BASICS ASPECTS OF MOLECULAR BIOLOGY AND DNA EXTRACTION.

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Regional Workshop on the prevention and diagnostic of *Fusarium* Wilt (Panama disease) of bananas and plantains caused by *Fusarium oxysporum cubensis* – Tropical Race 4 (TR4)
Port Spain, Trinidad and Tobago April 28th-May 9th, 2014

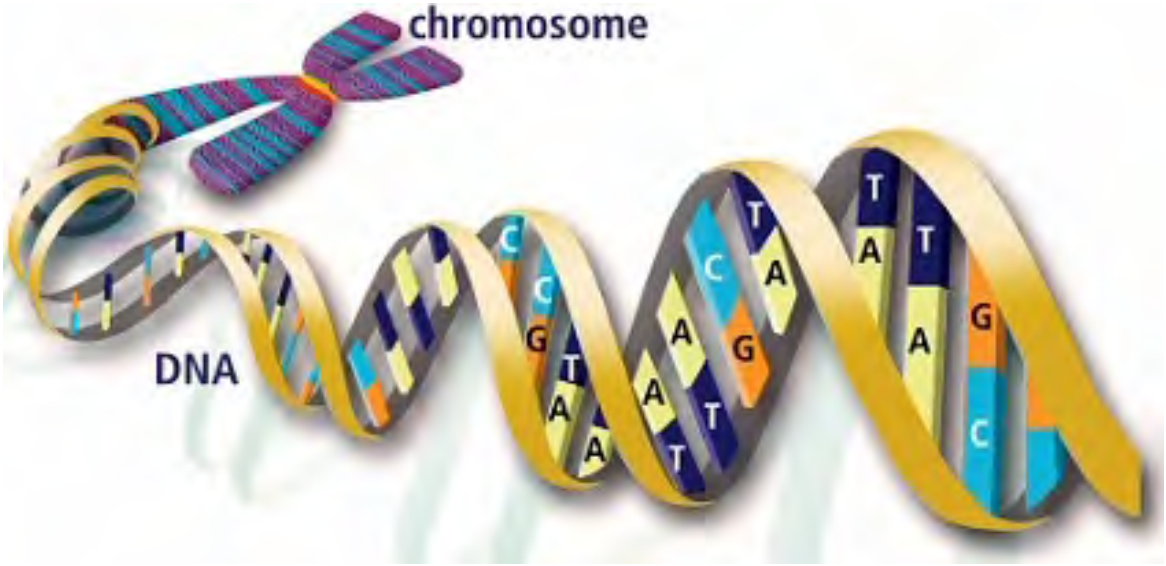
Molecular Biology

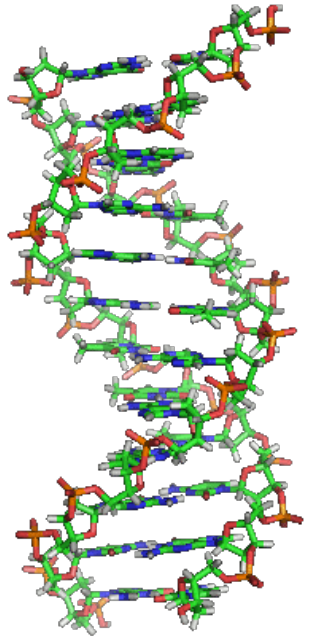
“Study of the structure, funtion and composition of biologically important molecules”

Mainly concerns itself with understanding the interactions between the various systems of a cell, including the interactions between the different types of DNA, RNA and protein biosynthesis as well as learning how these interactions are regulated to get a refined cell funtionability.

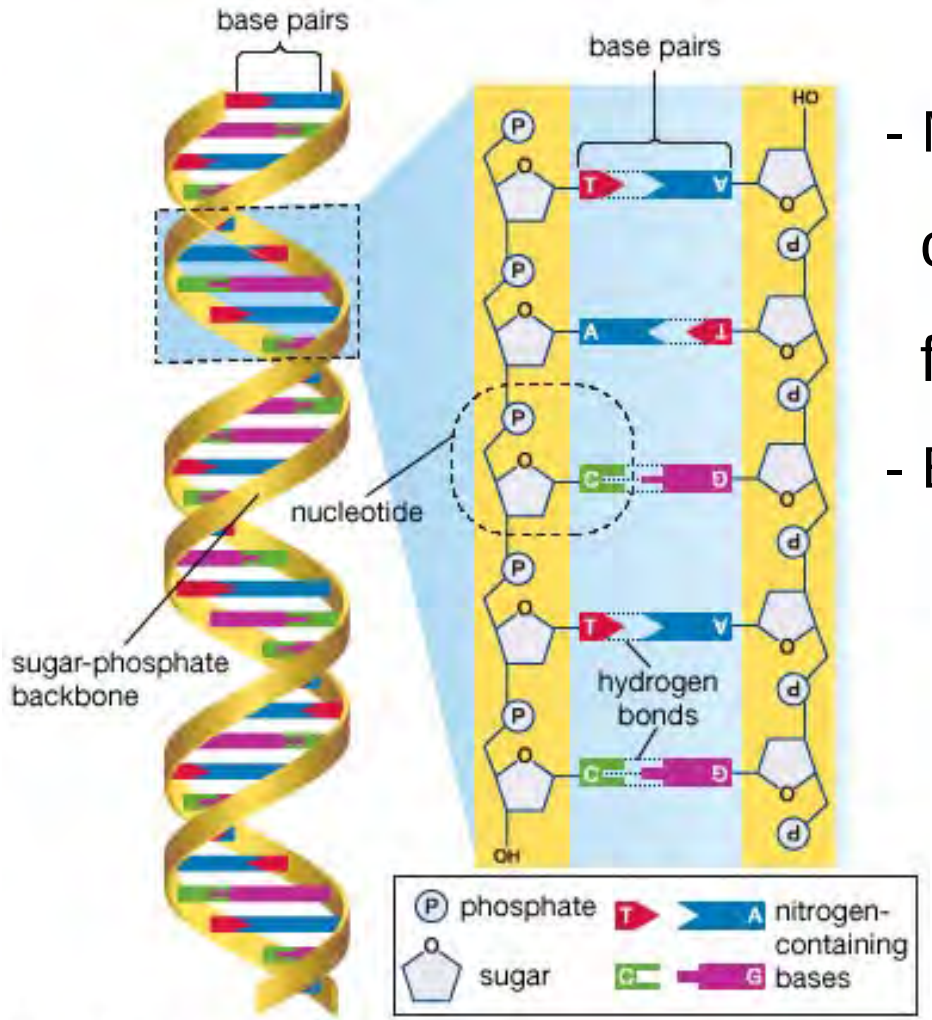
DNA Molecule (Desoxirribonucleic acid)

Is a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms and many viruses, being responsible of its hereditary transmission.





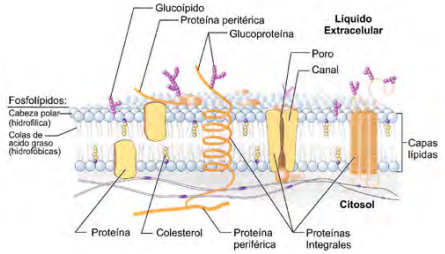
DNA Molecule



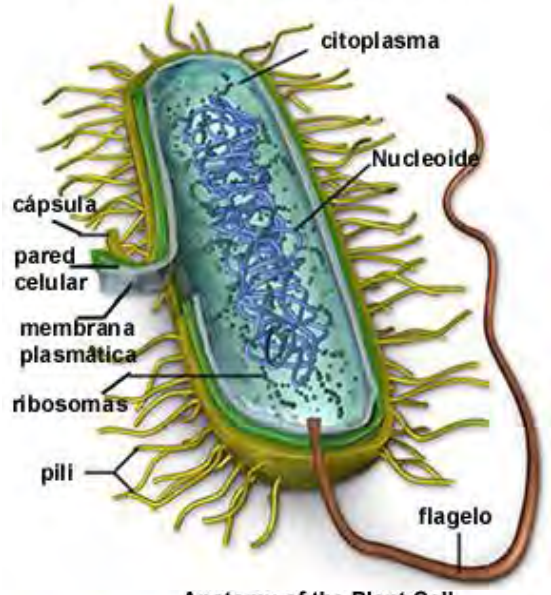
- Molecule of two strand strands coiled around each other to form a double helix
- Each strand is composed by:
  - Sugar
  - Phosphate
  - Base (adenine, thymine, cytosine, guanine)

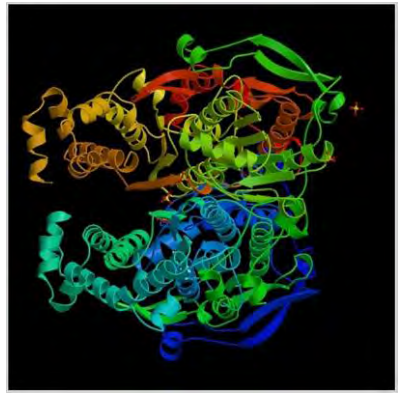
DNA EXTRACTION

LIPIDOS

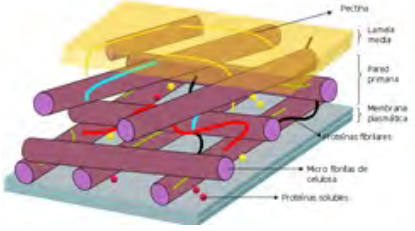


Procariotic cell structure





PROTEINS



Anatomy of the Plant Cell

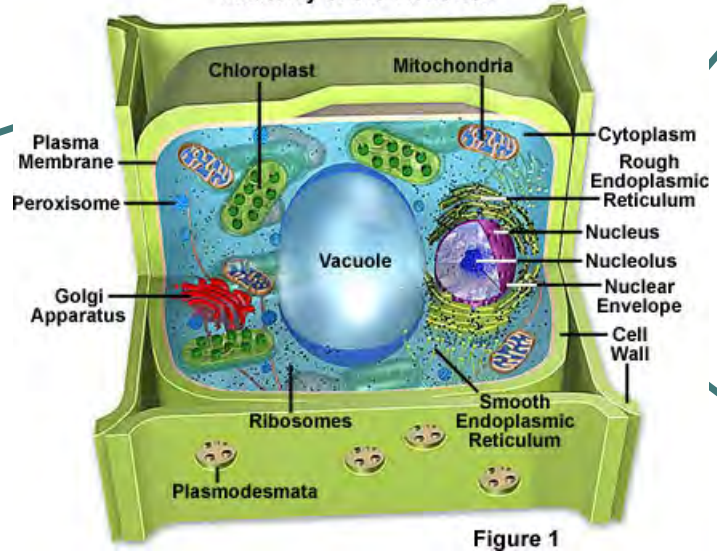
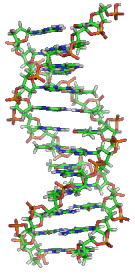


Figure 1



ATTGCCGTAACTG
TGGTAACCGTACC
GAAACCCTGCGTA

DNA EXTRACTION PROTOCOL

1.Lysis or cells disruption:

Extraction buffer and lysis buffer and incubation at 65°C:

NaCl (sodium chloride): phosphate of DNA molecule repel one molecule from others. Na+ ions form an ionic bond with phosphates and neutralized the negative charge allowing DNA molecules grouping.

EDTA (Ethylenediamine tetracetic acid): chelating agent with high affinity to metallic ions of Mg, DNAse cofactors (enzymes that degrade the DNA). EDTA bind to ions and overturn its effects.

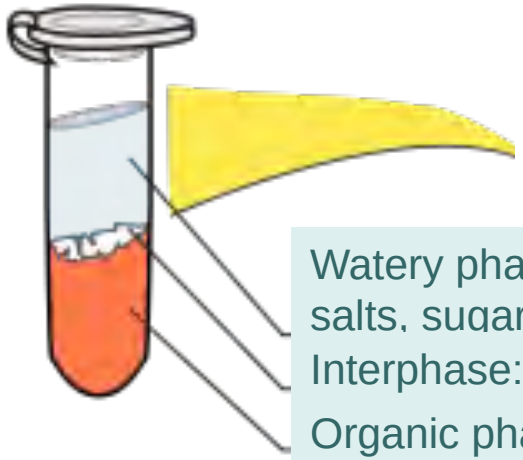
CTAB (Hexadecyl trimethyl-ammonium bromide): detergent used to brake cellular membranes and remove lipids

Other stabilizer agents : Tris HCl, sorbitol, sodium bisulphite, DTT, detergents: SDS (remove lipids), sarkosyl, triton, PVP (bind to polyphenols- components of vegetable cellular wall-removing), 2-mercaptoethanol (denature proteins).

DNA EXTRACTION PROTOCOL

2. Adition of chlorophorm-isoamyl alcohol (24:1) or phenol- chlorophorm-isoamyl alcohol (25:24:1). Centrifugation.

   Organic solvents, hydrophobics lysates keep trapped, eg. membrane lipids, proteins or polysacharids. Besides denature proteins.



Watery phase: hydrophilics compounds, nucleic acids, salts, sugars

Interphase: DNA

Organic phase: hydrophobic compounds

3. Proteins are eliminated adding a protease and increasing the osmolarity (sodium acetate or ammonium acetate)

DNA EXTRACTION PROTOCOL

4. DNA precipitates with alcohol – usually pure and could ethanol or isopropanol (2-propanol). Because DNA is non-soluble in alcohol, precipitate and form a pellet in the botton of the tube after centrifugation. This step also remove alcohol soluble salts.

DNA EXTRACTION PROTOCOL

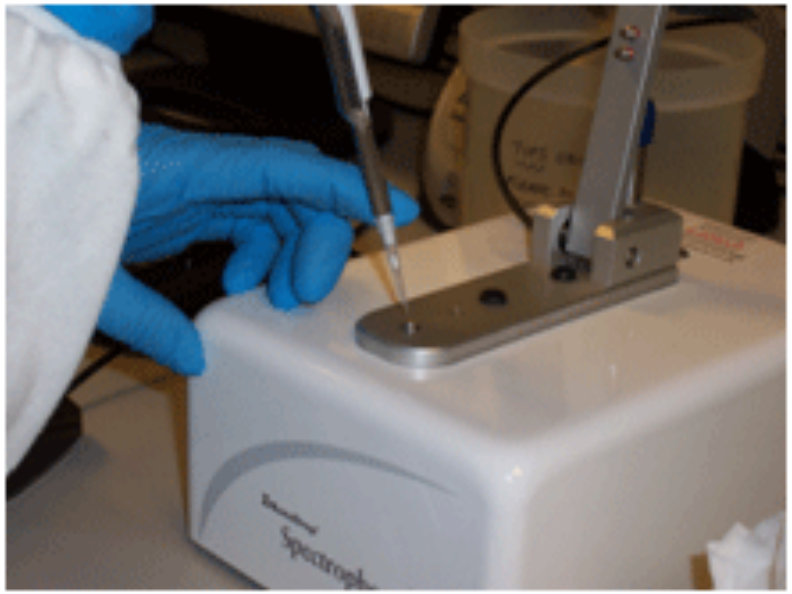
5. DNA cleans with 70% ethanol, dry and dilute in TE buffer (protect DNA from degradation) or sterile distilled water.

DNA Extraction kits

DNA Quantification

a. Espectrophotometer/ nanometer

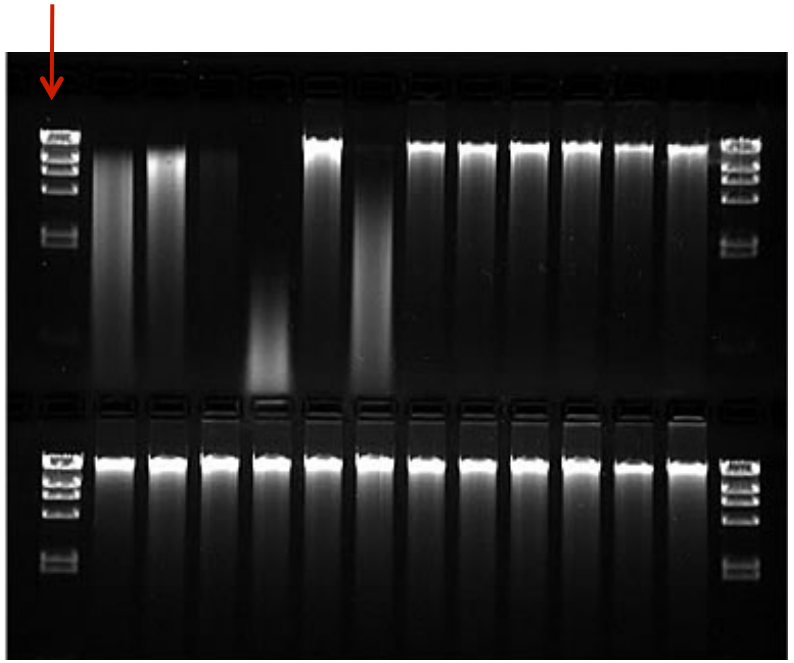
DNA/RNA absorb ultraviolet light with an absorption peak of 260 nm
Detector register the light that pass trough the sample (↑ absorption of light - ↑ concentration of nucleic acids)

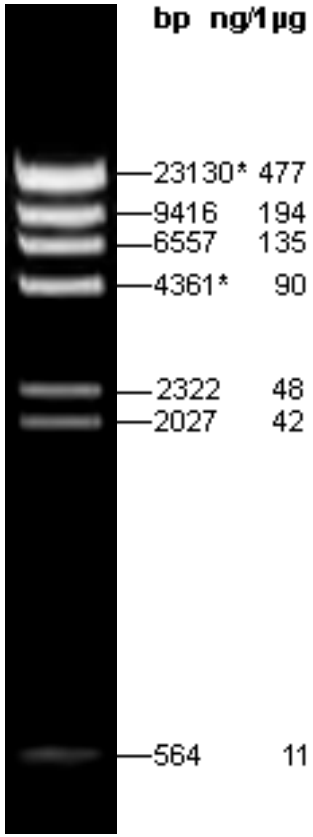


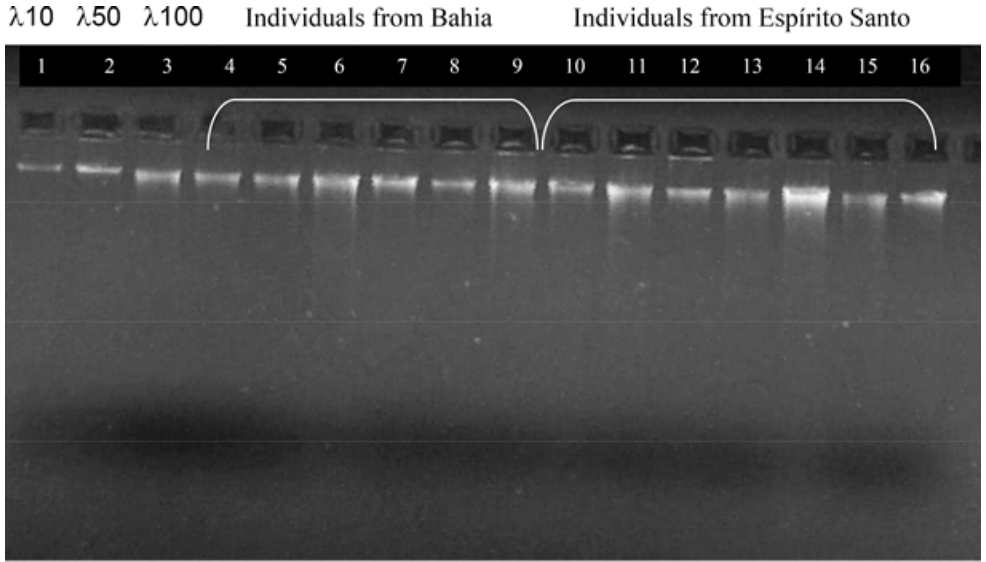
DNA/RNA is present
Results can be altered by contaminants (phenol, proteins)
260/280 = 1.8 stable
260/280 = 2.0 o >
(contamination with proteins)
260/280 = 1.6 o <
(contamination with con ARN)

DNA Quantification

b. Electrophoresis in agarose gels

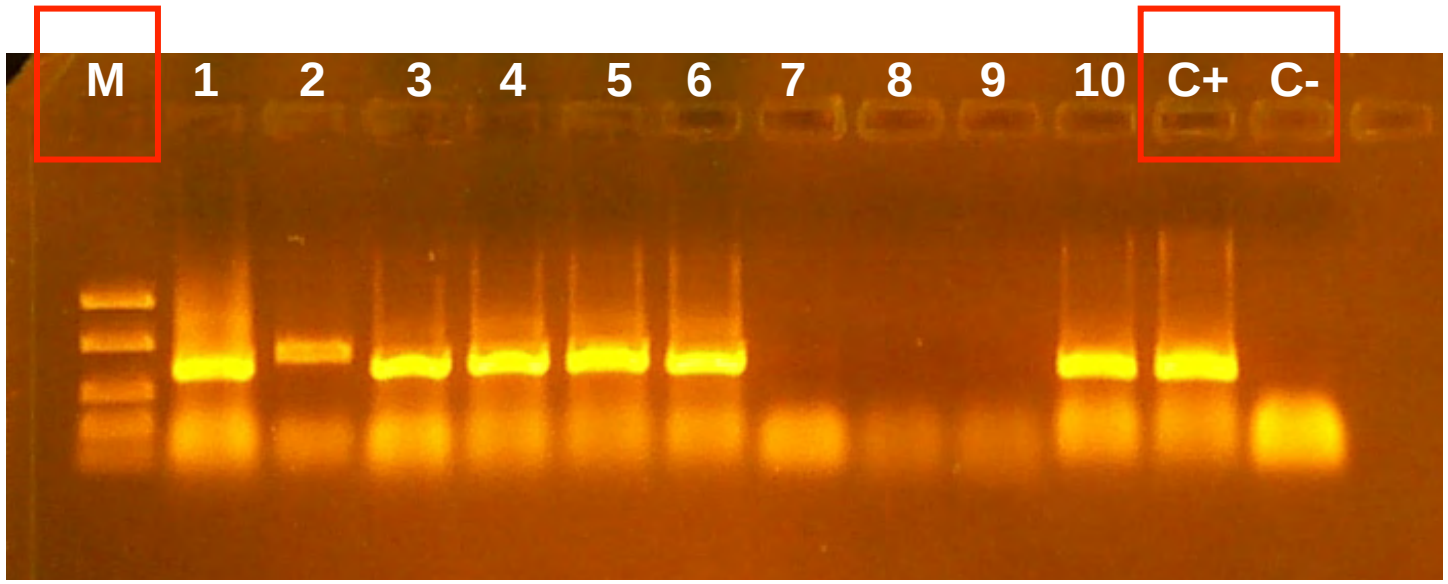






Electrophoresis in agarose gels

ATCGGTCATGGCCTAA
CTTATGGCCCAAAATG
GACTAGCTACGATAGC
TAGCTAGCTTAAACGAT
CGTAGCTAGTCGATATT
AAAAGCTATAGCTAGC
TAGCTAGTATATCGAAT
CGGTCATGGCCTAACT
TATGGCCCAAAATGGA
CTAGCTACGATAGCTA
GCTAGCTTAAACGATC
GTAGCTAGTCGATATTA
AAAGCTATAGCTAGCTA
GCTAGTATATCGAATCG
GTCATGGCCTAACTTAT
GGCCCAAAATGGACTA
GCTACGA



Positive result: presence of the fungi in the sample

Negative result: NO presence of the fungi in the sample

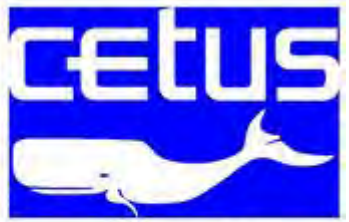
M: molecular weight ladder (indicate the base pares number of the band)

Polymerase Chain Reaction (PCR)

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., Arnheim, N. 1985. Primer-directed enzymatic amplification of *β*-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1230-1350.

Mullis, K.B. and Faloona, F. 1987. Specific sinthesis of DNA *in vitro* via polymerase catalysed chain reaction. *Methods Enzymol.* 55:335-350.

Kary Mullis (1944)

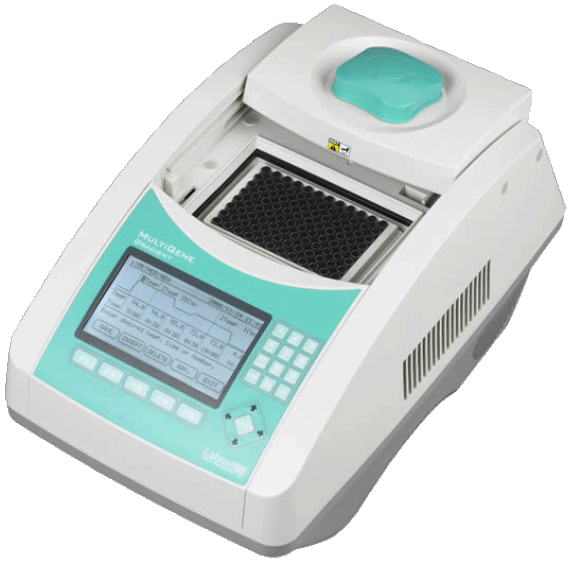


Primera compañía biotecnológica, fundada en 1971, en California.

Since then it´s included in more that 250000 scientific publications

THERMOCYCLER

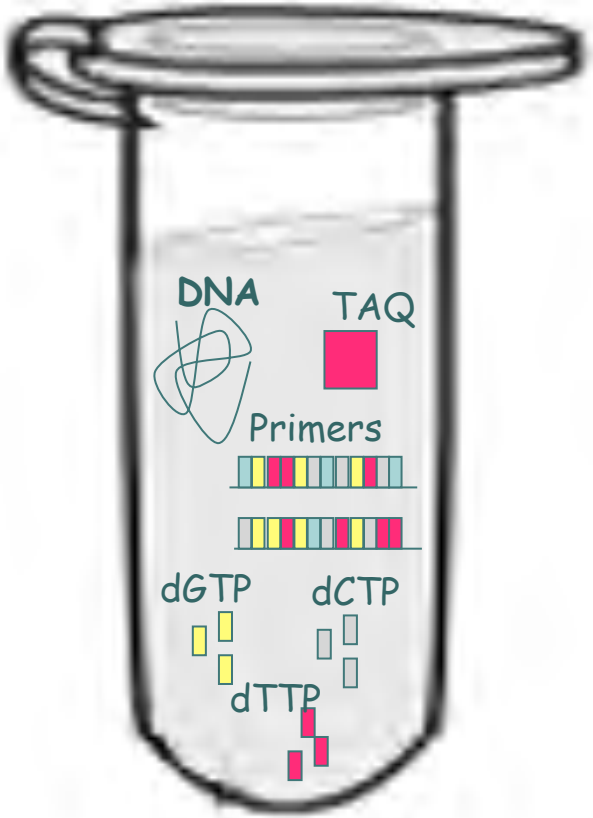
It´s a machine that heat and cool down the reaction in short periods of time

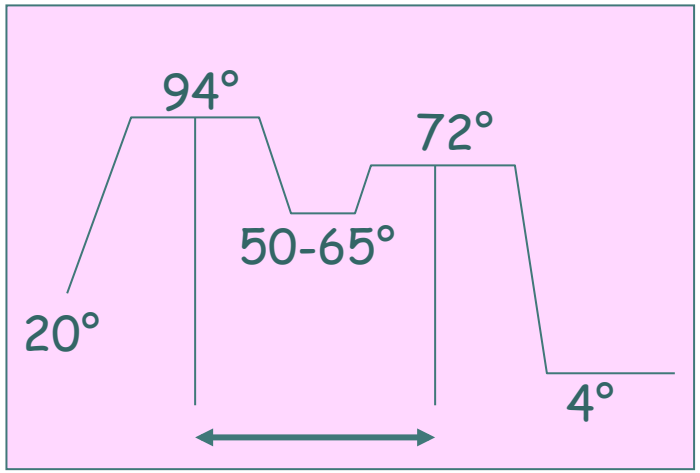


Reactive needed for a PCR reaction:

- ✓ DNA **template:** contains the DNA region (target) to be amplified. Can be used at different concentrations, but frequently at 10 ng.
- ✓ ***Buffer solution*** (Buffer 10x): provide a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- ✓ ***$MgCl_2$***: It´s used at 25mM -s a required cofactor for thermo-stable DNA polymerases, and magnesium concentration is a crucial factor that can affect the success of the amplification.
- ✓ ***Deoxynucleotide triphosphates*** (dNTP's: dATP, dGTP, dCTP, dTTP). Nucleotides containing triphosphate groups, the building-blocks from which the DNA polymerase synthesizes a new DNA strand
- ✓ ***Primers***: Short sequences of 20-24 nucleotides in length, that are complementary to the 3´ (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- ✓ Taq Polimerasa: DNA polimerasa de *Thermus aquaticus*

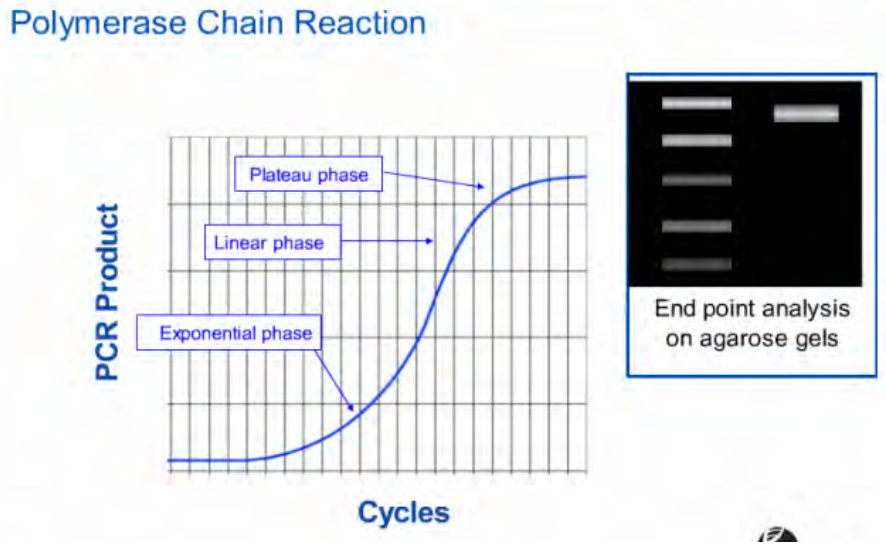
STEPS OF PCR REACTION:

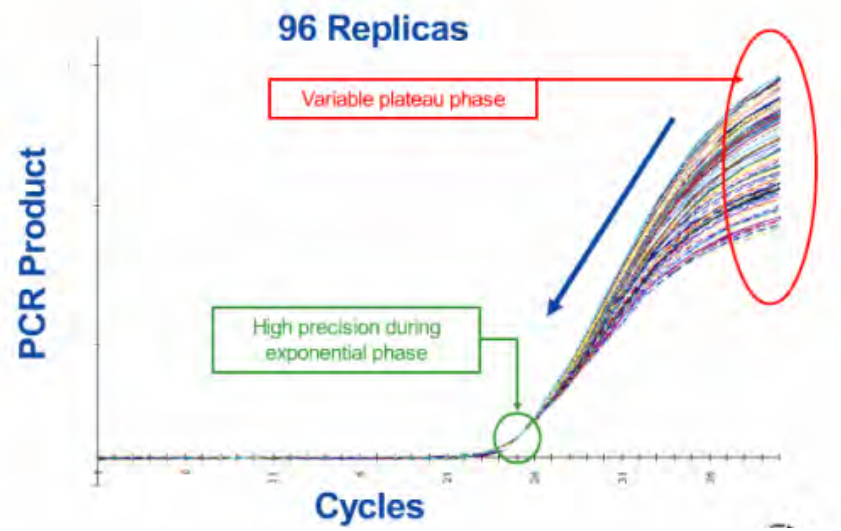




Other analysis: Real-Time PCR or quantitative PCR

The amplified DNA is detected as the reaction progresses in **"real time"**





http://www.youtube.com/watch?v=QVeVIM1yRMU

Other analysis:

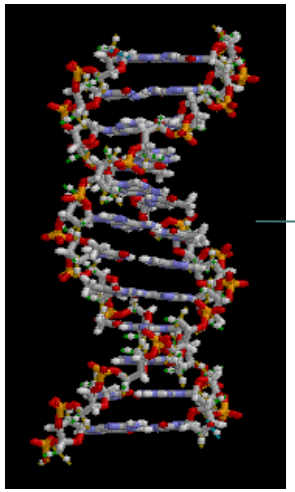
Sequencing:

- Amplicon generated in PCR is used

- Sample are cleaned or purified directly from PCR or band is extracted from the gel with special kits

- Websites like Gen Bank (www.ncbi.com) and Fusarium I-D (http://isolate.fusariumdb.org/index.php) allow to compare of sequence with other published in those sites (libraries)

Sequencing



CGGTCATGGCCTAACTTATGGCCCAAAAT

Unknown DNA

unknown: CGGTCATGGCCTAACTTATGGCCCAAAAT

***Pseudomonas* sp.:** CGGTCATGGCCTAACTTATGGCCCAAAAT

***Agrobacterium* sp.:** CGGTGTTGGCCTAACAAATGGCCCTCAAT

***Burkholderia* sp.:** AGGTCATCGCCTATGTTATGGGGGAAAAT

Result: unknown DNA has 100% of similarity with *Pseudomonas syringae* sequences

THANK YOU

Regional Workshop on the prevention and diagnostic of *Fusarium* Wilt (Panama disease) of bananas and plantains caused by *Fusarium oxysporum cubensis* – Tropical Race 4 (TR4)
Port Spain, Trinidad and Tobago April 28th-May 9th, 2014