Basics of Polymerase Chain Reaction (PCR)

Agenda

PCR Basics Part I	**Basics of Molecular Biology**
	Definition of PCR
	The phases of PCR
PCR Basics Part II	Definition of Real Time PCR
	Qualitative Real time PCR
	Quantitative Real Time PCR
PCR Basics Part III	Definition of Melting Temperature
	Melt Curve Analysis

Learning Objectives

The General Objective of this Module Is to Give You an Understanding of the PCR methods used with the GeneXpert

At the end of the training, you will be able to:

- List the elements involved in the PCR Process
- Explain the PCR Process and describe the PCR steps
- Define “RT-PCR” (2 possible meanings)
- Describe the RT-PCR curves, define the Ct
- Explain how quantitation can be performed with RT-PCR
- Define Melting Temperature
- Explain how Melting Curve Analysis allows to identify microbial resistance

Basics of Polymerase Chain Reaction (PCR) - I

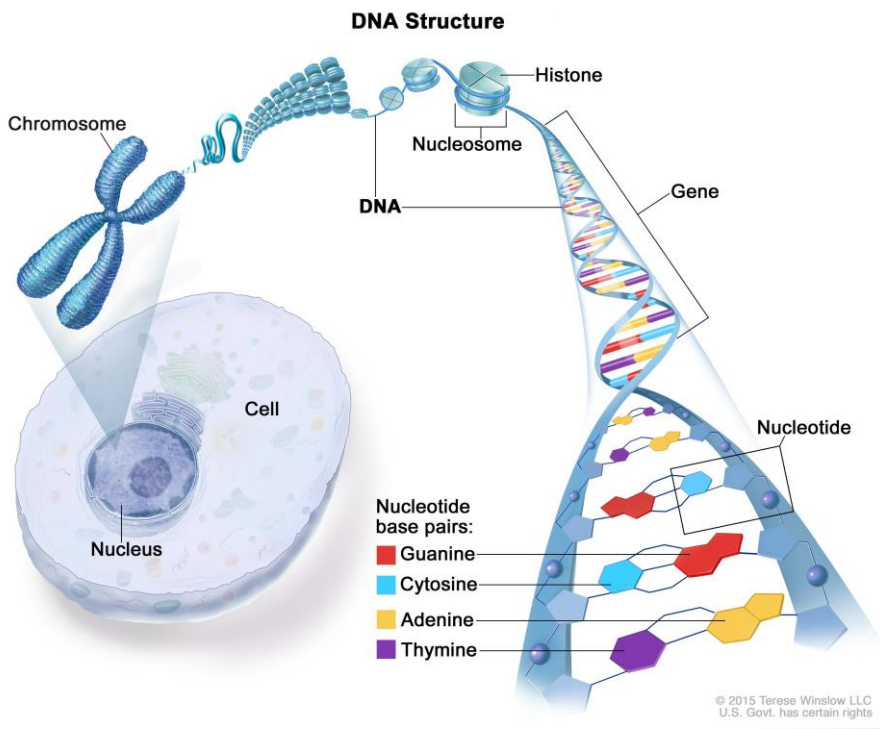
Basics of Molecular Biology

A quick reminder

Basics of Molecular Biology

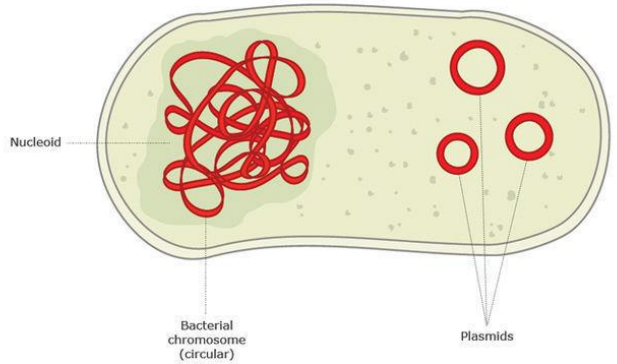
Genetic information contained in DNA

- DNA is built in a double helix
- DNA encodes the genetic information (different genes, different information)
- DNA is organized into a long chain called chromosomes
- 23 pairs of chromosomes are in the nucleus of human cells



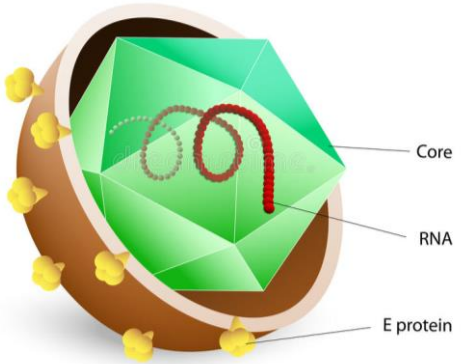
Genetic material in bacteria

- Genetic information in bacteria is encoded in DNA
- Most bacteria have a genome that consists of a single circular DNA molecule, located in a region called nucleoid (not bound by a membrane)
- Extrachromosomal genetic elements such as plasmids and bacteriophages often determine resistance to antimicrobial agents, production of virulence factors, or other functions.



Genetic material in viruses

- A virus is a small parasite that cannot reproduce by itself. It relies on the host cell machinery.
- Viral genome can be found in various forms : RNA or DNA, single or double stranded, linear circular or even segmented



Ex: Hepatitis C virus

Dreamstime

DNA building blocks

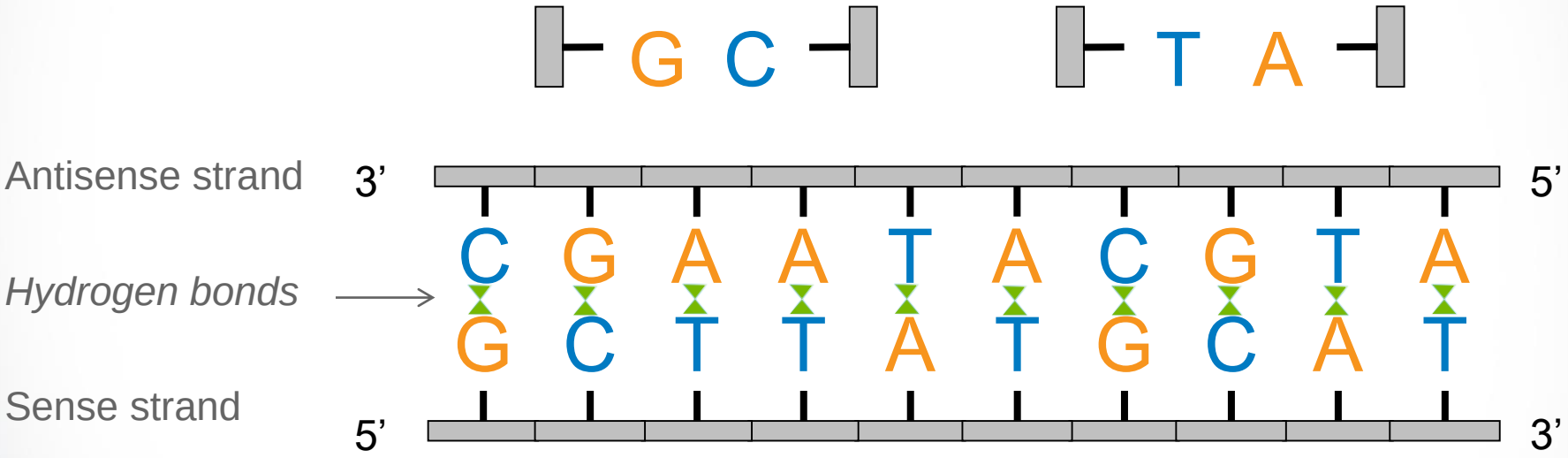
DNA is made up of 4 nucleotides

- A = Adenine
- T = Thymine
- C = Cytosine
- G = Guanine

The 4 bases are linked together to form a sequence (single strand of DNA)

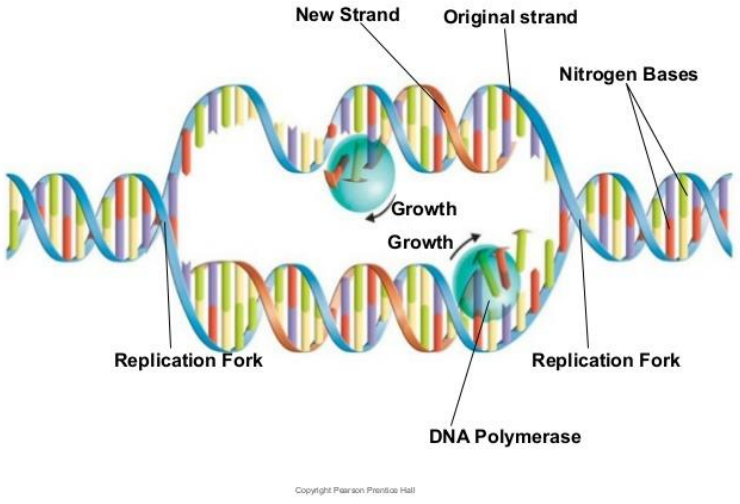
Basic Molecular Biology

Most DNA is double stranded and pairs in a unique way:

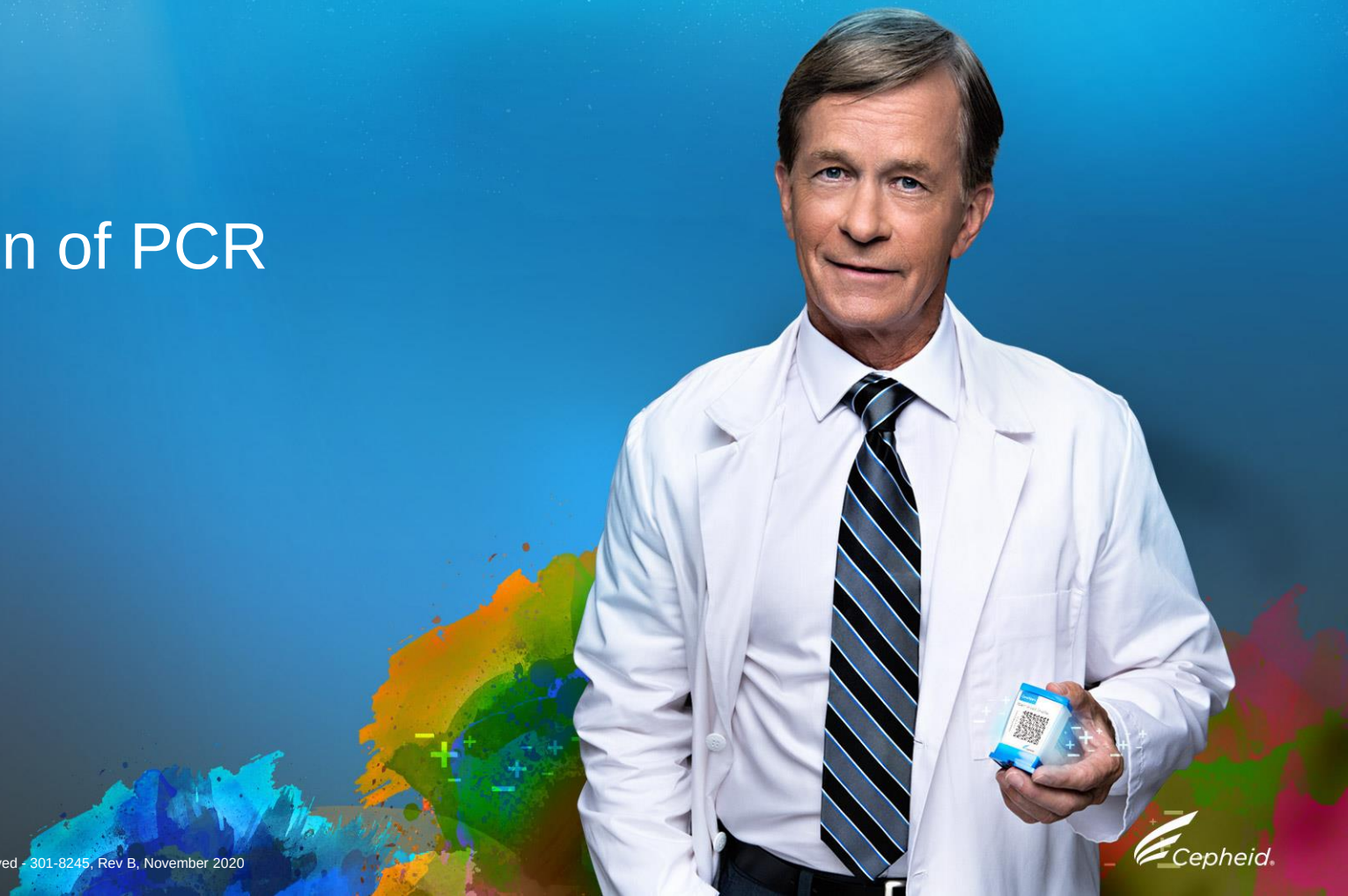


DNA replication

- New DNA is made by enzymes called **DNA polymerases**. They synthesize DNA in the 5' to 3' direction only.
- Another enzyme called primase makes an RNA **primer** to prime the Polymerase.
- Once the RNA primer is in place, DNA polymerase "extends" it, adding nucleotides one by one to make a new DNA strand that is complementary to the template strand.



Definition of PCR

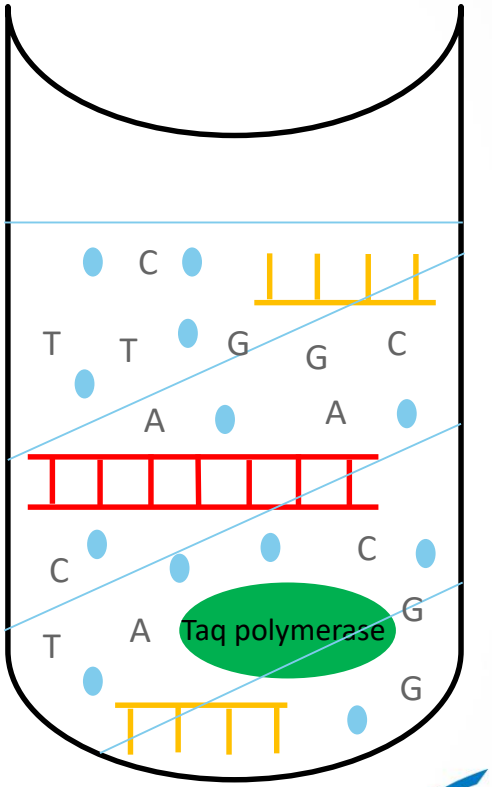


What is PCR ?

1. PCR (**Polymerase Chain Reaction**) is a chain reaction which generates multiple copies of a specific sequence of DNA present in the sample.
2. DNA amplification occurs by repeated thermal cycles
3. The number of copies of the specific sequence doubles after each cycle
4. After forty cycles, a single copy becomes around 2 trillion copies

Components of a PCR reaction

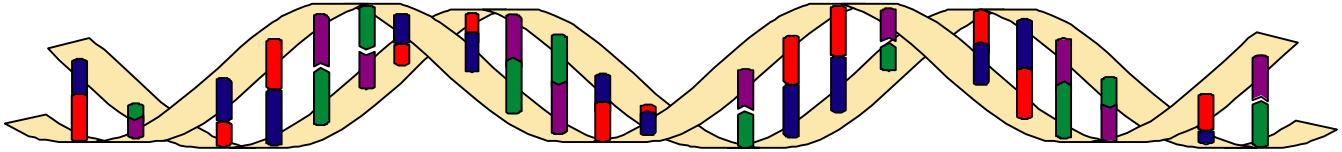
- **DNA template** (virus, bacterial or human gene)
- **dNTP's** (a mix of all four nucleotides required to build new DNA strands: A, T, C, G)
- **Primers** (oligonucleotides of about 20 nucleotides, which will anneal to the target DNA)
- **Polymerase** (natural thermostable Taq polymerase that can function at an optimum temperature of about 70°C)
- **Buffer** (Mg2+, Tris-HCl, Triton: provides the optimal conditions for the polymerase to work)



The phases of PCR

The first phase of a PCR cycle - denaturation

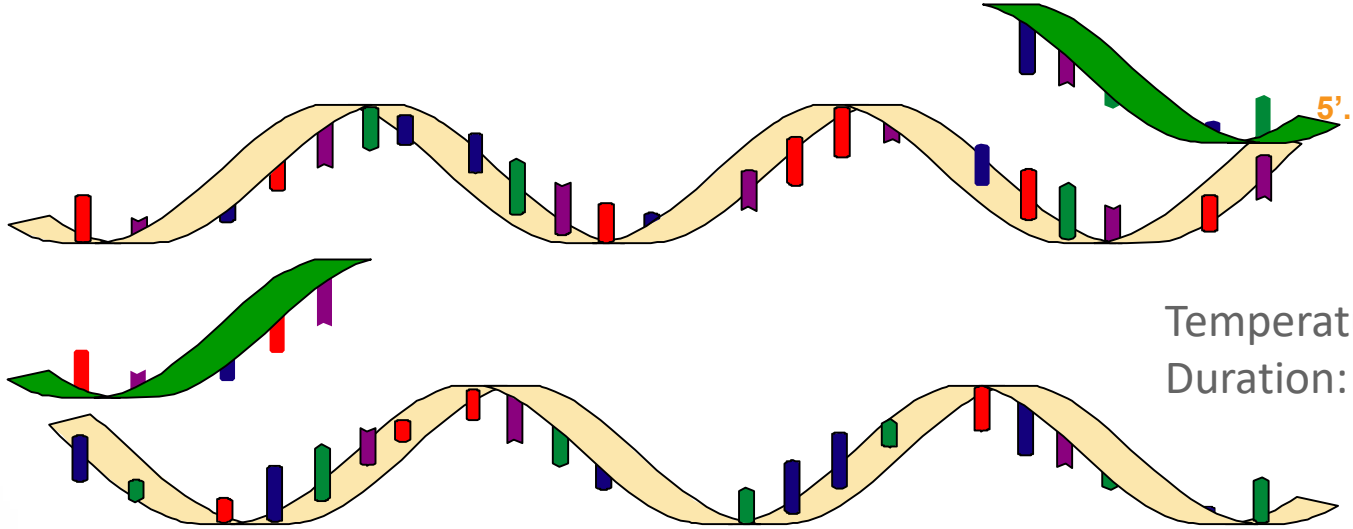
separation of DNA strands



- 90-95 ºC
- 20-30 sec.

The second phase of a PCR cycle - annealing

specific primer binding



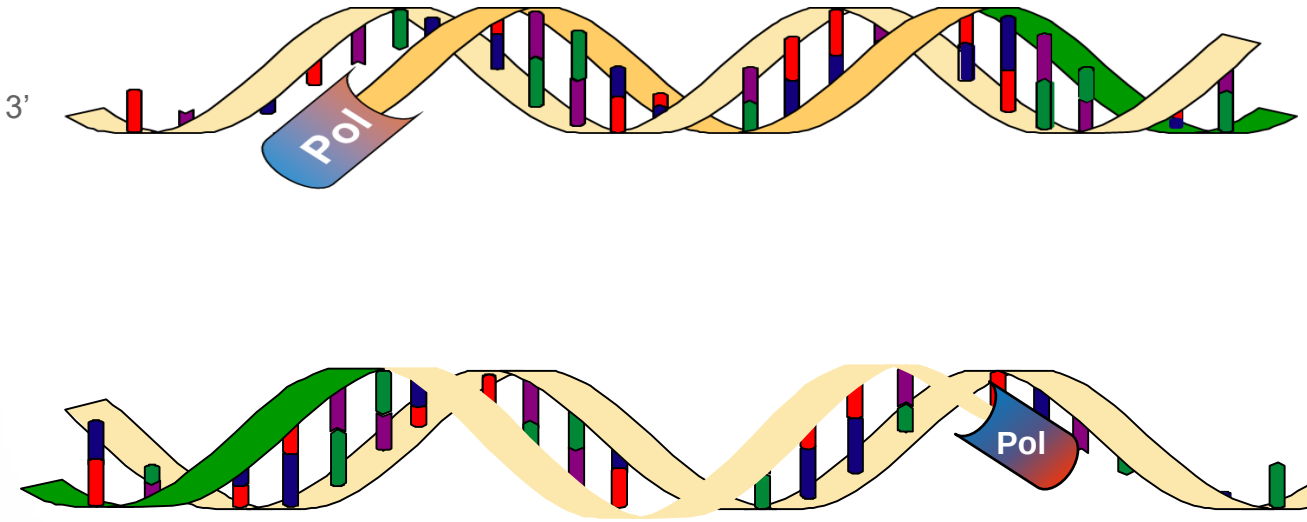
5’. Reverse primer

Temperature: 40-65 ºC
Duration: 20-40 seconds

5’ Forward primer

The third phase of a PCR cycle- extension

DNA strand synthesis



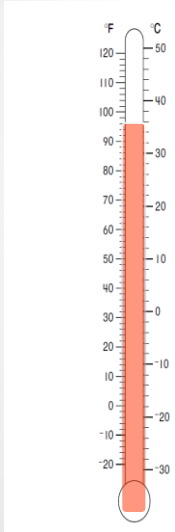
5’. Reverse primer

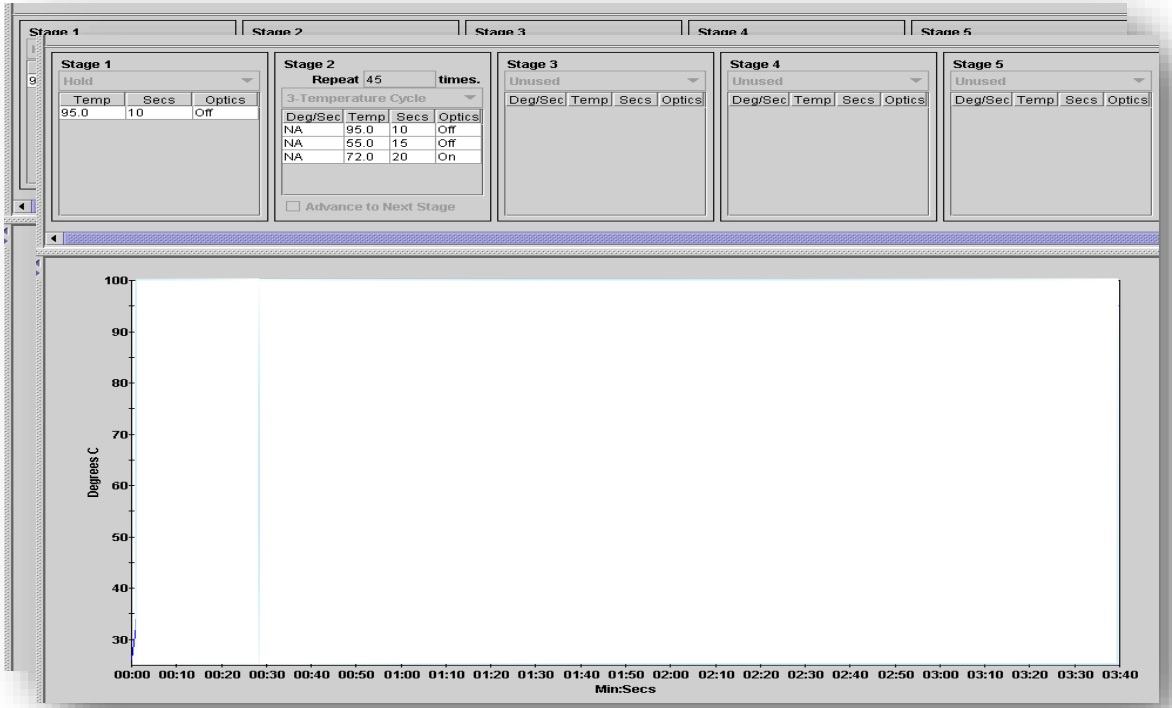
- 60-75 ºC

Duration depends on the sequence size to be amplified

5’ Forward primer

Thermal profile of PCR cycling

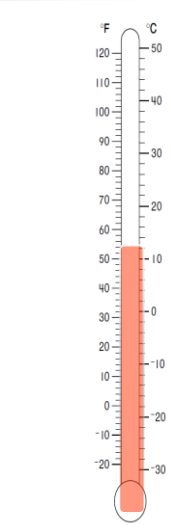


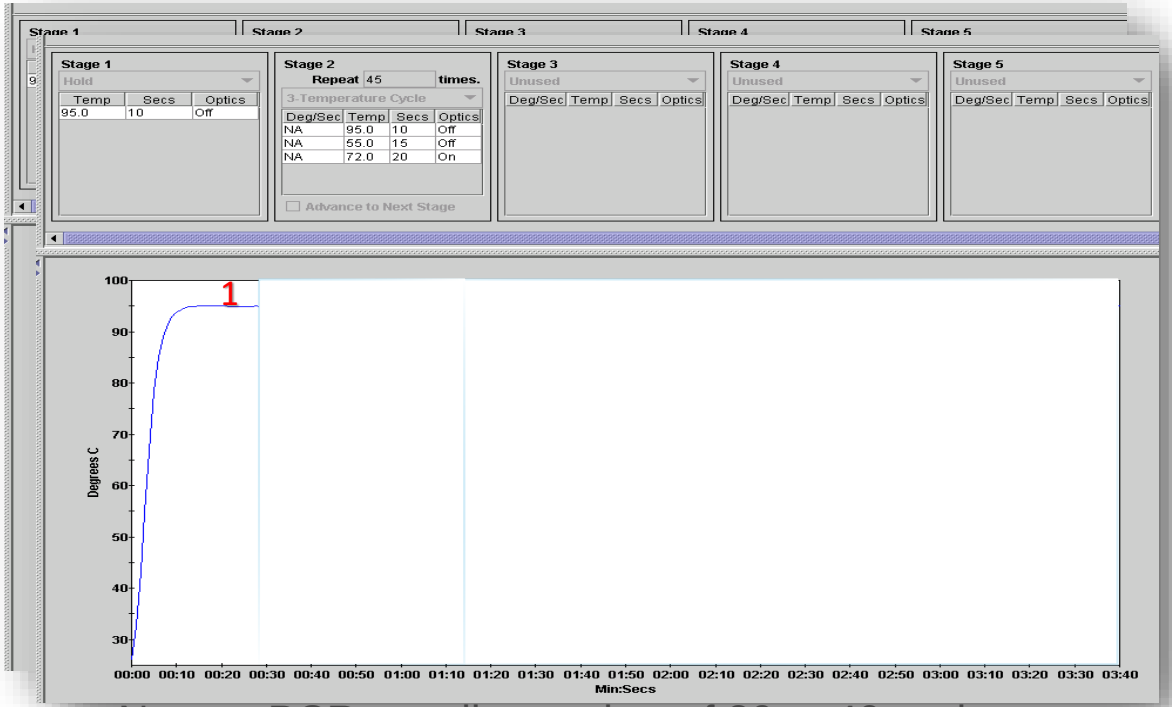


1. Denaturation

Note: a PCR usually consists of 30 to 40 cycles

Thermal profile of PCR cycling

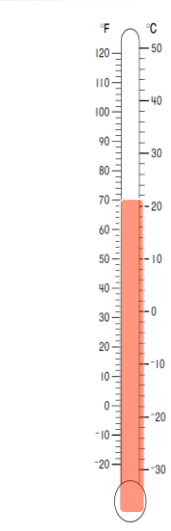


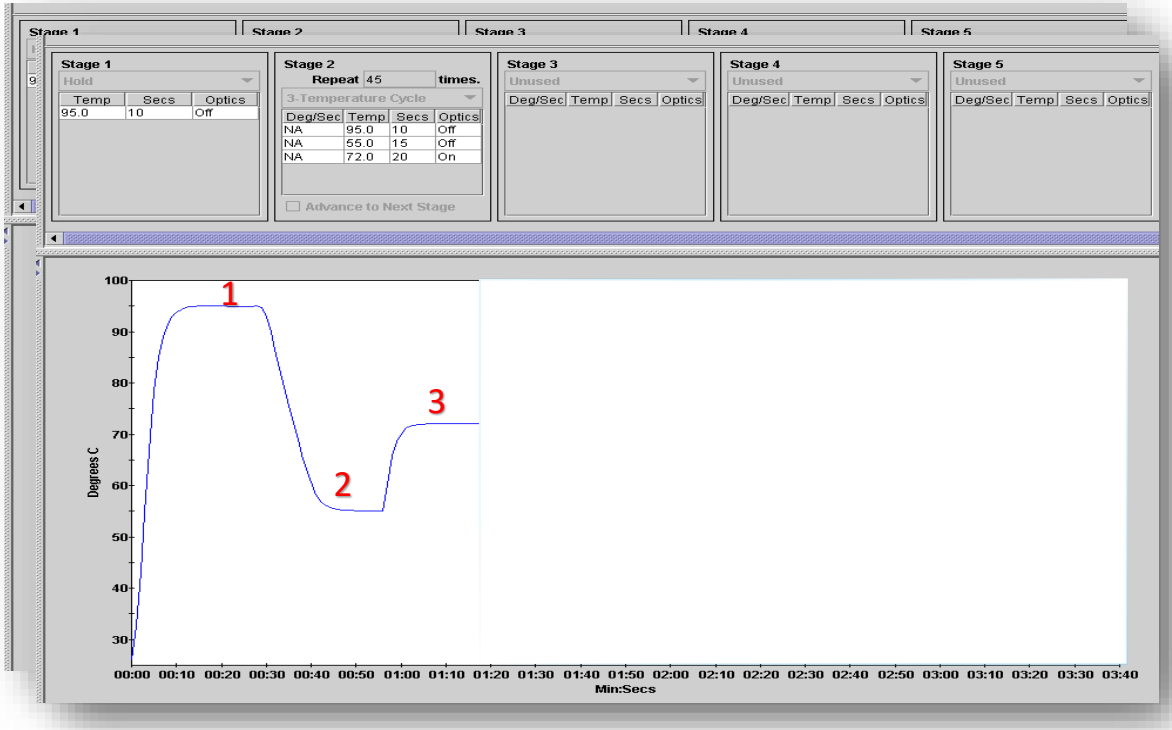


2. Annealing

Note: a PCR usually consists of 30 to 40 cycles

Thermal profile of PCR cycling





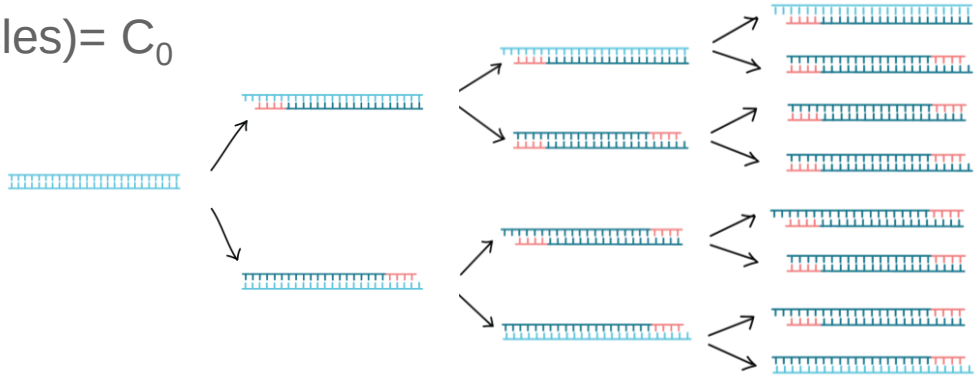
3. Extension

Note: a PCR usually consists of 30 to 40 cycles

Number of DNA copies obtained by PCR

– **In theory**, the number of copies of target DNA doubles with each cycle, which means a PCR efficiency factor E = 2

- Starting concentration (0 cycles)= C_0
- After one cycle: C_0 x 2
- After 2 cycles: C_0 x 4
- After 3 cycles: C_0 x 8
- **After n cycles: C_0 x 2^n**



– **In reality**, this replication rate cannot be sustained forever and the doubling becomes less than a doubling , then no replication at all

Factors influencing PCR efficiency

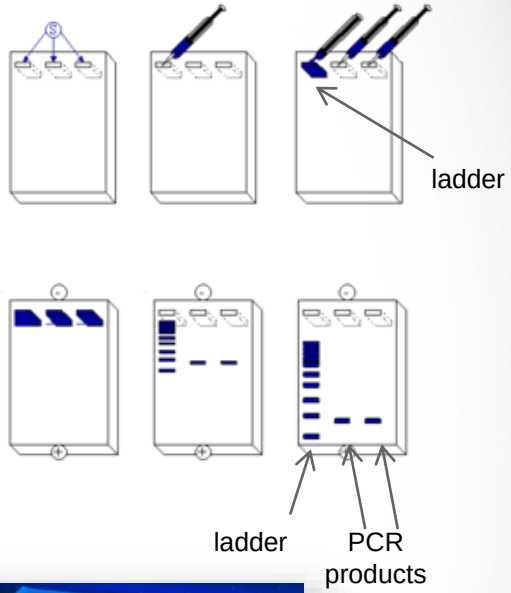
- Test design (Manufacturer)
  - Primer/Probe design
  - Type of DNA polymerase
  - Quality of reagents
  - Master mix
  - PCR cycling conditions: temperatures and duration of the phases

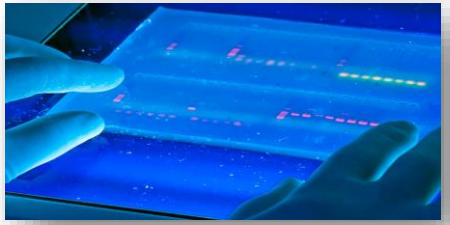
- Pre-analytics (Laboratory technician)
  - Quality of Reagents, due to transport and storage conditions
  - Sample quality : presence of PCR inhibitors

Product detection at end point

In the classical PCR, the detection is performed at <u>end-point</u>
(end of PCR)

1. A mix of fragments of known sizes (ladder) is loaded into an agarose gel, as reference, to calculate the size of the PCR products.
2. The PCR product is also loaded into the gel
3. An electric field is applied, so negatively charged molecules migrate toward the positive pole
4. The PCR product migrates according to size
5. The DNA is stained using ethidium bromide, visible under UV lamp
6. If the target we are looking for is present in the sample, a PCR product of the expected size is present



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