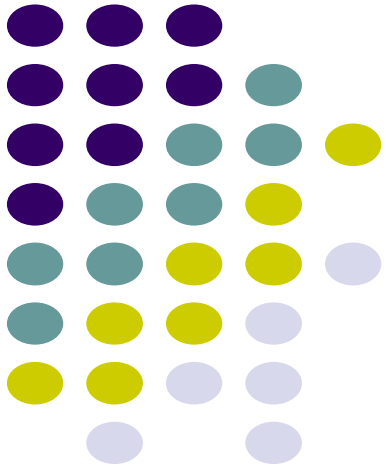
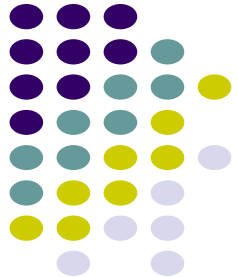
Nucleic acid based techniques



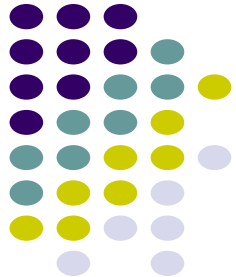


- Some pathogen detection methods are DNA/RNA based and they overcome uncertain diagnosis or pathogen taxonomy, enabling a rapid and accurate detection and quantification of pathogens
- In 1993, Nobel prize was awarded to K. Mullis for amplification of nucleic acid sequences using the technology of polymerase chain reaction (PCR).
- Based on the fidelity of DNA hybridization and replication PCR is used for highly specific detection of fungi, bacteria, viruses, phytoplasma, etc.

- PCR technique can provide high sensitivity and specificity due to the fidelity of DNA amplification. Success of PCR depends on the efficacy of DNA extraction and the performance is affected by inhibitors present in the sample assay, polymerase activity, PCR buffer and concentration of deoxynucleoside triphosphate.
- In addition, application of PCR for pathogen detection requires designing a primer to initiate DNA replication, which could limit the practical applicability of this technique for new or unknown pathogens.

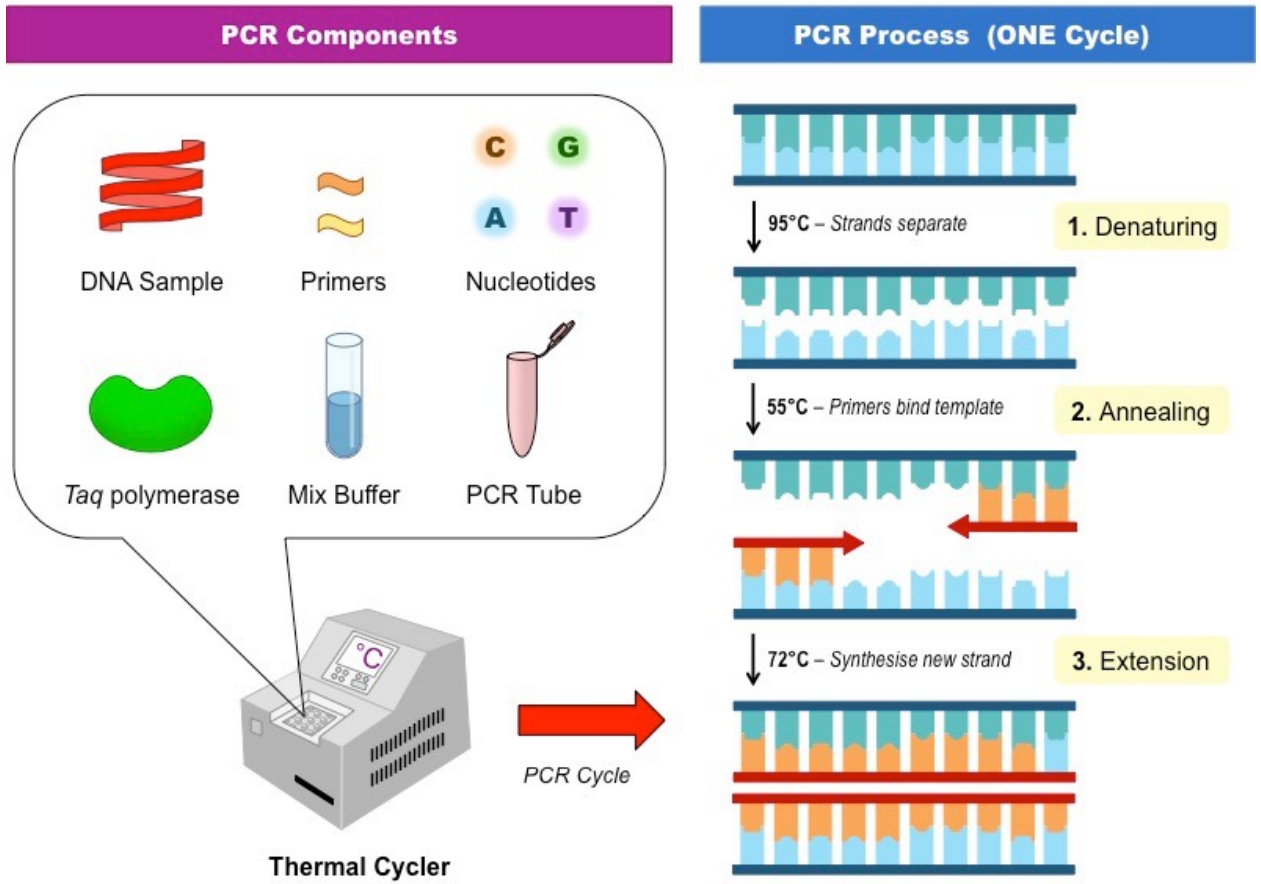
PCR based methods

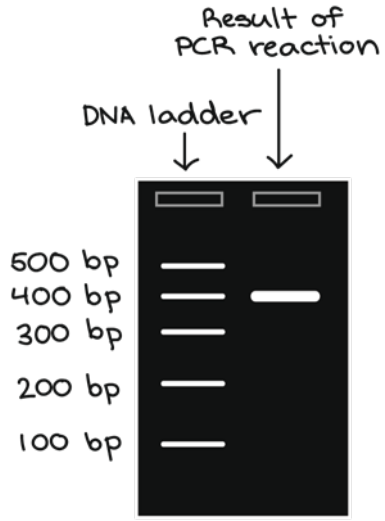
- PCR based methods can be used for different genomes: ssRNA, ssDNA, or dsDNA
- PCR offers several advantages:
  - the capability to detect a single target in complex mixtures,
  - rapid and specific detection of multiple targets, and
  - the potential to detect unculturable pathogens such as viruses or some bacteria and phytoplasma.
- Genome extraction of pathogens could be done either following manual techniques or using commercial kits specifically designed to extract nucleic acids from different types of plant material.



- All molecular detection methods for detecting plant pathogens are based on the accurate design of oligonucleotides and probes. Target sequences can be found using the GenBank® Nucleotide Sequence Search program provided by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).
- Conserved regions for each target can be identified using the Basic Local Alignment Search Tool (BLAST), with the BLASTn program designed for analysis of nucleotides. Specific nucleotide regions are selected and primers specific for DNA or RNA targets can be easily designed.

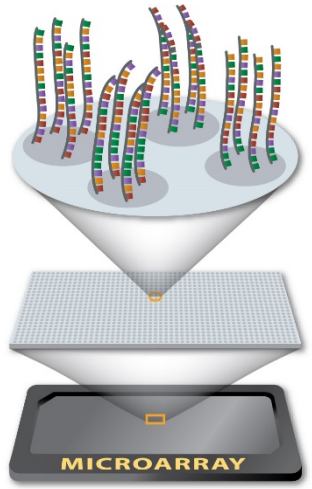
- Primers are designed to pair with unique DNA regions from target organisms for DNA amplification and detection. The presence of the amplification product confirms the presence of the organism in the tested sample.
- The amplified product is visualized through agarose gel electrophoresis with ethidium bromide (EtBr) staining.
- Now less toxic and more sensitive SYBR GREEN detection method is used under UV irradiation. Generally, PCR can be performed in 2–3 h, but more advanced systems can deliver a result in minutes.


PCR Components
PCR Process (ONE Cycle)
DNA Sample
Primers
C
G
A
T
Nucleotides
Taq polymerase
Mix Buffer
PCR Tube
°C
Thermal Cycler
PCR Cycle
95°C – Strands separate
1. Denaturing
55°C – Primers bind template
2. Annealing
72°C – Synthesise new strand
3. Extension


Result of PCR reaction
DNA ladder
500 bp
400 bp
300 bp
200 bp
100 bp

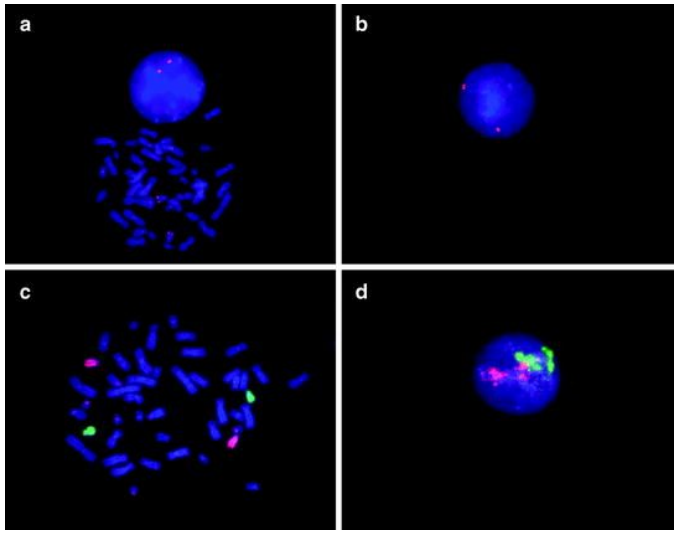
DNA microarray

- DNA microarrays are promising high-throughput tools for multiple pathogen detection.
- A microarray consists of a solid matrix, usually a glass slide, on which oligonucleotide probes or other DNA fragments are placed in very precise locations at high density. Target DNA sequences in a sample are then hybridized to the probes and detected by fluorescence.
- The advantage of microarray-based detection is the combined powerful nucleic acid amplification strategies with a massive screening capability, resulting in a high level of sensitivity, specificity, and high throughput capacity; it can detect many different pathogens in a single assay.



FISH

- A hybridization technique used for detection of plant pathogens is the *Fluorescence In situ Hybridization* (FISH).
- This technique use 16S or 23S rDNA oligonucleotide probes labelled with a fluorescent dye in combination with fluorescence microscopy.
- FISH probes (20-30mers) recognize the pathogens in plant tissues/cells fixed in a microscopic slide and hybridize with target gene in of the pathogen in the plant samples. The probe-target hybridization can be visualized by fluorescent light.
- FISH was used with probes to target the 23S rDNA to detect *Ralstonia solanacearum* in potato peels



- FISH could also be used to detect fungi and viruses and other endosymbiotic bacteria that infect plants.
- The high affinity and specificity of DNA probes provide high single-cell sensitivity in FISH, because the probe will bind to each of the ribosomes in the sample.
- However, the practical limit of detection lies in the range of around 10^3 CFU/mL.
- In addition to the detection of culturable microorganisms that cause the plant diseases, FISH could also be used to detect yet-to-be cultured (so called unculturable) organisms in order to investigate complex microbial communities.

Visualization of non-symptomatic downy mildew pathogen in Impatiens using FISH