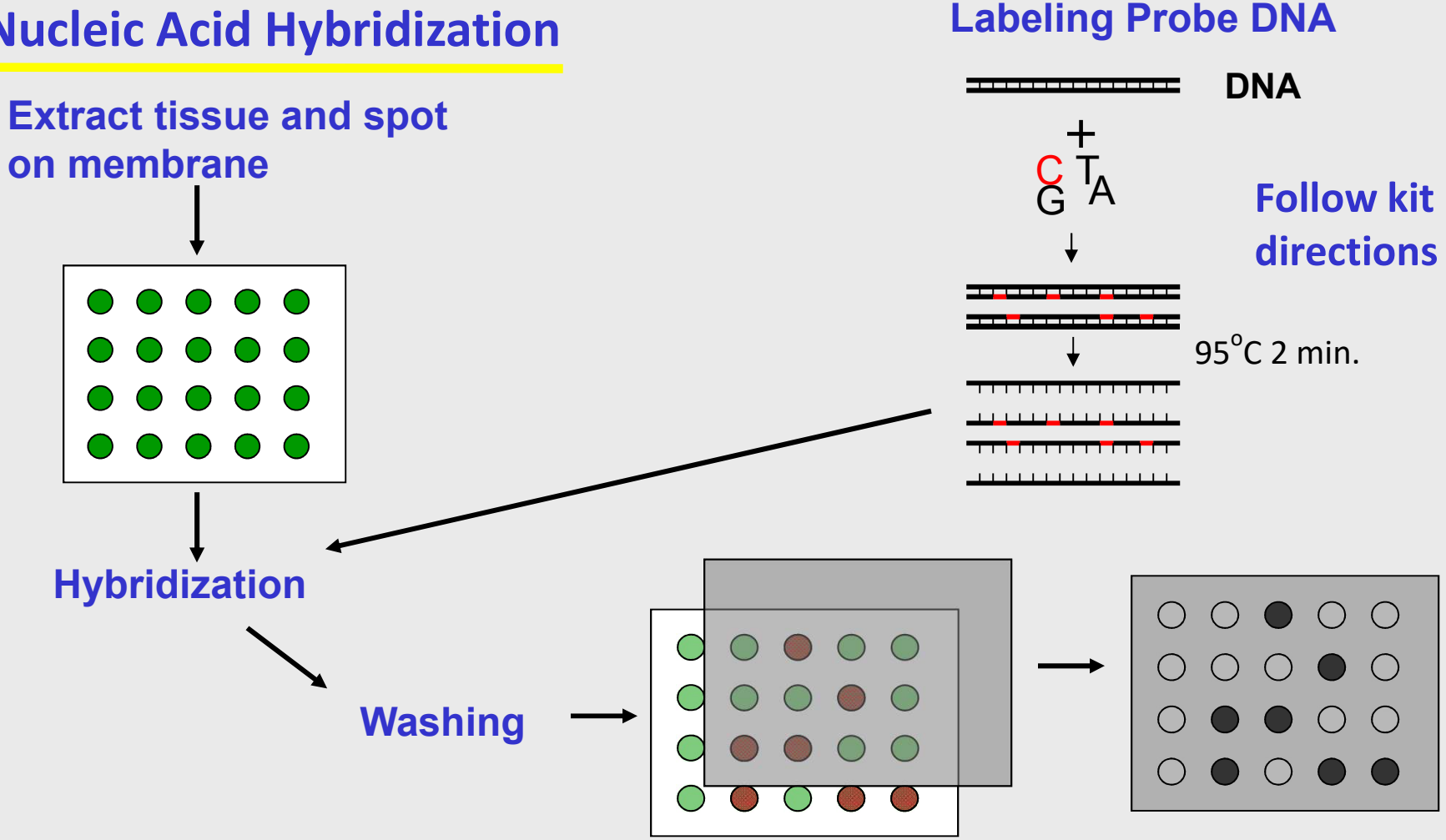


Target nucleic acid bound to membrane

Probe nucleic acid binds to complimentary sequences of the target nucleic acid

Probe stays bound to complimentary sequences

Nucleic Acid Hybridization



Nucleic Acid Hybridization

Extraction:

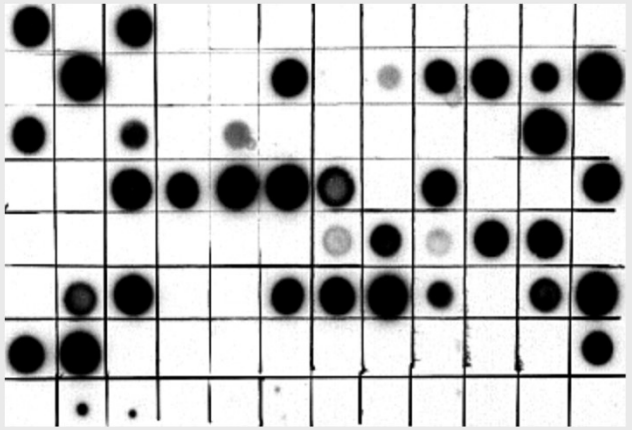
Sample may be applied by:

- Grinding tissue and spotting homogenate

- Holding a freshly cut plant tissue on the surface

- Squashing plant tissue on the membrane

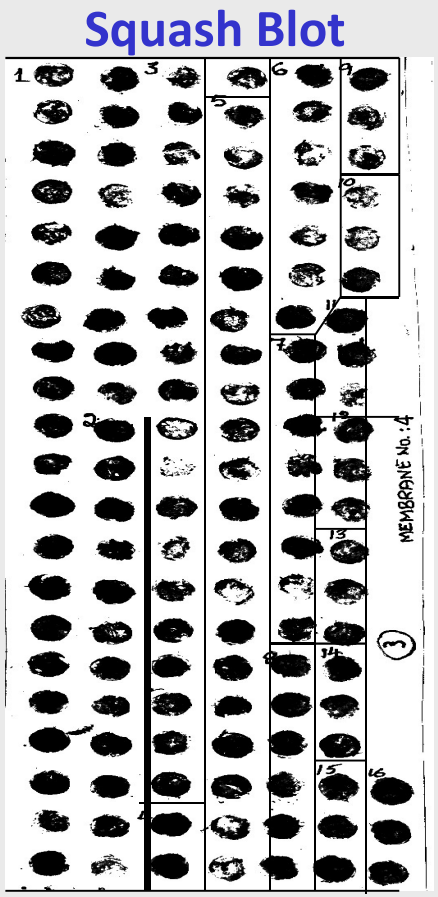
Non-radioactive labels
available which work well

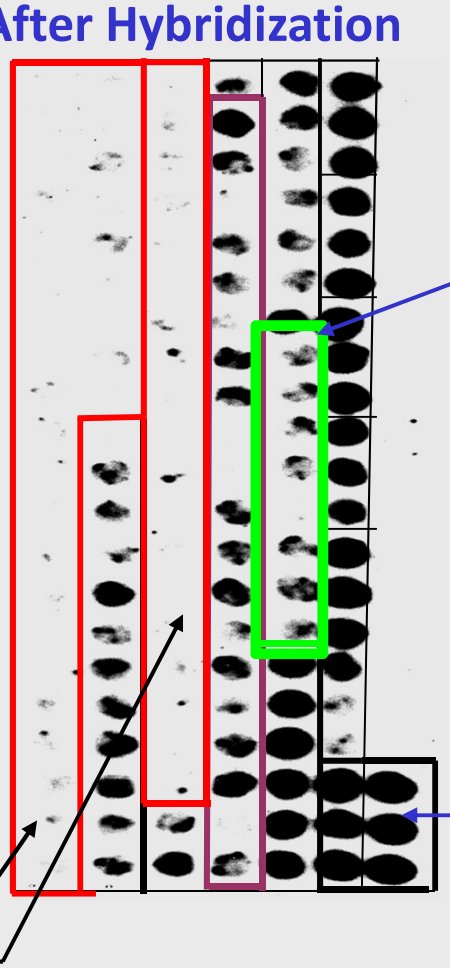


Example of grinding and spotting

Nucleic Acid Hybridization - Squash Blots

Spots are visible due to plant pigments on the membrane





Spots are visible due silver halide grains in x-ray film emulsion that changed by energy emitted by P-32

Nucleic Acid Hybridization

Strengths:

- More sensitive than the best serological techniques (at least 1000x more sensitive than ELISA)
- Very specific-probes can be made which distinguish between targets differing in just a few nucleotides
- Broad-spectrum probes can be made which detect all members of a strain, the species or the genus
- Results can be quantitative
- Good for large numbers of samples
- Portable - samples can be spotted onto the membrane in the field and then forwarded to a processing laboratory far away.

Nucleic Acid Hybridization

Limitations:

- Requires somewhat special facilities, equipment, expertise, reagents
- Too expensive and time consuming for small numbers of samples

Commercially available as a service through diagnostic companies for some viruses/viroids (ex. AgDia)

4. Nucleic Acid Microarrays

Nucleic Acid Microarray:

- A collection of microscopic DNA spots attached to a solid surface.
- Each DNA spot contains picomoles (10^{-12} moles) of a specific DNA sequence, known as probes (reporters or oligos).
- These can be a short section of a viral genome (cDNA) - they hybridize to the DNA or RNA sample (called the *target*) under high-stringency conditions.
- Probe-target hybridization is detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Microarray-based detection and genotyping of viral pathogens

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Departments of *Biochemistry and Biophysics, †Microbiology and Immunology, and ‡Medicine, and §Howard Hughes Medical Institute, University of California, San Francisco, CA 94143

The detection of viral pathogens is of critical importance in biology, medicine, and agriculture. Unfortunately, existing techniques to screen for a broad spectrum of viruses suffer from severe limitations. To facilitate the comprehensive and unbiased analysis of viral prevalence in a given biological setting, we have developed a genomic strategy for highly parallel viral screening. The cornerstone of this approach is a long oligonucleotide (70-mer) DNA microarray capable of simultaneously detecting hundreds of viruses. Using virally infected cell cultures, we were able to efficiently detect and identify many diverse viruses. Related viral serotypes could be distinguished by the unique pattern of hybridization generated by each virus. Furthermore, by selecting microarray elements derived from highly conserved regions within viral families, individual viruses that were not explicitly represented on the microarray were still detected, raising the possibility that this approach could be used for virus discovery. Finally, by using a random PCR amplification strategy in conjunction with the microarray, we were able to detect multiple viruses in human respiratory specimens without the use of sequence-specific or degenerate primers. This method is versatile and greatly expands the spectrum of detectable viruses in a single assay while simultaneously providing the capability to discriminate among viral subtypes.

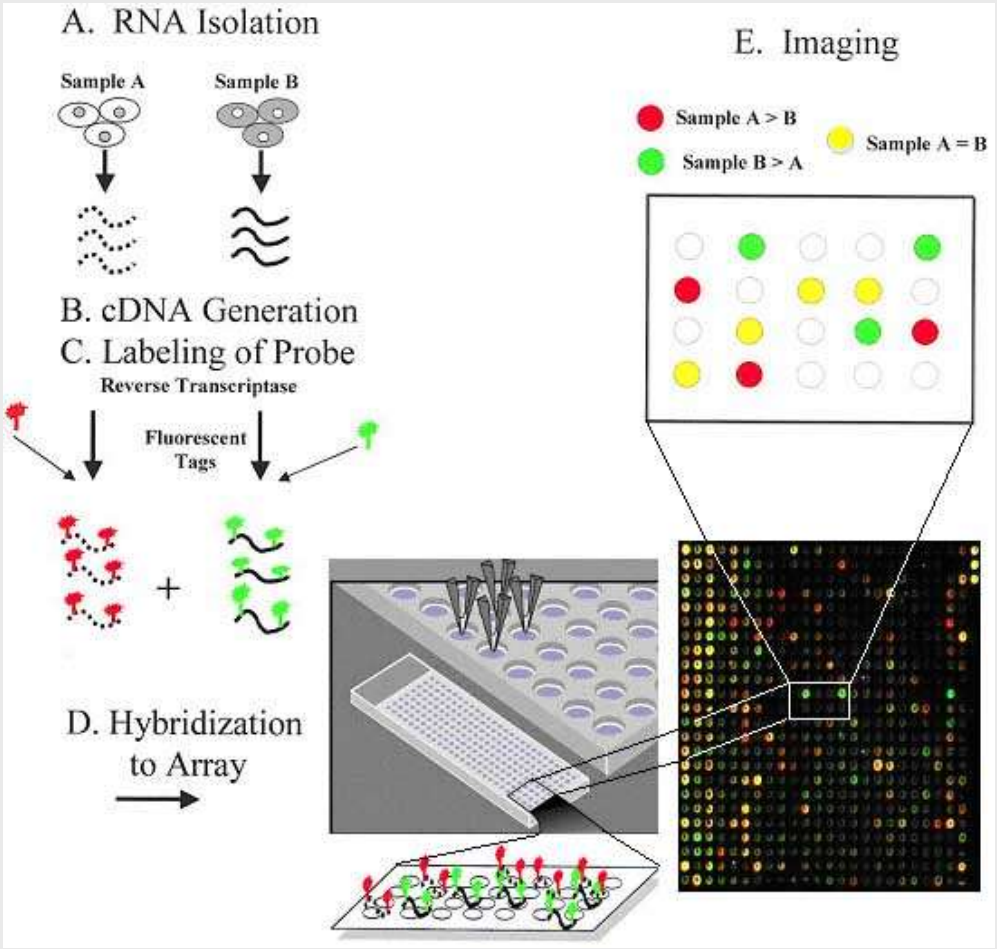
www.pnas.org/cgi/doi/10.1073/pnas.242579699 PNAS | November 26, 2002 | vol. 99 | no. 24 | 15687–15692

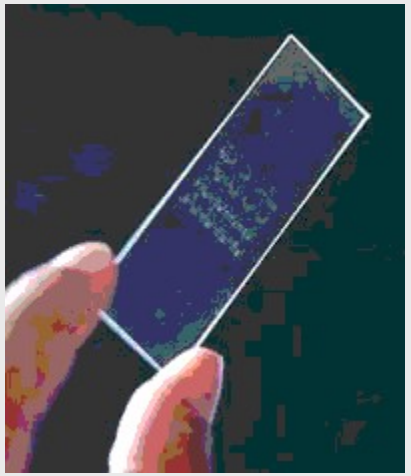
Similar to nucleic acid hybridization assay (NASHA) except that the probe is fixed to a solid matrix rather than the unknown being fixed

To identify cause of orphan diseases:

- Coat microarray with short sequences from known viruses
- Label unknown sample and hybridize to microarray

4. Microarrays





Microarray Technology

Strengths:

- Can screen for a large number of viral genera/virus species/strains
- Rapid
- Might be good at identification of new species within known genera

Limitations:

- Very expensive technique
- Lots of false positives
- Requires specialized laboratory and highly trained operators
- Not very good at identifying new genera

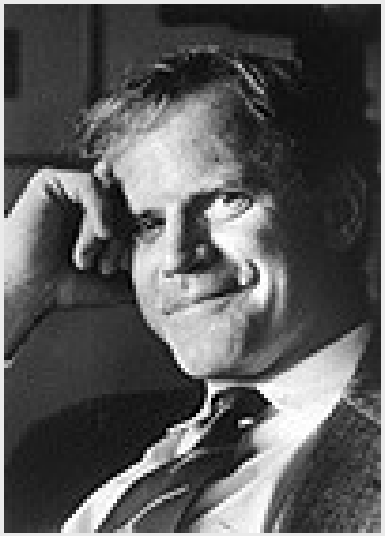
Amplification Assays

PCR, RT-PCR

RCA

RPA

Polymerase Chain Reaction



Kary Mullis

Received a Nobel prize for improving on PCR

"One of several scientists who, after success in their area of research, go on to make unfounded, sometimes bizarre statements in other areas" Denies Aids/HIV connection, global warming and promotes astrology.

Polymerase Chain Reaction - Animations

(PCR animation**):** DNA replication can be harnessed to increase the abundance of specific sequences, and these selectively amplified sequences can then be identified by the use of the appropriate label, e.g. radioactivity or fluorescence.

More animations (with voice):
http://www.youtube.com/watch?v=eEcy9k_KsDI&feature=related

Polymerase Chain Reaction (PCR)-Based Assays

Specificity of PCR can be adjusted based on the sequence of the primer

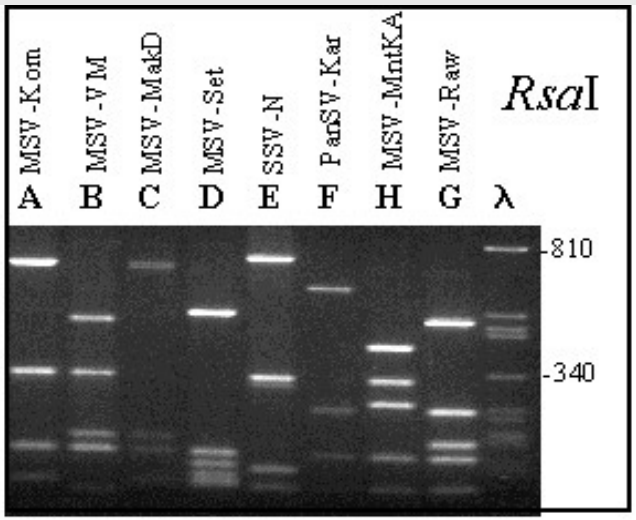
- **Primers can be designed to detect one virus/viroid or**
- **Primers can be designed to be genus-specific or family-specific**

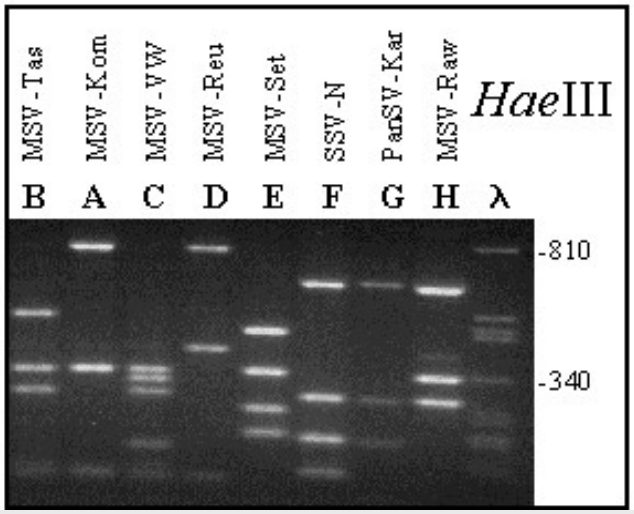
Polymerase Chain Reaction

PCR lends itself to downstream applications

- Amplified DNA may be digested to analyze RFLP pattern

- Amplified DNA can be sequenced and compared with known sequences to determine the identity of the virus





Polymerase Chain Reaction

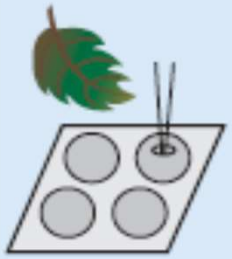
One problem with PCR for us is the sensitivity of *Taq* to plant compounds:

Sources of viral nucleic acid for detection:

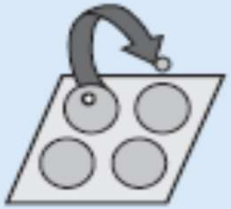
- Fresh, frozen, dried plant tissue
- Nucleic acid extracts from tissue
- Cloned DNA in *E. coli*
- Nucleic acid on FTA cards

- Nucleic acid on FTA cards

FTA Plant Protocol Overview



Sample Application
Press plant tissue onto the card or apply homogenate. Allow to dry completely.



Disk Removal
Punch a disk out of the FTA matrix impregnated with plant material.

FTA Purification Reagent Washes
Place the disk in PCR tube and wash twice with FTA Purification Reagent. Discard used reagent after each wash.

TE⁻¹ Rinses
Wash twice with TE⁻¹ buffer (10 mM Tris, 0.1 mM EDTA pH 8.0) and discard used buffer after each wash.

Drying Step
Dry disk in PCR tube.

Direct to PCR
Add PCR master mix directly to the disk and amplify.

WHATMAN CATALOG ORDERING INFORMATION

Cat. No.	Description	Qty/Pack
WB120205	FTA Classic Card	100
WB120206	Indicating FTA Classic Card	100
WB120055	FTA Mini Card	100
WB120056	Indicating FTA Mini Card	100
WB120210	FTA Micro Card	100
WB120211	Indicating FTA Micro Card	100
WB120208	FTA Gene Card	100
WB120028	CloneSaver Card	10
WB120204	FTA Purification Reagent	500 mL
WB100030	FTA Gene Card Tray	20
WB100005	Harris Micro Punch 1.2 mm (with Mat)	1
WB100006	Replacement Tip 1.2 mm	1
WB100007	Harris Micro Punch 2.0 mm (with Mat)	1
WB100008	Replacement Tip 2.0 mm	1
WB100020	Replacement Cutting Mat	1
WB100028	Harris Uni-Core 1.25 mm punch	4
WB100029	Harris Uni-Core 2.0 mm punch	4
WB100010	Multi-Barrier Pouch, Large	500
WB100011	Multi-Barrier Pouch, Small	500
WB100024	CloneSaver™ Resealable Multi-Barrier Pouch	50
WB100016	FTA Card Mailer	50
WB100003	Desiccant Packets (1 gm)	1000

Polymerase Chain Reaction & FTA Cards

Example: Detection of Begomoviruses by PCR from FTA cards

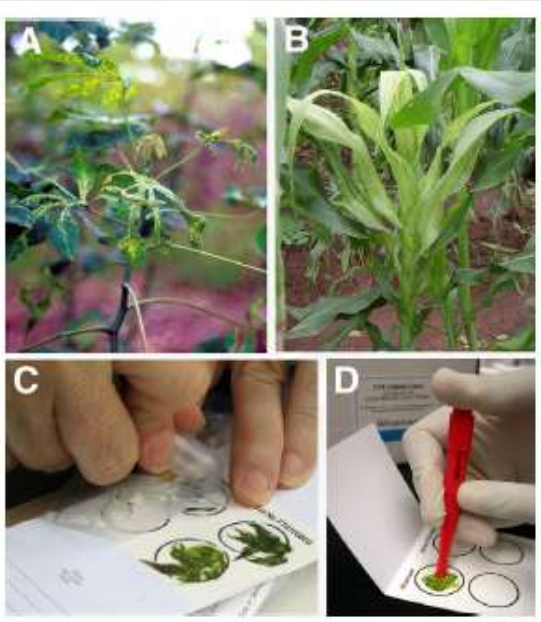
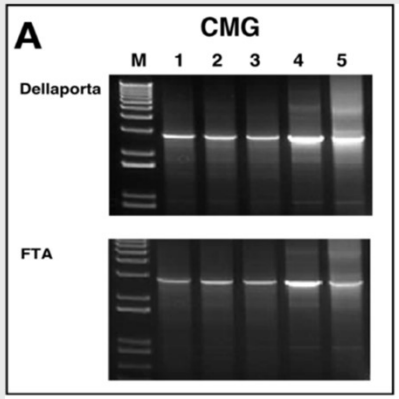
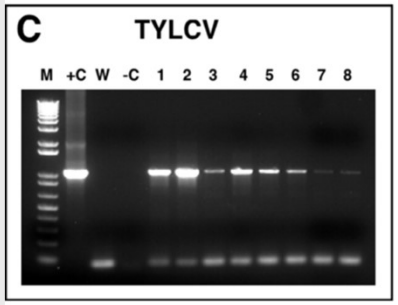
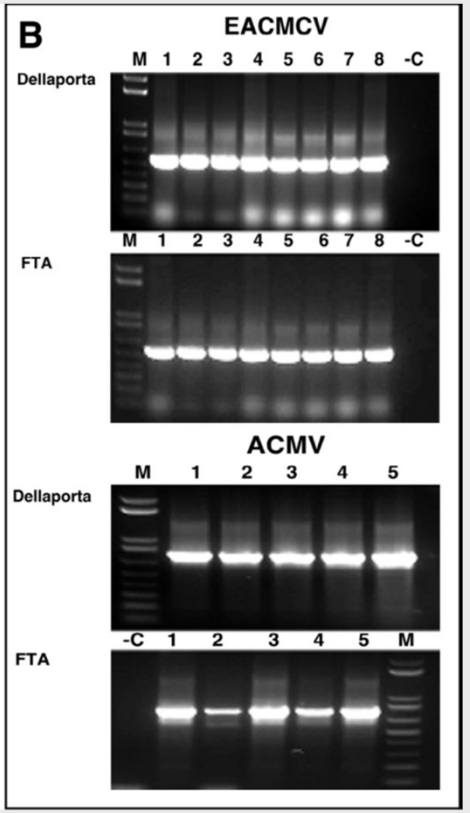


Figure 1
Disease symptoms on field grown cassava and maize plants and FTA sampling method. (a) cassava plant in Western Kenya showing severe cassava mosaic disease symptoms (b) severe maize streak virus symptoms on maize plant in farmer's field in Malawi (c) symptomatic leaves are pressed into FTA Classic Cards (d) 2 mm diameter punches being removed from FTA Classic Card for subsequent viral DNA elution and molecular analysis







•Ndunguru et al 2005. Virology Journal 2:45

Polymerase Chain Reaction (PCR)-based Assays

Strengths:

- More rapid than some assays
- Highly sensitive
- Suitable for handling few to mid-range no. of samples
- PCR products suitable for cloning and sequencing
- Multiplexing possible (simultaneous detection of several different viruses) utilizing primer mixtures (Dietzen et al., 2001, Plant Disease 85: 989-992)
- Lends itself to addition assays

Polymerase Chain Reaction (PCR)-Based Assays

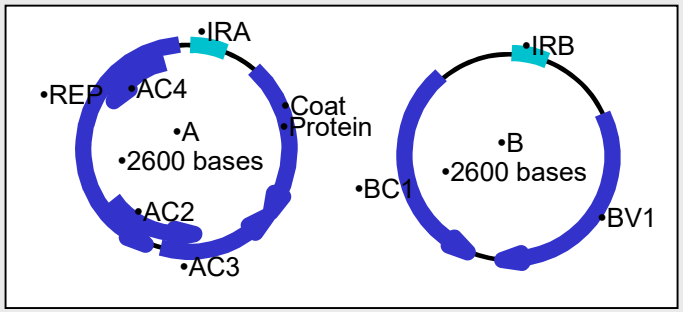
Limitations:

- Extreme sensitivity creates problems with contamination from other samples, equipment, aerosols, etc.
- Taq polymerase is sensitive to plant compounds so nucleic acid needs to be free of plant chemicals (polysaccharides, tanins, polyphenols, etc..)
- Requires special and expensive equipment, reagents, expertise
- Need to have a sequence of a virus to design the primers
- Amplified PCR product may not be representative of the dominate sequence in the virus population.

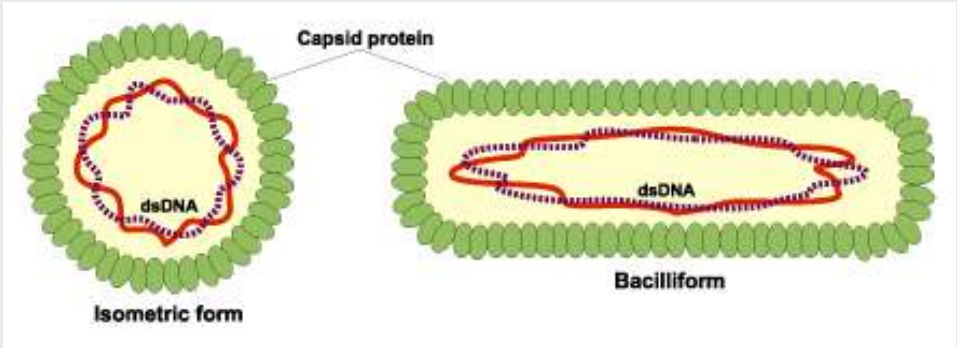
Rolling circle amplification (RCA)

Best for detection of viruses that have circular DNA genomes

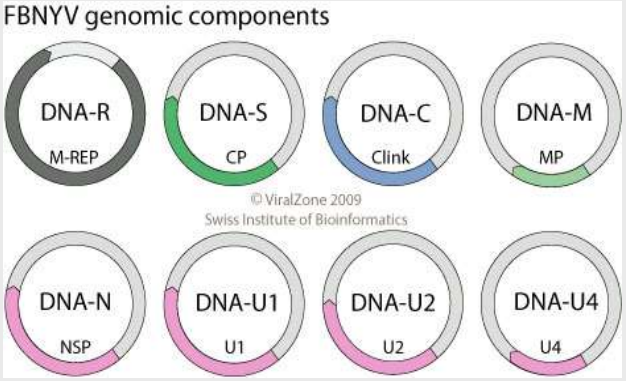
Geminiviridae



Caulimoviridae



Nanoviridae



Amplification Scheme:

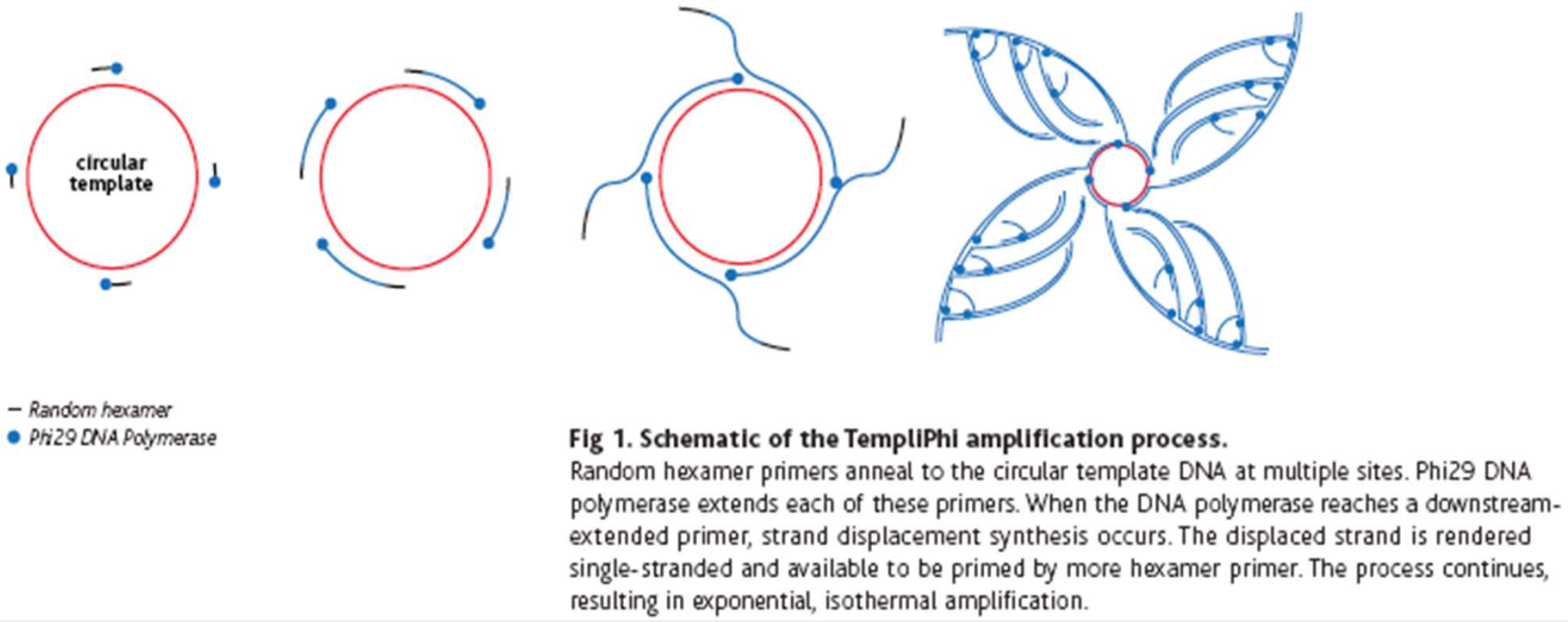
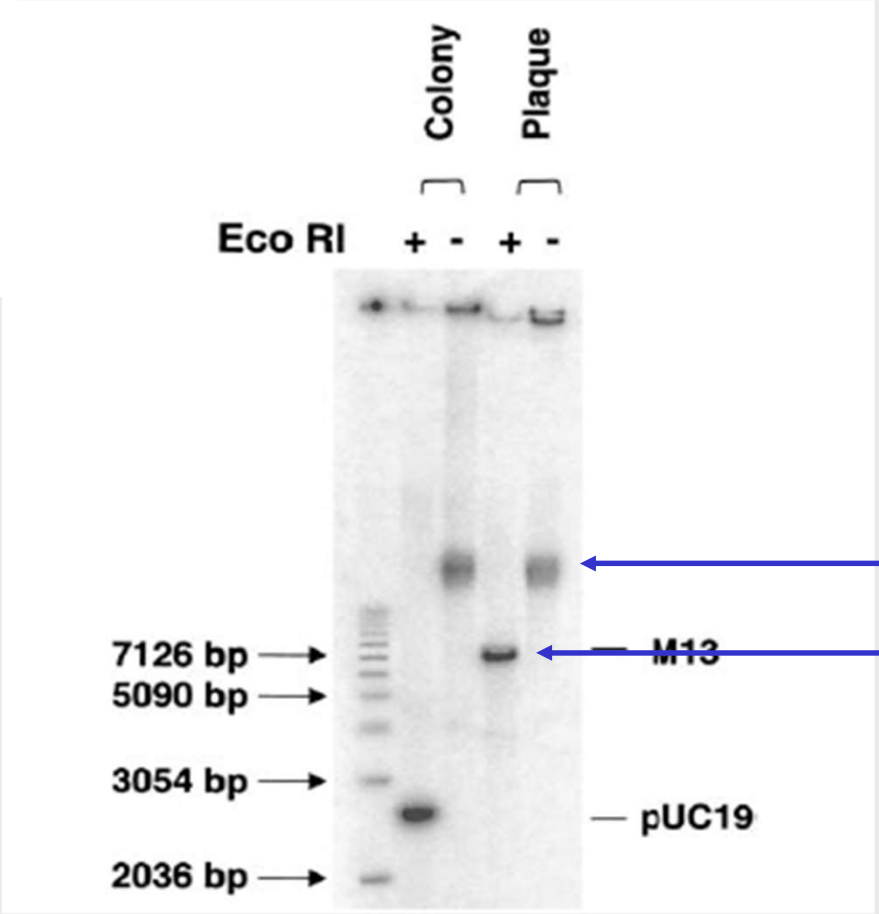


Fig 1. Schematic of the TempliPhi amplification process.
Random hexamer primers anneal to the circular template DNA at multiple sites. Phi29 DNA polymerase extends each of these primers. When the DNA polymerase reaches a downstream-extended primer, strand displacement synthesis occurs. The displaced strand is rendered single-stranded and available to be primed by more hexamer primer. The process continues, resulting in exponential, isothermal amplification.

- Relies on the replication polymerase from *Bacillus phage Phi29 (Podoviridae, Phi29likevirus)*
- This polymerase has high fidelity and preferentially amplifies circular DNAs

Result:

Example using M13 a bacteriophage



Amplification results in concatemers: long strands of DNA which are multiple copies of target DNA

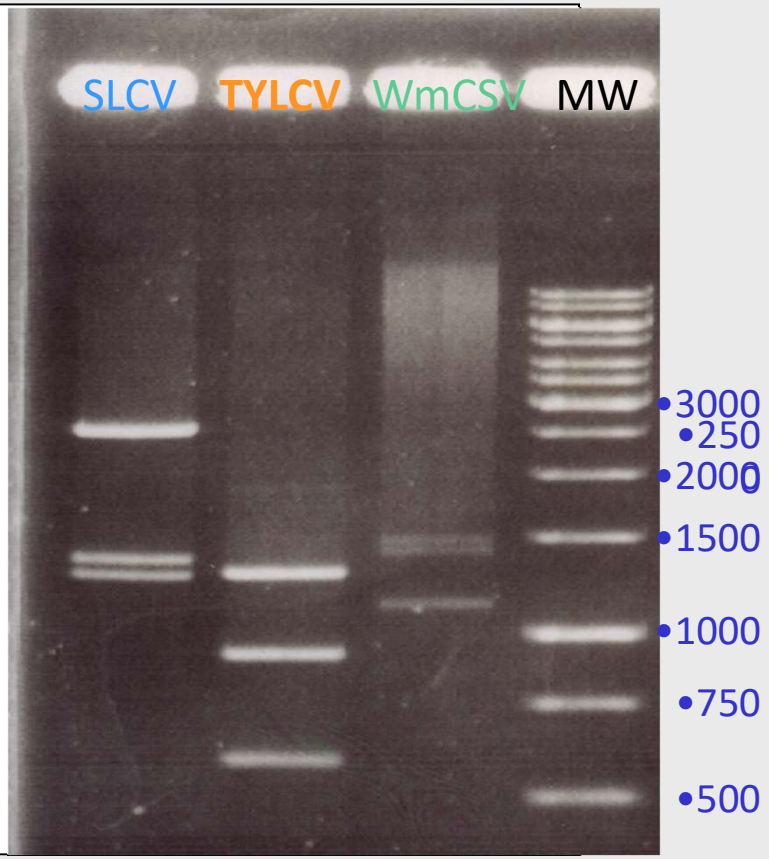
Unrestricted

Restricted (full length genome of M13 ~6400 nt

Rolling circle amplified DNA can be used for downstream applications: cloning, RFLPs, etc…

- Following rolling-circle amplification (RCA), total DNA was restricted with *Apa* I.

- The following bands are expected:
- SLCV = 2611, 1363, 1278
- TYLCV = 1300, 900, 600
- WmCSV = 1494, 1406, 1126, 1121



Rolling Circle Amplification

- Strengths:

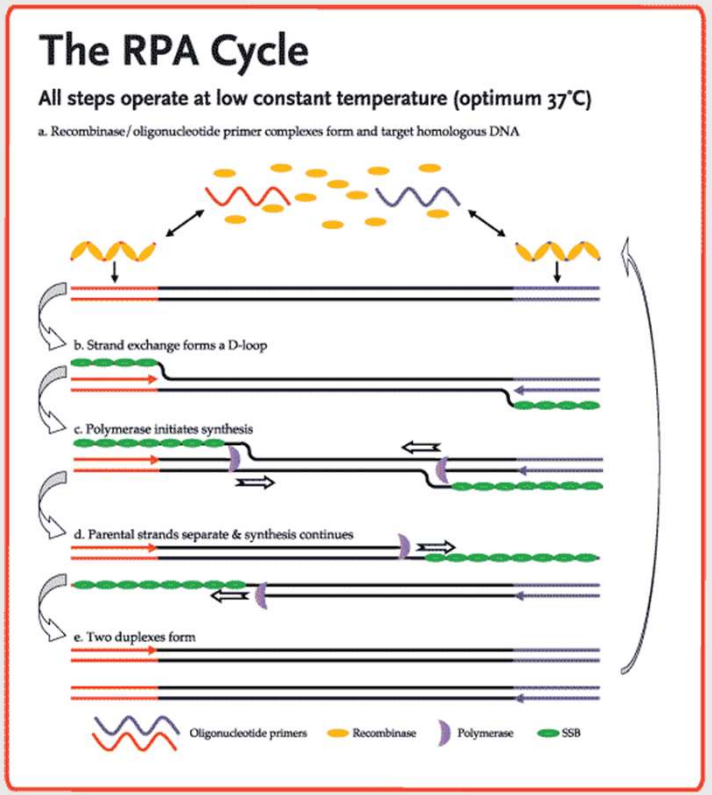
- Very rapid with simple sample preparation
- Needs no specialized equipment
- Highly sensitive
- Suitable for handling moderate number of samples
- RCA lends itself to downstream application

- Limitations:

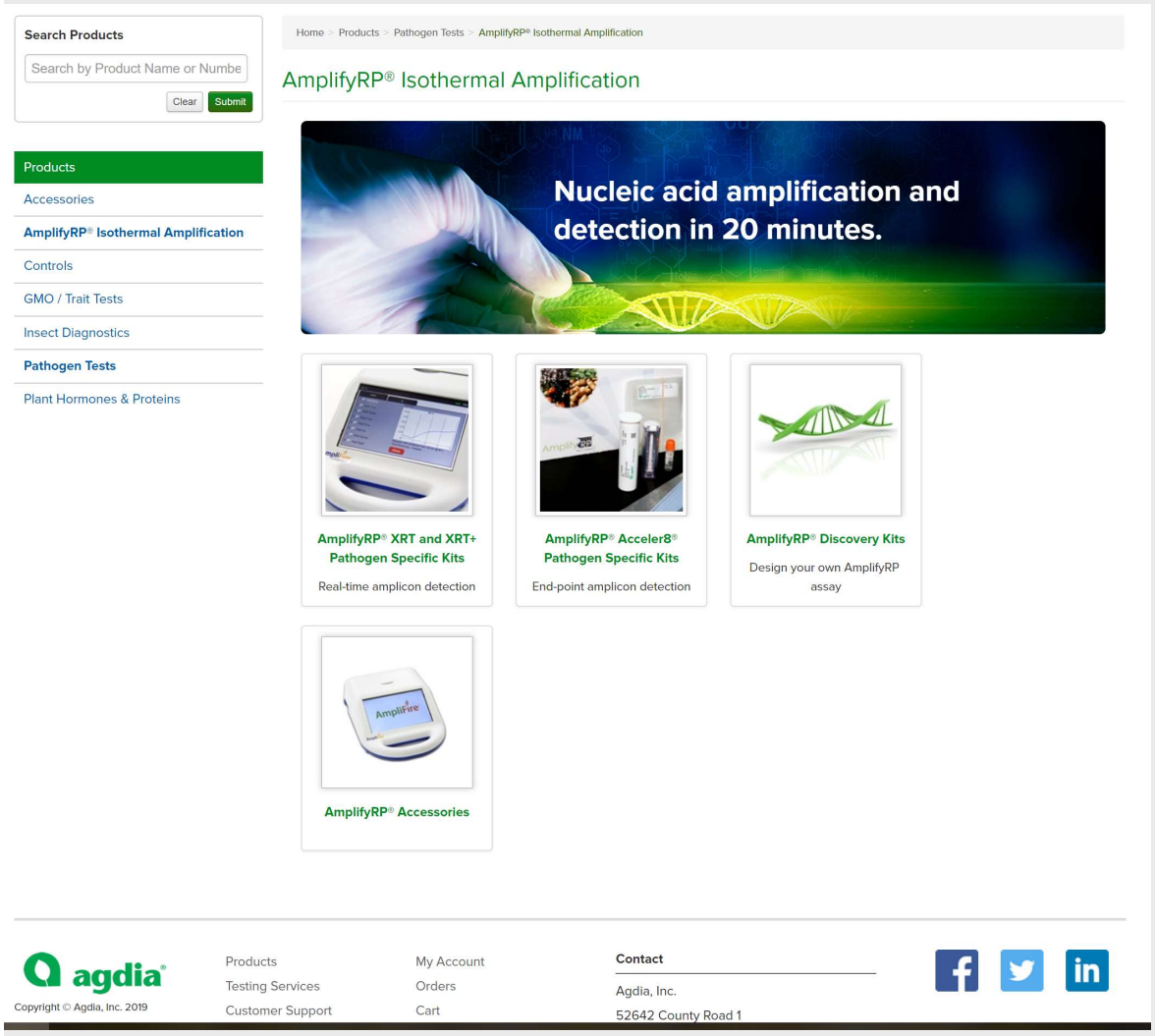
- Kit is relatively expensive
- Extreme temperature sensitivity, poor long term stability of Phi29 DNA polymerase
- Limited to use with DNA genomes
- Due to variations in sequence, there many be some variation in the results

Recombinase Polymerase Amplification

- Relatively new type of assay - first published in 2006 (Piepenburg et al 2006)
- Used more in medical than plant sciences but no. of available assays is growing
- Uses primers, recombinase, various nucleic acid binding proteins and a polymerase to amplify sequence
- Isothermal reaction, room temperature, 15 min incubation



SSB: Single-stranded DNA-binding proteins. Have high affinity to ssDNA and participate in DNA replication, recombination, and repair as accessory protein. SSB plays a role in separating DNA strands during replication and prevent ssDNA from re-forming a double helix.



Various options:

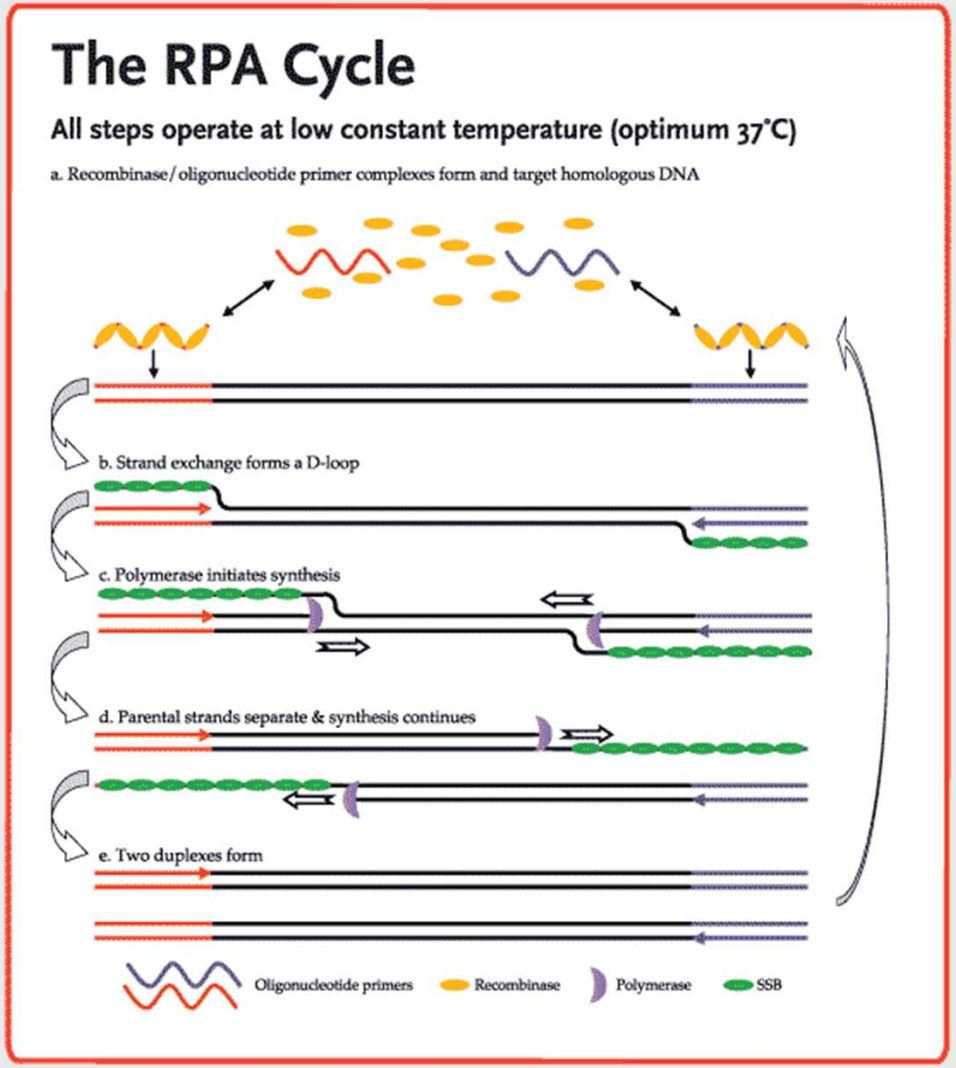
1) Kits for detection of specific pathogens (species of bacteria and viruses)

2) Design your own RPA assay

Explanation: How RPA works

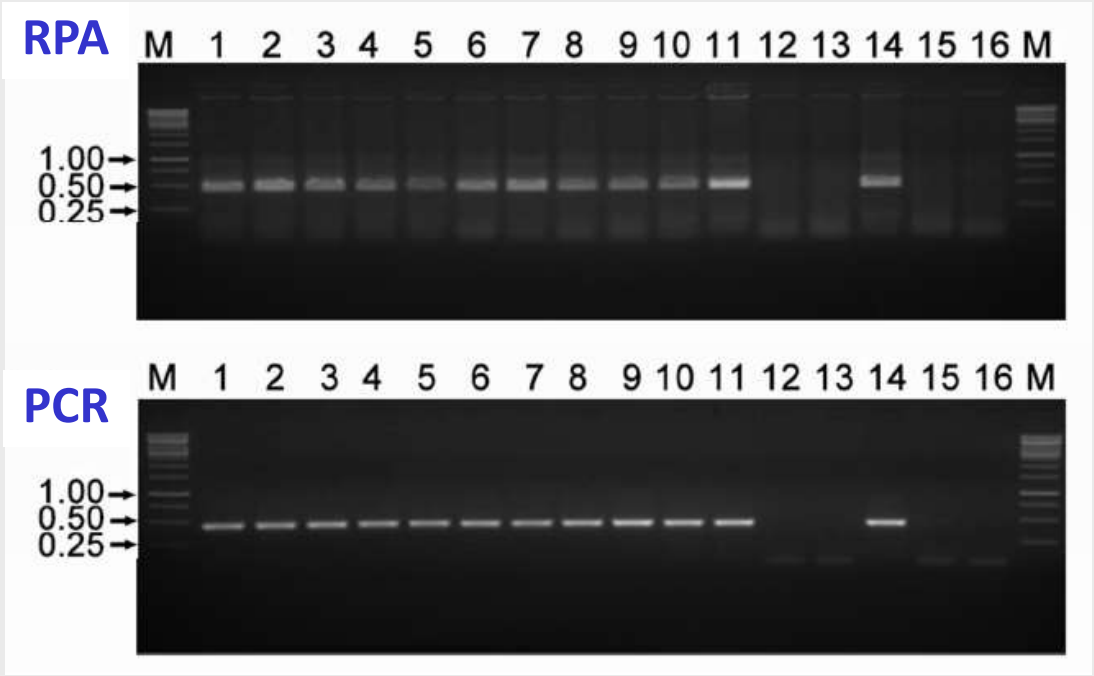
- Recombinase/oligonucleotide primer complexes form and target homologous target DNA sequences
- Strand exchange forms a D-loop (triple strands of DNA)
- Polymerase initiates synthesis
- Parental strands separate and synthesis continues
- Two duplexes form
- “Rinse and repeat”

Video: https://www.youtube.com/watch?v=x6AbsOrSAoY&vl=en



Recombinase Polymerase Amplification

- Primers are longer than those in PCR (30-40 nt)
- Amplicon must be smaller than 500 bp usually but can be modified to amplify larger amplicons
- Less sensitive than PCR when tested against pure DNA
- **BUT** more sensitive than PCR in the presence of plant contaminants
- Faster than PCR
- Many downstream applications: Amplicons can be cloned, restricted, direct sequenced, etc



13 samples of tomato, 1-11 with symptoms typical of TYLCV, 12-13 showing questionable symptoms, 14 pos. control, 15-16 water controls

Recombinase Polymerase Amplification

Strengths:

- Very rapid with simple sample preparation (20 min reaction time)
- Needs no specialized equipment or expertise
- Highly sensitive
- Suitable for handling few to moderate number of samples
- RPA lends itself to downstream applications
- Tolerant of plant contaminants (unlike PCR, RT-PCR)

Limitations:

- Its new
- Kit is relatively expensive ($5 per reaction) compared to some other assays, about the same as PCR
- Primers are longer so a bit more expensive

Identification by Sequencing the Viral Genome

- By sequencing part or all of the genome using DNA generated by PCR, RCA, RPA or cDNA from dsRNA
- Using Ecogenomic methods (covered in lab lecture on metagenomics)
- Sequencing the genome of a purified virus
- Compare new sequence with known sequences using appropriate algorithms to know if virus is new, known, or related to known viruses

Diagnosis By Sequencing The Viral Genome

Strengths:

- Necessary to establish the relationship of a new virus within a genus
- Only method that allows for the defining of a virus as a species
- Probability of misleading results very low
- Allows you to determine relatedness to other viruses

Limitations:

- Relatively expensive technique
- Must have personnel with appropriate and specific training
- Time consuming especially for viruses with larger genomes
- Sequence variation may not be readily related to the biological characters (silent mutations).
- Best if sequence is based on infectious clones of the virus (not always available)