Detection of Covid-19 by Molecular Techniques

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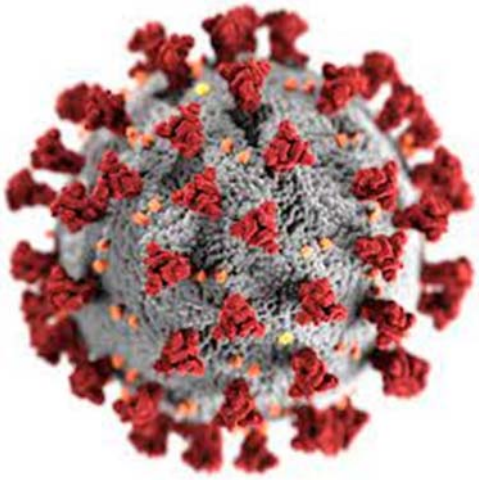
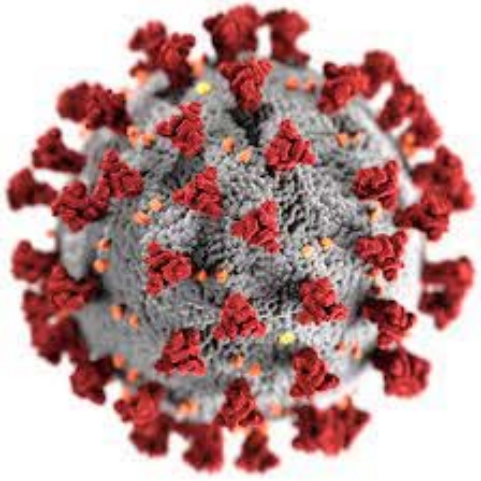
Gorgan ,August 2021

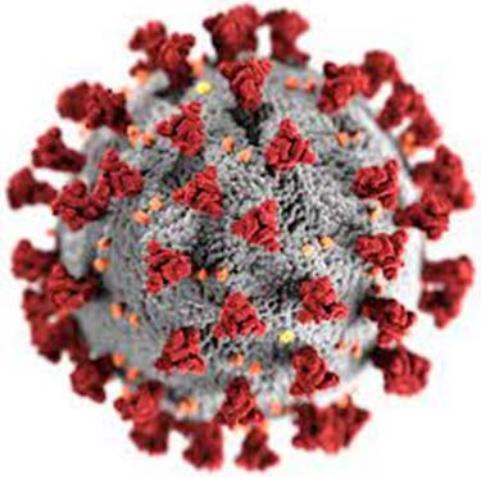


Coronaviruses RNA viruses
Realm Riboviria
Order Nidovirales
Family Coronaviridae
Subfamily Orthocoronavirinae,



Pathogenicity of Coronaviruses :

- Respiratory tract infections (from mild to lethal). Mild illnesses in humans include some cases of the common cold while more lethal varieties can cause SARS, MERS, and COVID-19. In cows and pigs they cause diarrhea, while in mice they cause hepatitis & encephalomyelitis.



Swab-based SARS-CoV-2 testing :

Nasopharyngeal specimen preferred choice

Oropharyngeal sample should also be collected

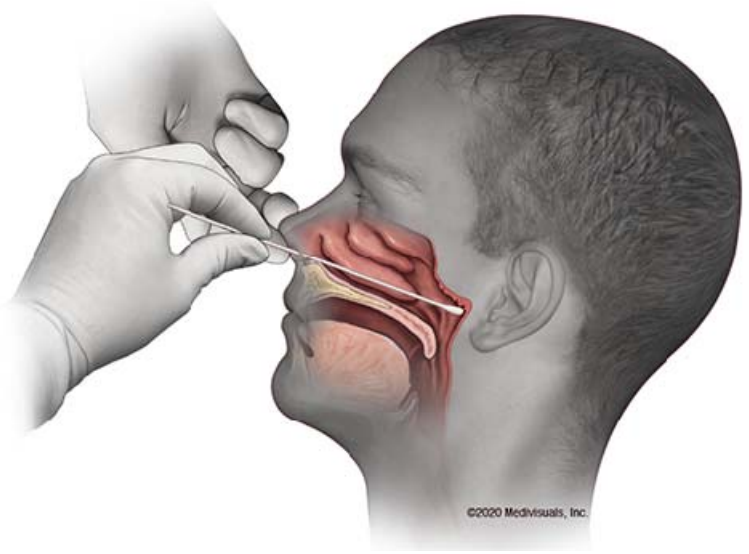
Collecting both NP and OP swab has shown to improve sensitivity by up to ***two-fold.***

Sputum,

bronchoalveolar lavage

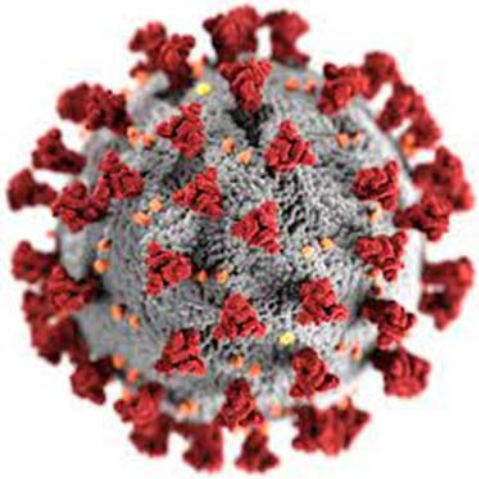
endotracheal aspirate during intubation.

self-collected saliva specimen.



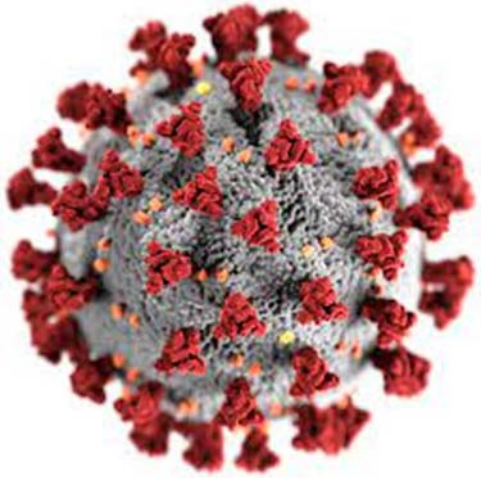
EQUIPMENT AND PREPARATION

- Viral transport medium (VTM),
- swab sticks,
- personal protective equipment (PPE)
- tongue depressor,
- ice-pads
- ice-box,
- marker,
- requisition form,



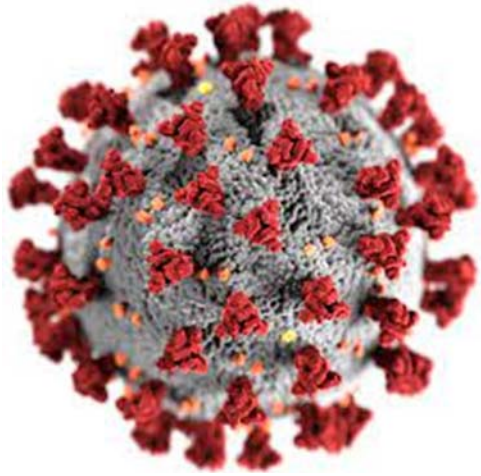
Swabs Characteristics:

- Swabs should be made up of rayon or dacron with plastic shaft.
- Cotton or calcium alginate swabs should not be used since it may inhibit PCR reaction.
- Stick with wooden shaft may cause trauma during sample collection



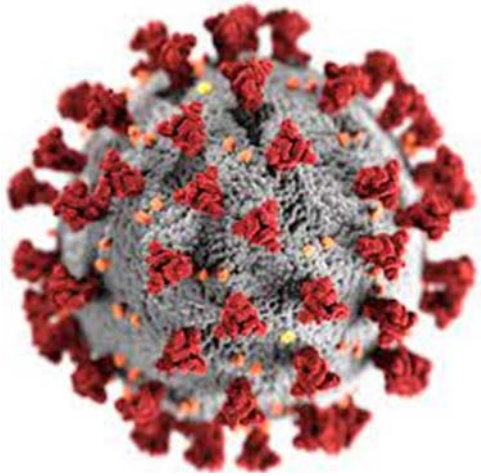
VTM:Viral Transport Medium

- 3 ml fluid composed of gelatin and antimicrobial agents in a buffered salt solution.
- It helps to prevent the specimen from drying, maintains the viability of the virus, and avoids the growth of contaminants
- Other solution as DW :Must be rejected
- Dry Swab :Must be rejected

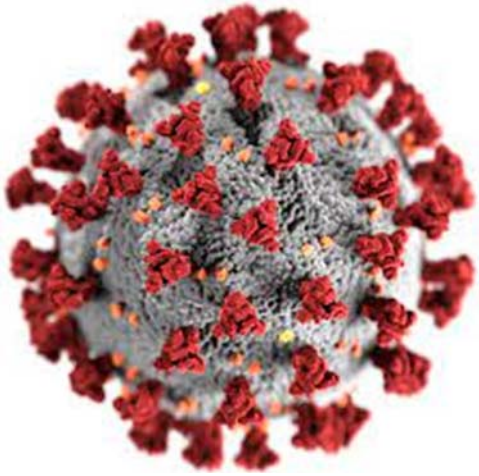


SPECIMEN TRANSPORTATION & MAINTENANCE :

- The specimen should be transferred to the laboratory maintaining cold chain (2-4°C) throughout
- Maintenance of specimen for 72 hours(in some references for 5 days)in 2-4°C
- If there is delay of more than 72 hours, sample should be stored at -70°C

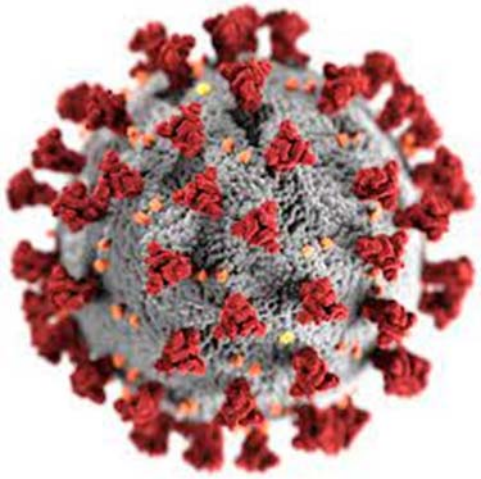



Welcome
to our Lab



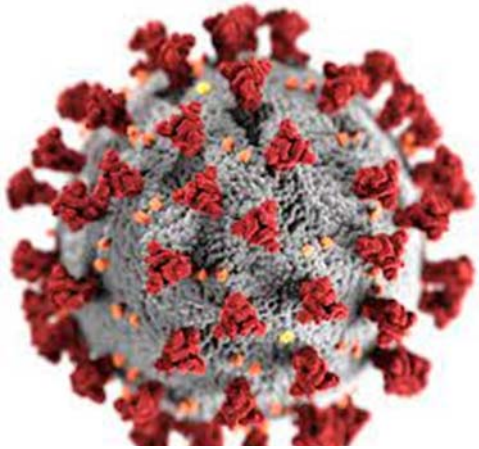
Performance of PCR depends upon:

- Sample type
- Different stage of infection in patient,
- The skill of the collection
- The quality and consistency of the PCR assays being used.

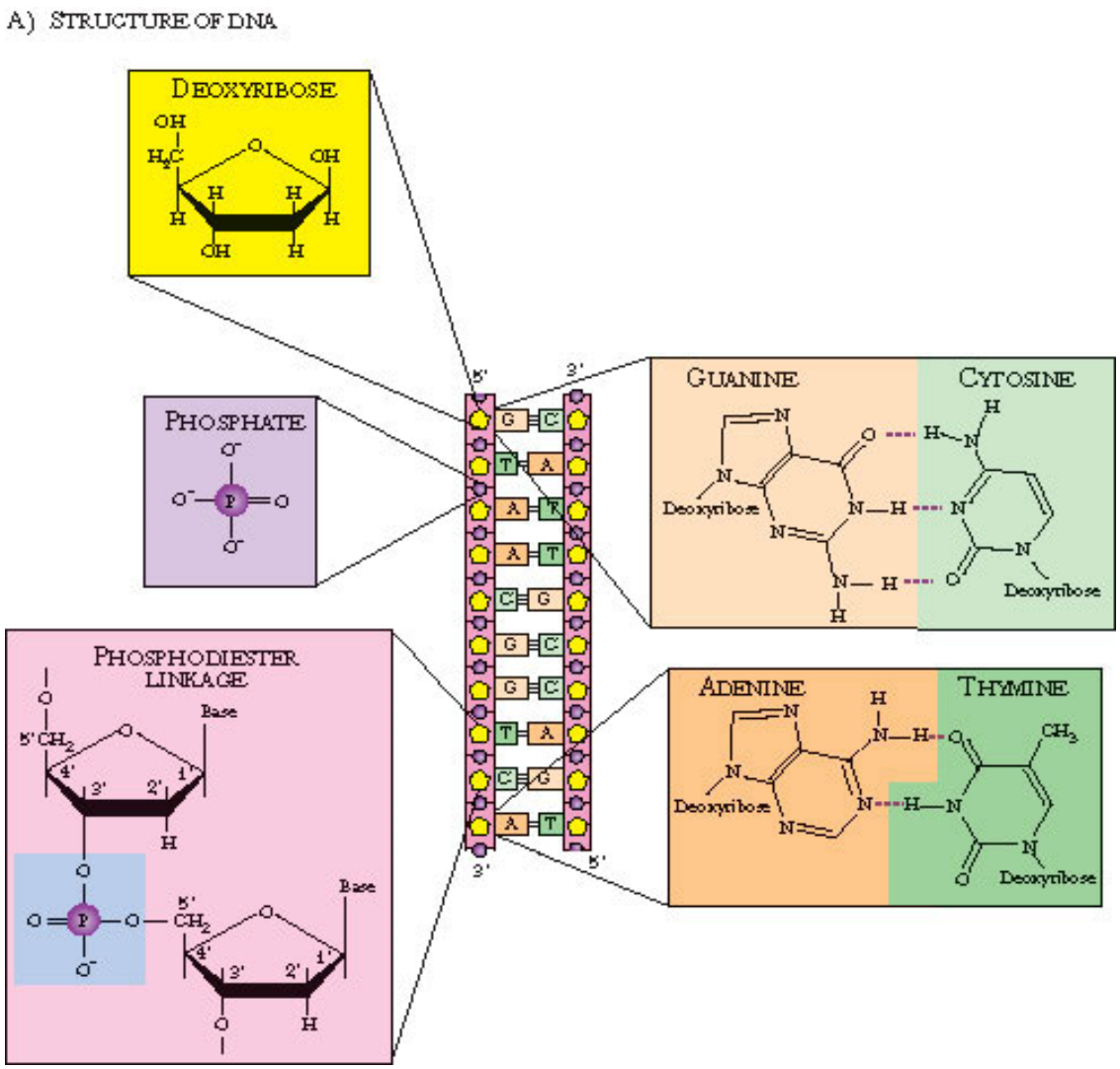


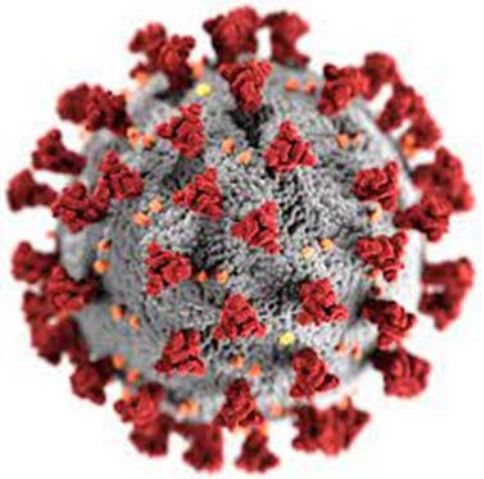
How PCR Works

- Protocol
  - Put all reagents into a PCR tube
  - Break the DNA ladder down the middle to create two strands, a 5' to 3' strand and a 3' to 5' strand
    - Melting or heat denaturation
  - Bind each primer to its appropriate strand
    - 5' primer to the 5' to 3' strand
    - 3' primer to the 3' to 5' strand
      - Annealing
  - Copy each strand
    - DNA polymerase
      - Extending



Structure of DNA



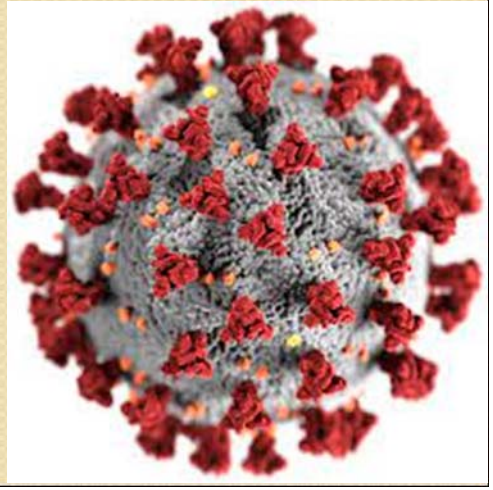


DNA is built from a series of nucleotides

Nucleotide consists of a phosphate, sugar, & nitrogenous base

The phosphate and sugar groups are sides of the ladder and are called the sugar-phosphate backbone.

- Unattached phosphate group, which then attaches to the carbon 3 present in one side. while the other one goes in the opposite direction antiparrallel

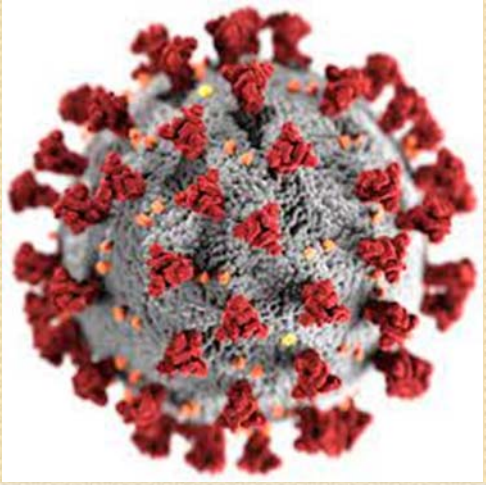


The nitrogenous bases are in the middle and they carry the information in genes.

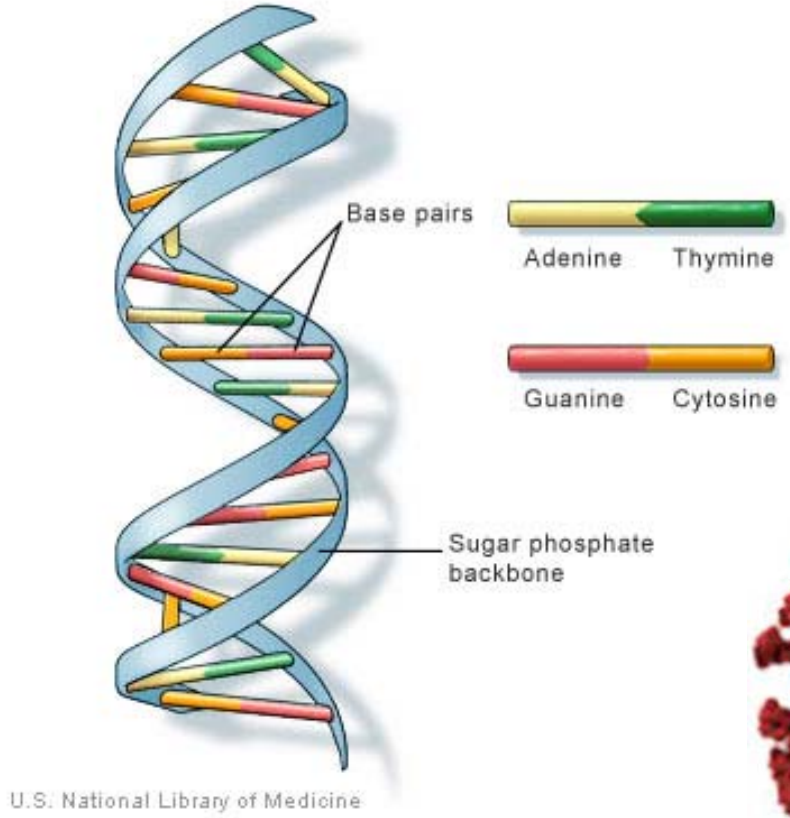
There are four bases:

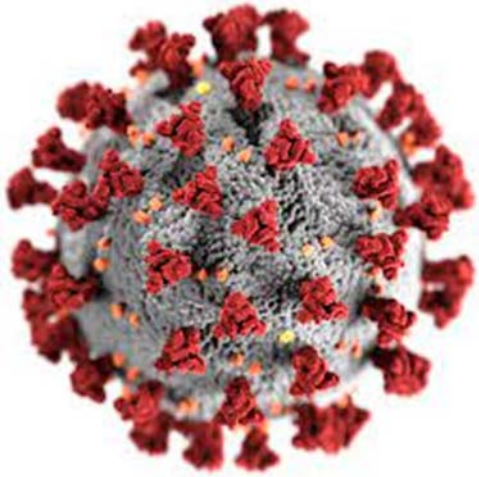
cytosine, guanine, adenine and thymine, (CGTA)

The connect via hydrogen bonds (C attached to G and A to T)



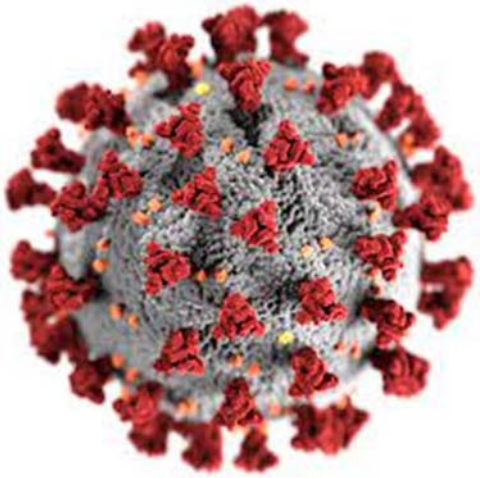
DNA structure





Reagents Needed For PCR

- DNA sample which you want to amplify
- DNA polymerase
  - Taq DNA polymerase – Works at high temps .A special DNA polymerase (Taq) is used to make many copies of a short length of DNA (100-10,000 bp) defined by primers
- Nucleotides
  - Called dNTPs
- Pair of primers
  - One primer binds to the 5' end of one of the DNA strands
  - The other primer binds to the 3' end of the anti-parallel DNA strand
  - Delineate the region of DNA you want amplified
- Water
- Buffer



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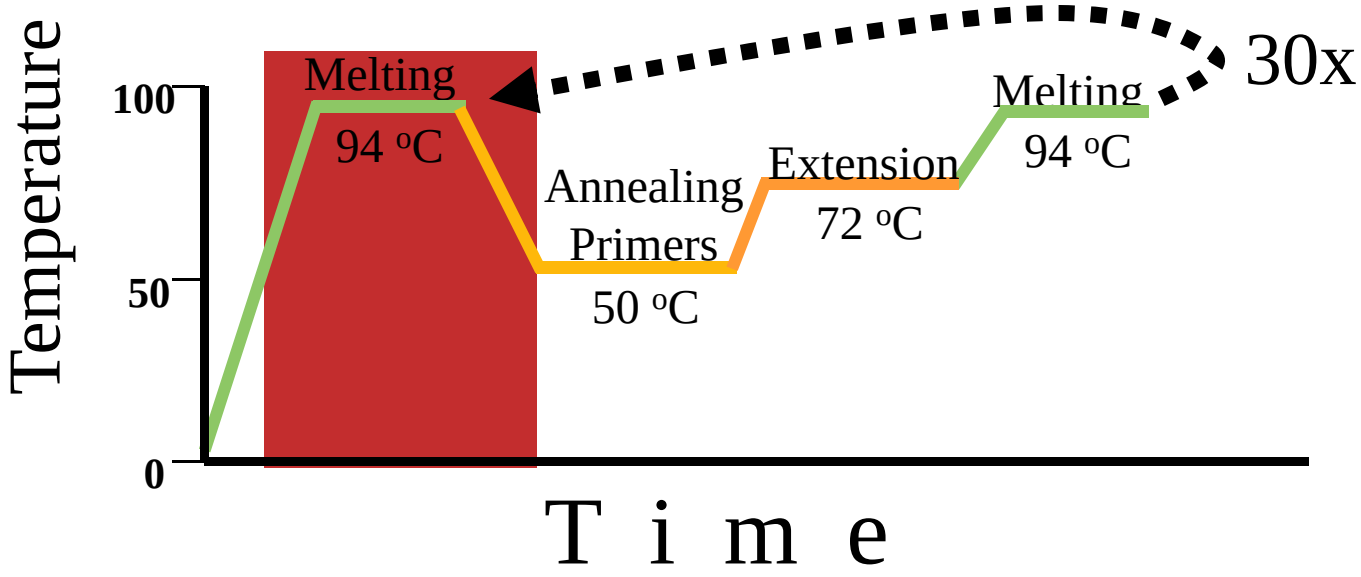
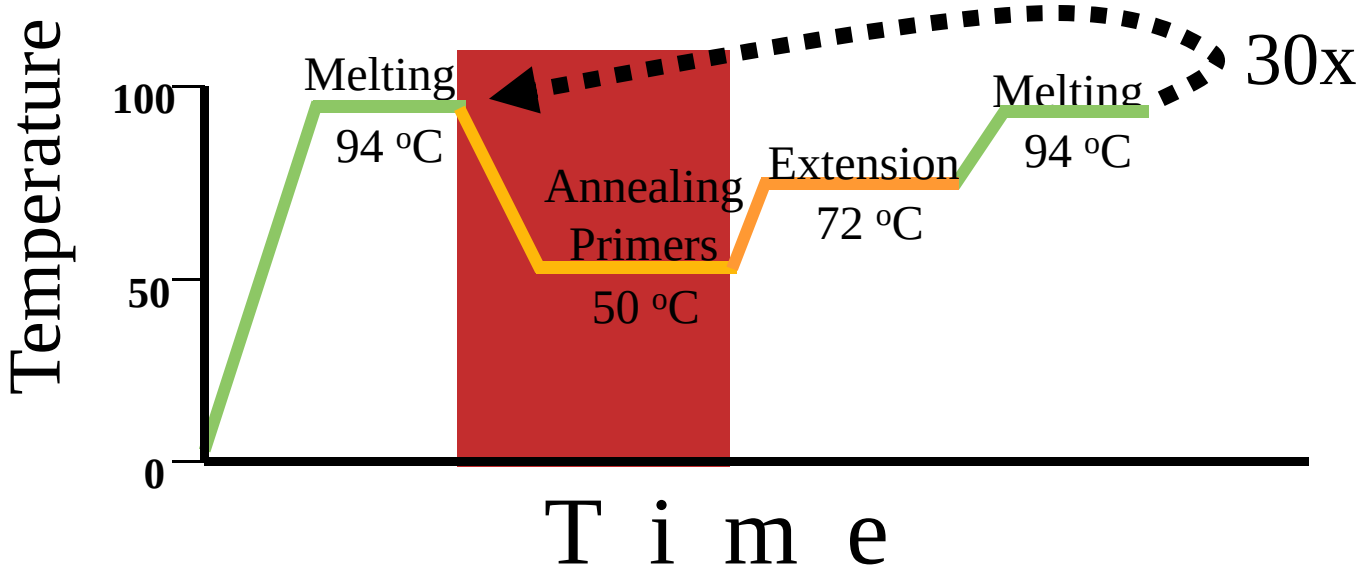
PCR


Temperature
100
50
0
Melting
94 °C
Annealing
Primers
50 °C
Extension
72 °C
Melting
94 °C
30x
T i m e

PCR


Temperature
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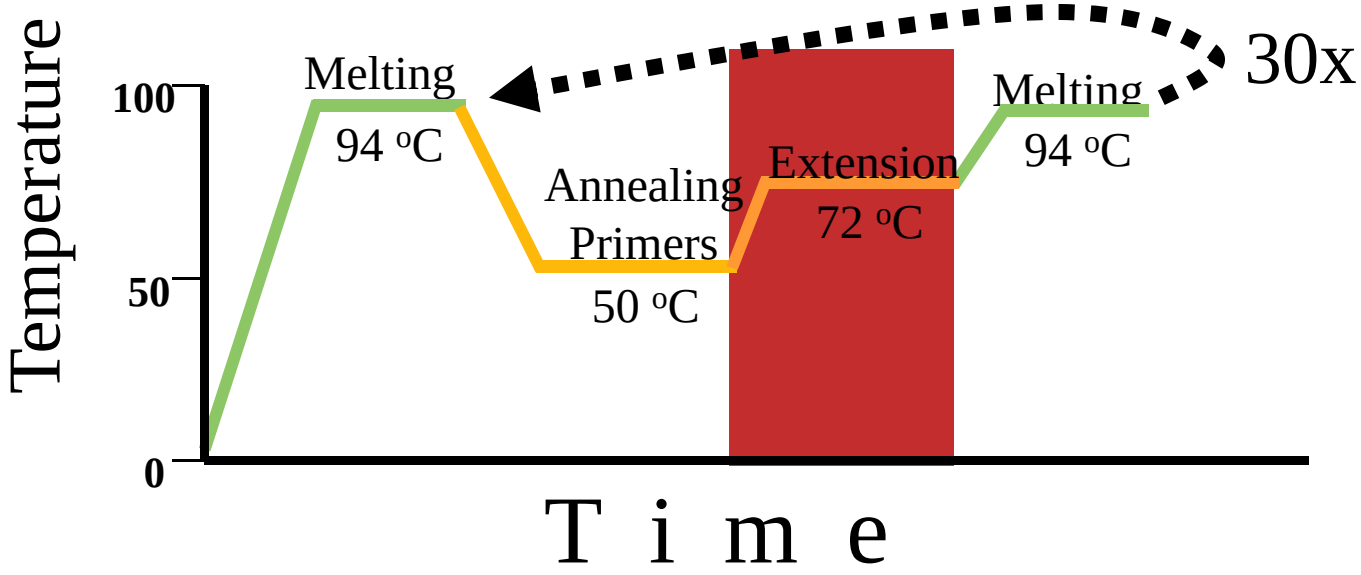
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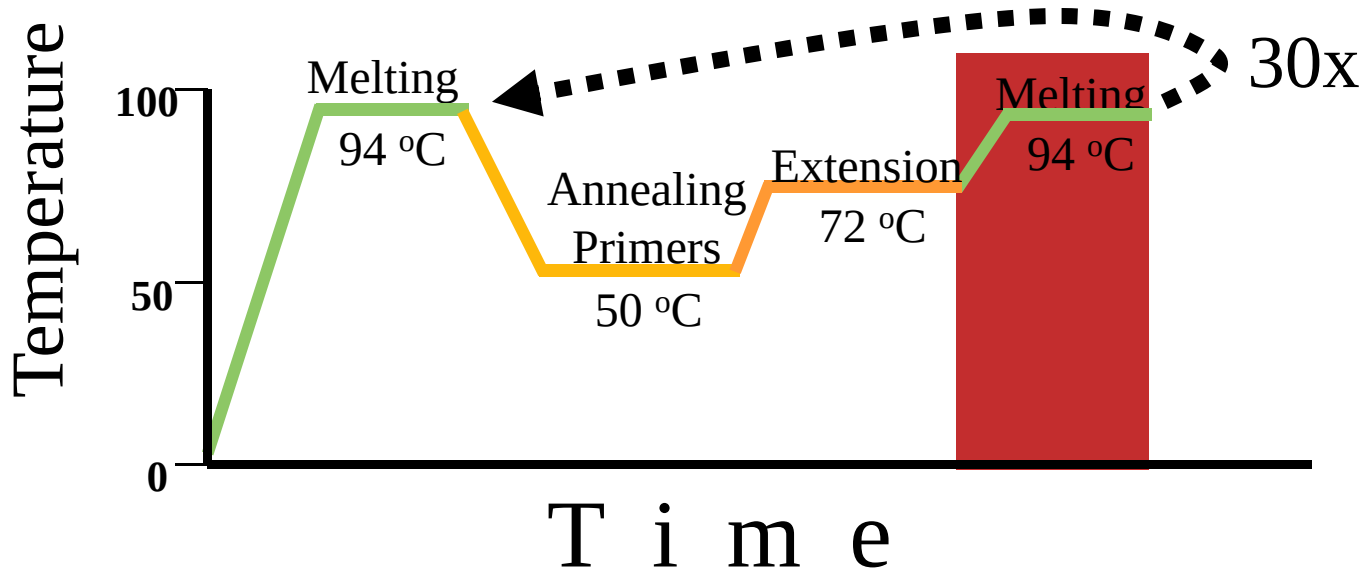
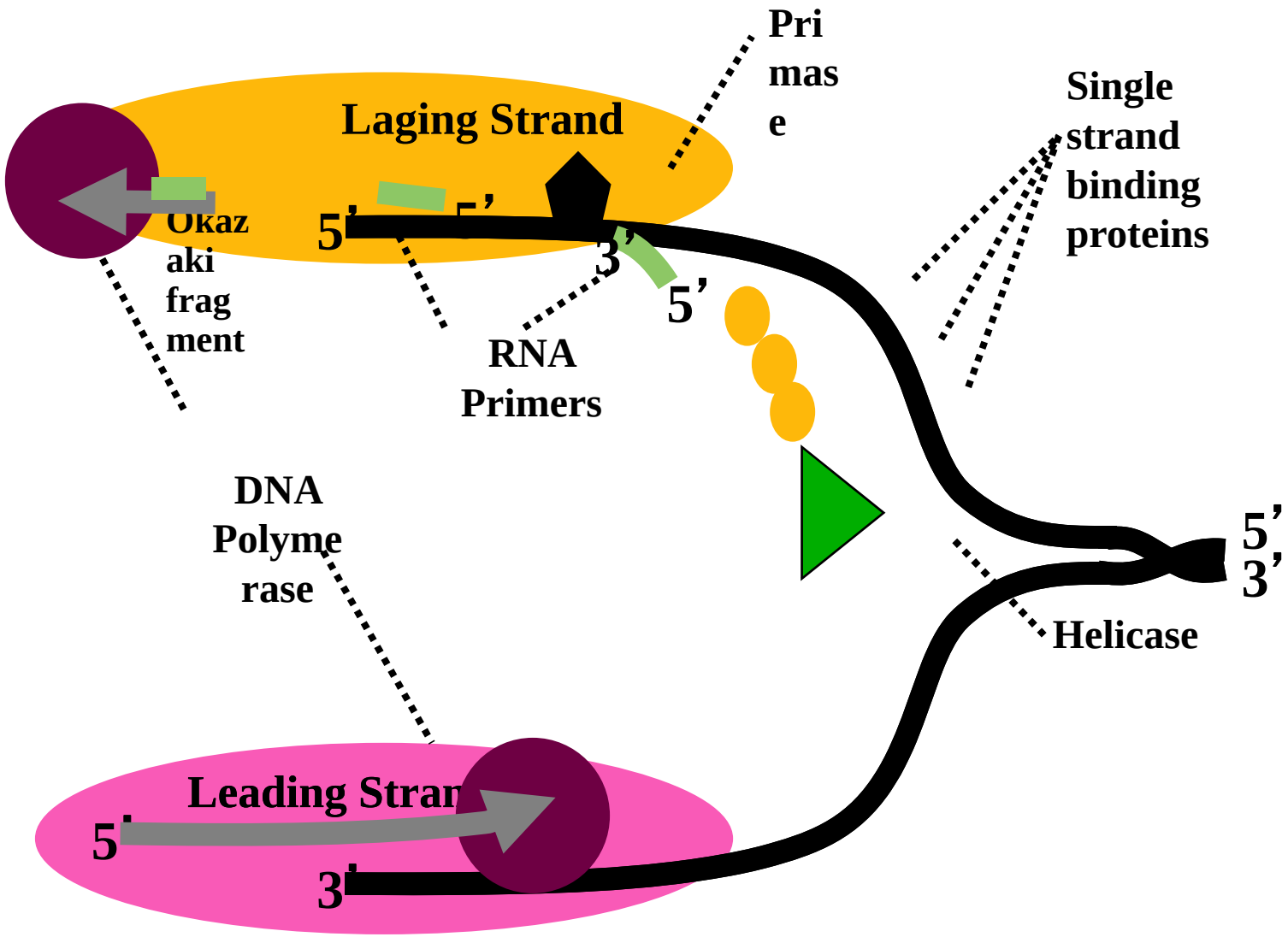
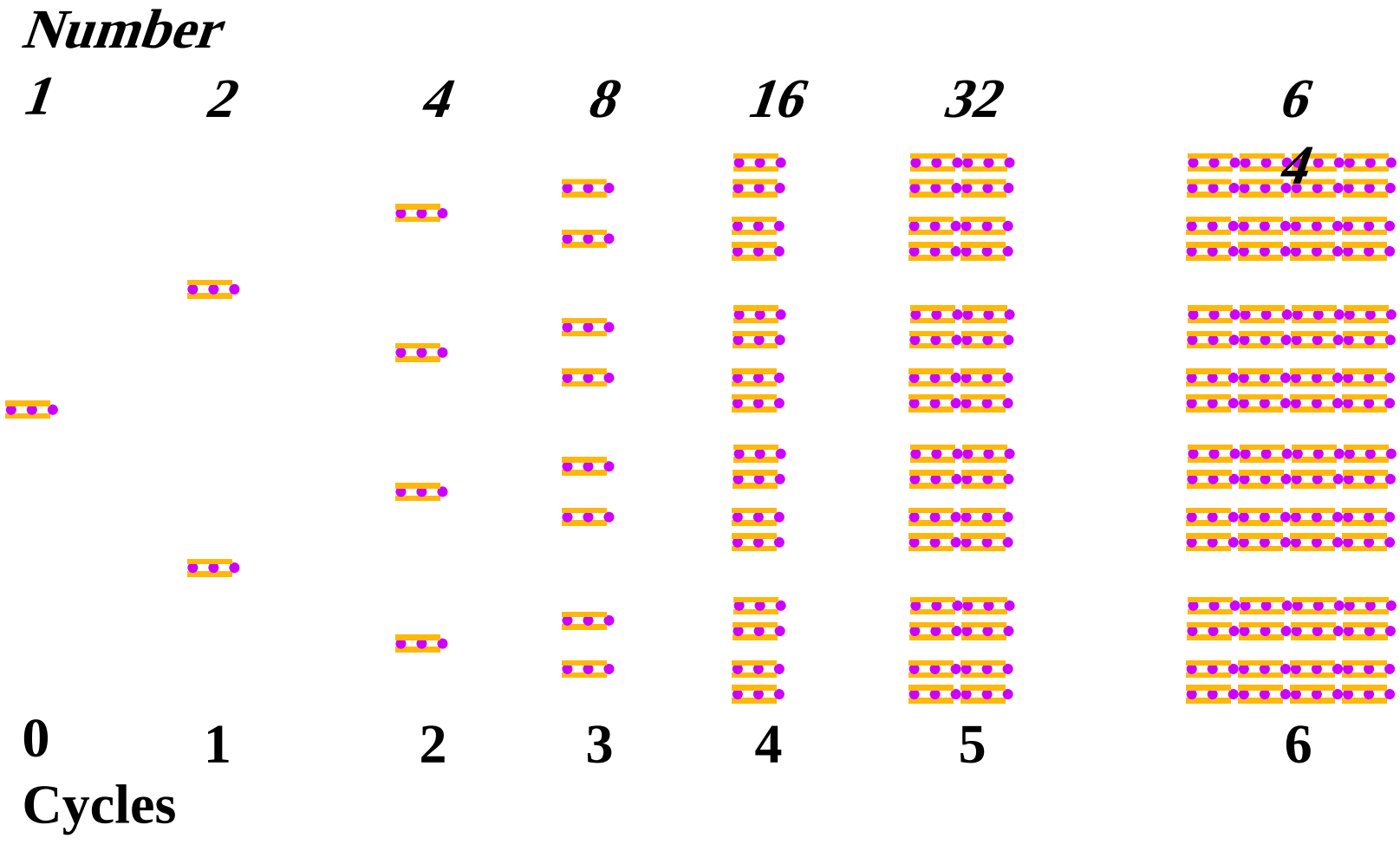
PCR


Temperature
100
50
0
Melting
94 °C
Annealing
Primers
50 °C
Extension
72 °C
Melting
94 °C
30x
T i m e

Extension - The Replication Fork



DNA Between The Primers Doubles With Each Thermal Cycle



Theoretical Yield Of PCR

Theoretical yield = 2^n x y

Where y = the starting
number of copies and
n = the number of thermal cycles

If you start with 100 copies, how many copies are made in 30 cycles?

2^n x y
= 2^{30} x 100
= 1,073,741,824 x 100
= 107,374,182,400

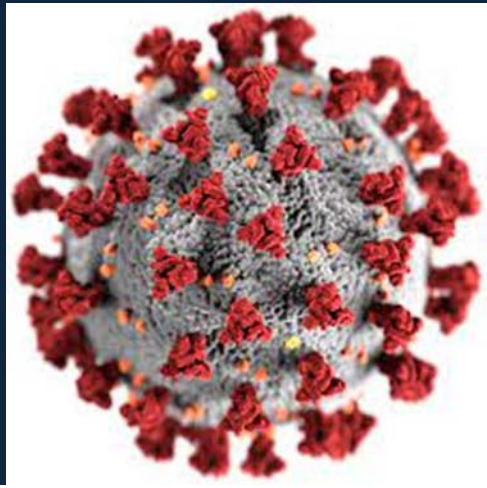
Real-time PCR

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection

- Real-time PCR describes methods by which the target amplification and
- detection steps occur simultaneously in the same tube (homogeneous).
- These methods require special thermal cyclers with precision optics that
- can monitor the fluorescence emission from the sample wells.

- Real-time PCR steps:

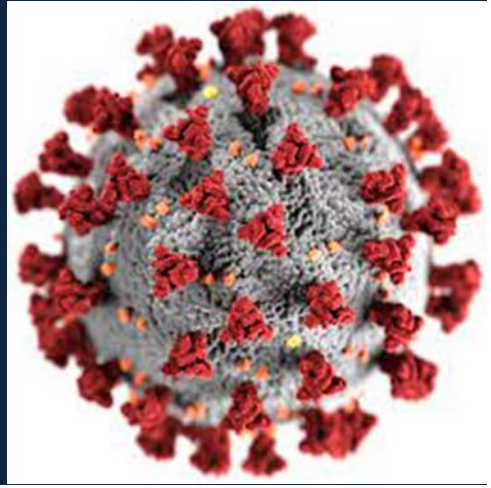
1. Denaturation
2. Annealing
3 . Extension



- Real-time PCR steps:

1. Denaturation

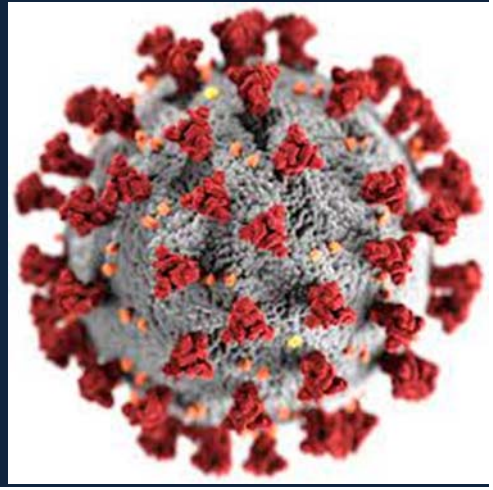
high-temperature incubation is used to “melt” dsDNA into single strands and loosen secondary structure in single-stranded DNA (ssDNA). The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.



- Real-time PCR steps:

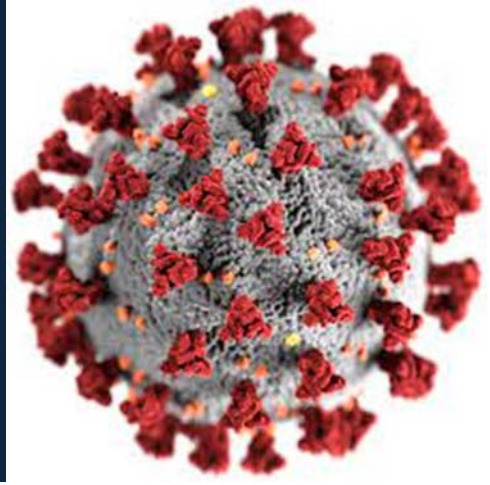
  2:Annealing:

  during annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (typically 5°C below the Tm of the primer).



- Real-time PCR steps:

3. Extension : at 70–72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step, using 60°C as the temperature



- The computer software supporting the thermocycler monitors the data throughout
- the PCR at every cycle (kinetic) and generates an amplification plot for
- each reaction.

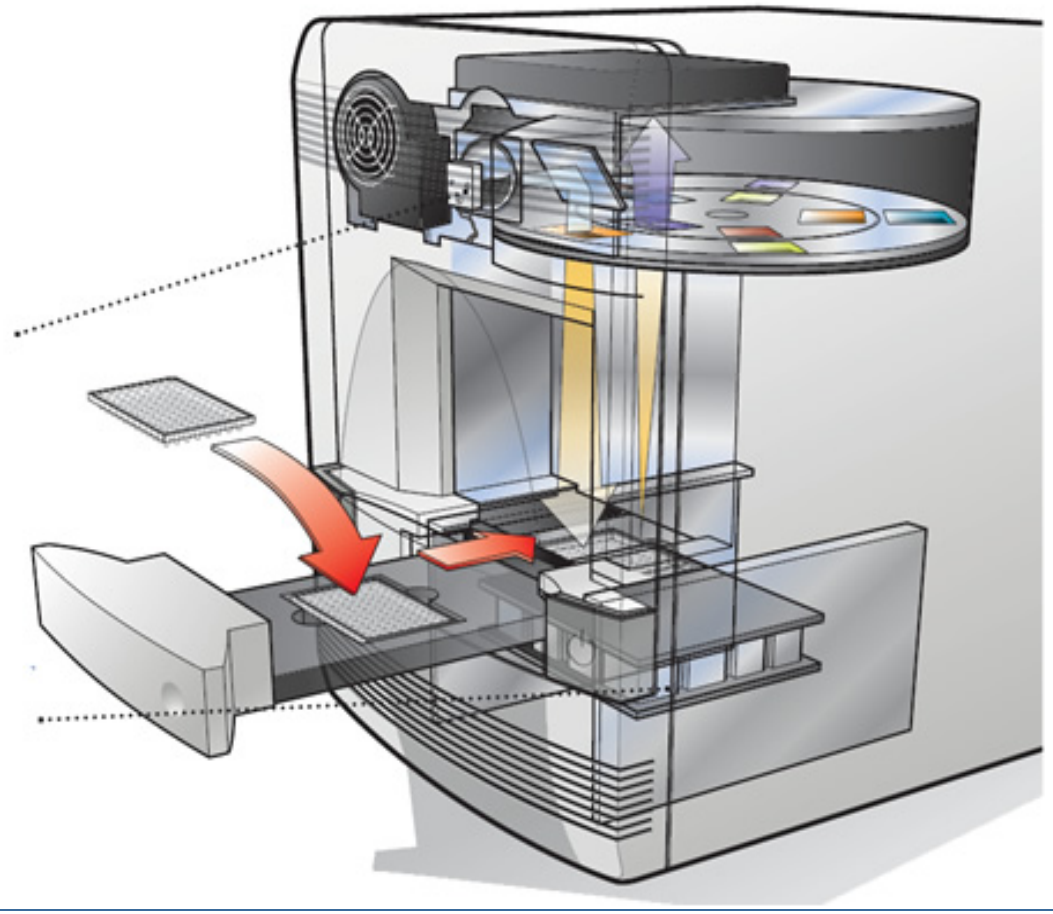
How it Works: Real Time PCR

Brendan Maher

The instrumentation is basic: a thermal cycler for amplification, a light source for excitation of fluorescent probes (see chemistries below), a camera for recording, and a computer to control the instrument and record data. Increasingly sophisticated instruments, such as those capable of multiplex experiments, are becoming affordable in academic labs.

The light source in the Applied Biosystems 7500 (represented here) is a simple halogen lamp shone through one of five different excitation filters over the entire sample. A CCD camera positioned above the sample records fluorescence from behind one of five emission fi lters. Some makes and models use a scanning head that moves over the plate, exciting and reading fluorescence in the wells individually.

Many qPCR instruments including the ABI 7500 use a Peltier element for heating and cooling. Peltier coolers use electron flow between semiconductor couples to heat or cool one side of a plate depending on the direction of current. Other systems use liquid or air fl ow or mechanical transition between blocks of different temperatures to cycle the samples.



(www)

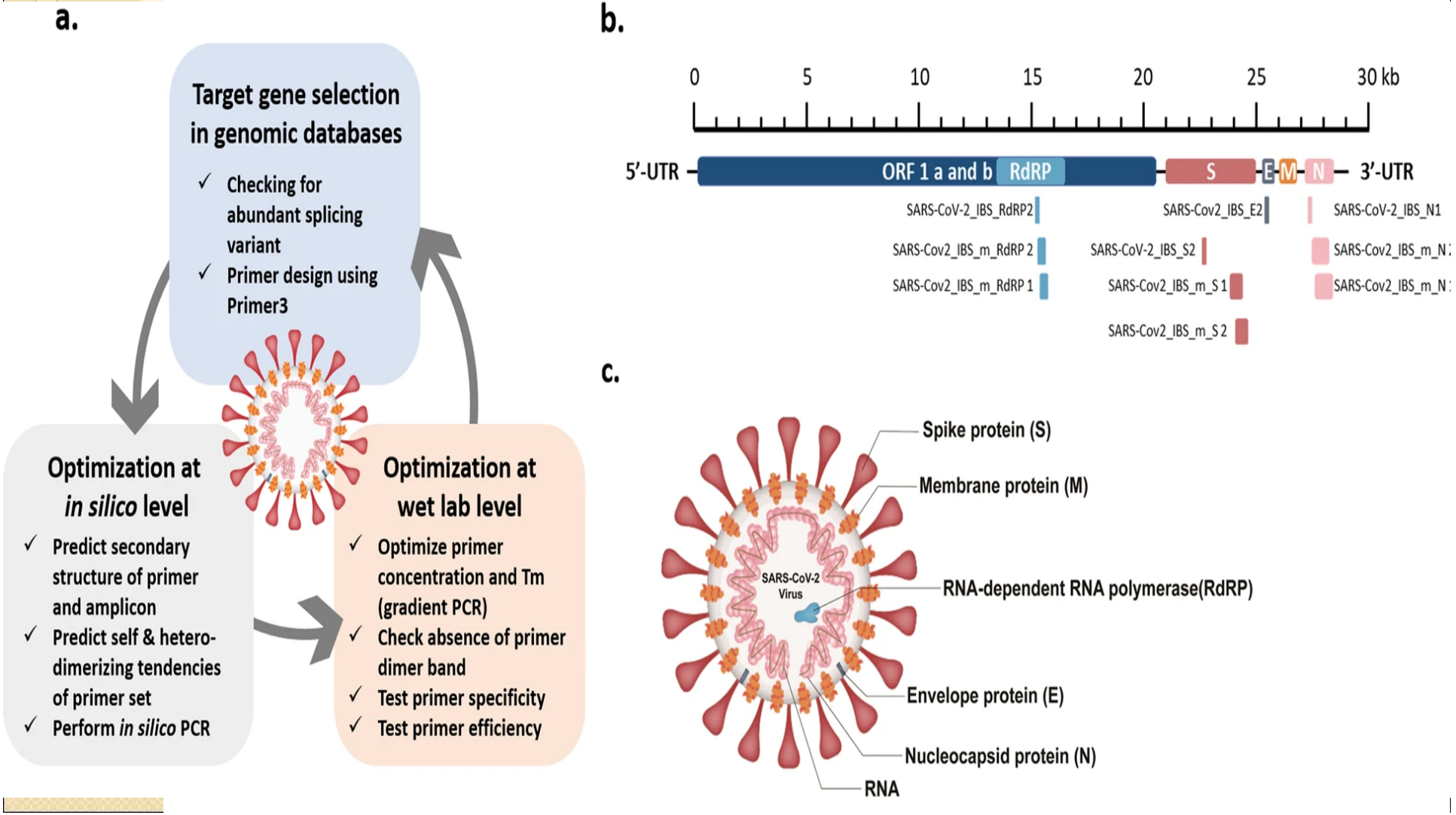
What Type of Instruments are used with Real-Time PCR?

Real-time PCR instruments consist of THREE main components:

1. **Thermal Cycler (PCR machine)**
2. **Optical Module (to detect fluorescence in the tubes during the run)**
3. **Computer (to translate the fluorescence data into meaningful results)**

Target Genes in Covid Rt- PCR

- E , S , Orflab , N , RdRp



Real-Time Principles

- **Three general methods for the quantitative assays:**
  - **Hydrolysis probes (TaqMan)**
  - **Hybridizing probes**
  - **DNA-binding agents (SYBR Green)**

What is Real-Time PCR?

The Polymerase Chain Reaction (PCR) is a process for the amplification of specific fragments of DNA.

Real-Time PCR a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses.

As we will see, Real-Time PCR allows us to measure minute amounts of DNA sequences in a sample!

A typical amplification plot is shown in Figure 66-3. The amplification plot shows the normalized fluorescence signal from the

reporter (Rn) at each cycle number. In the initial cycles there is little change in the amount of fluorescence. This defines the baseline of the plot. An increase above the baseline indicates detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline to call positive samples. The PCR cycle at which the sample fluorescence passes the threshold is defined as the cycle threshold (CT). There is an inverse linear relationship between the log of the initial target concentration and the CT. Alternatively, the cycle number corresponding to the maximal rate change in fluorescence, the second derivative maximum, has a similar relationship to initial target concentration.

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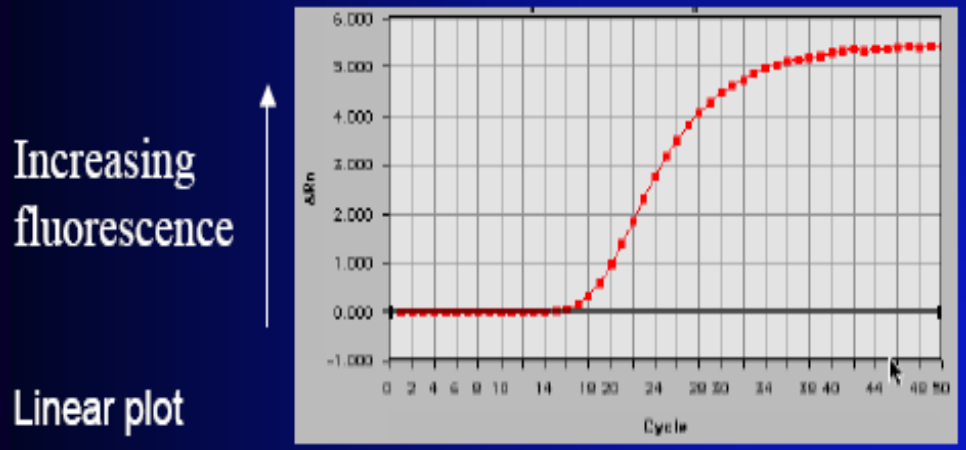
Real time PCR in comparison with other technical methods

Less time to getting results

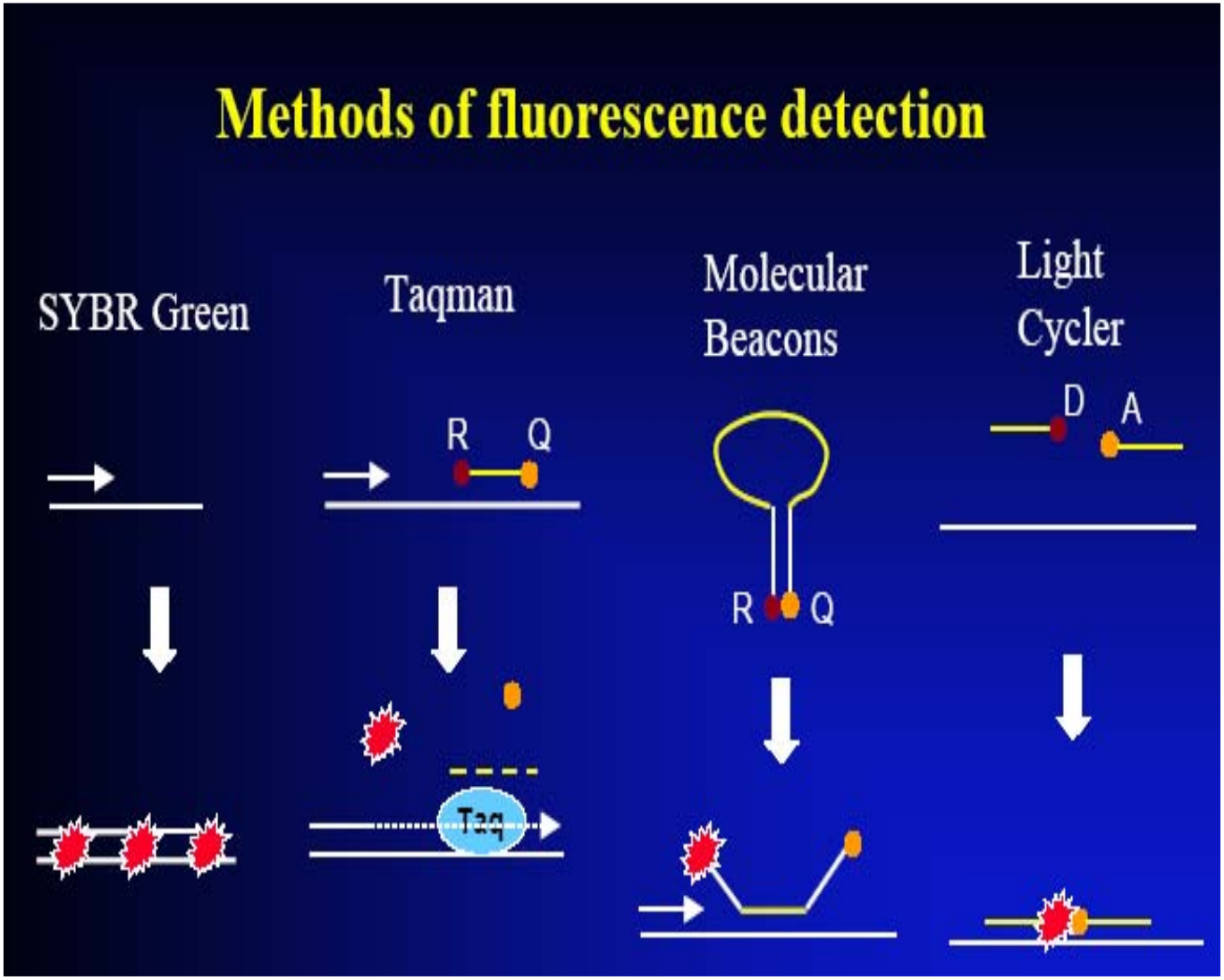
Detection of "amplification-associated fluorescence" at each cycle during PCR

No gel-based analysis at the end of the PCR reaction

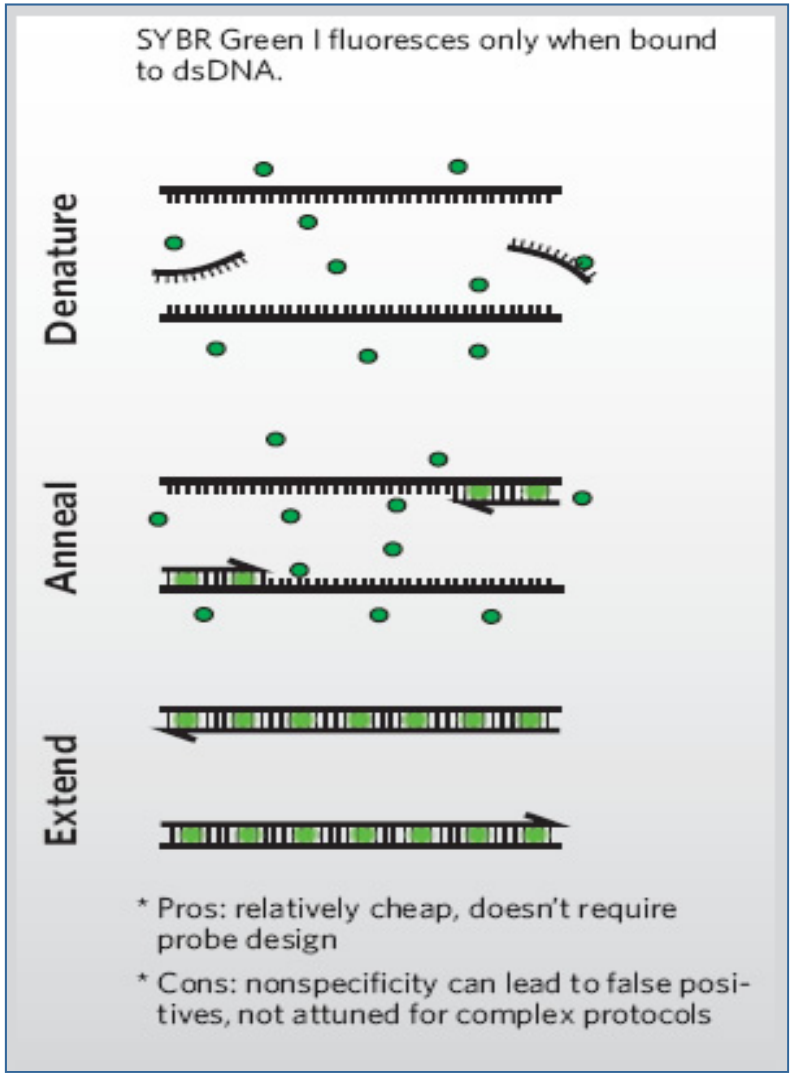
Computer based analysis of the cycle-fluorescence time course



Detection in real time PCR



SYBR® green


SYBR Green I fluoresces only when bound to dsDNA.
Denature
Anneal
Extend
* Pros: relatively cheap, doesn't require probe design
* Cons: nonspecificity can lead to false positives, not attuned for complex protocols


Introduction
Advantage
disadvantages
Real Time PCR
Product Detection
in medicine
Conclusion
Reference

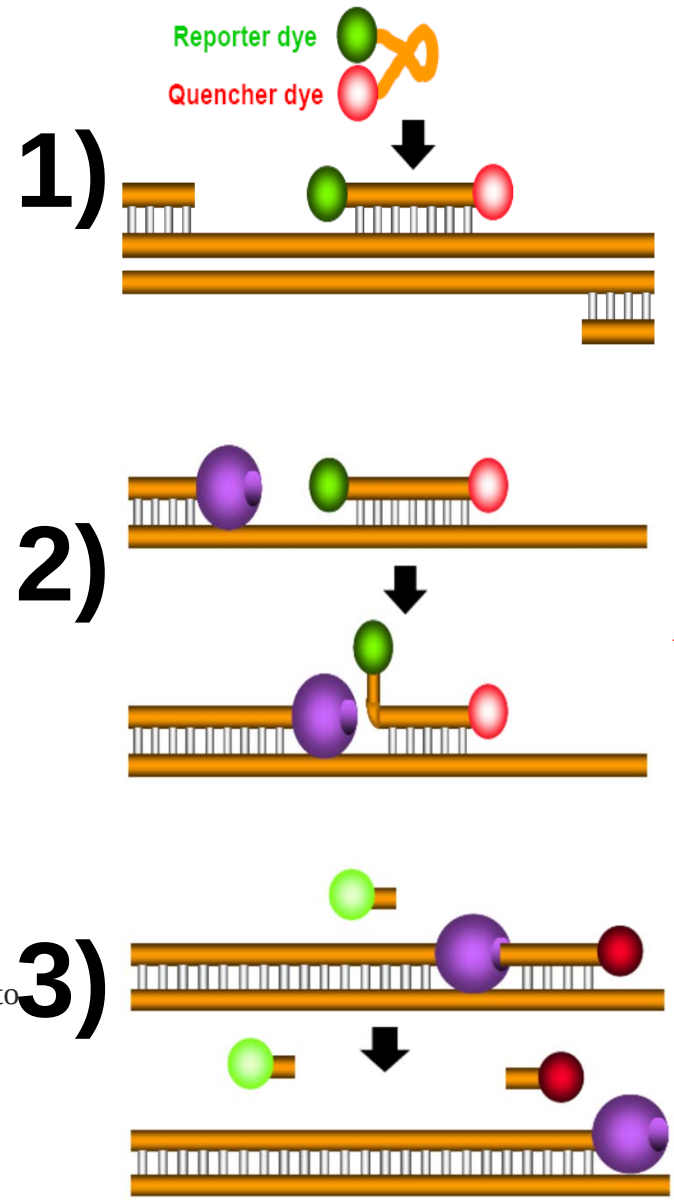
Taqman

1) Denaturation and hybridization of probe.

2) Extension of primer and strand displacement of probe.

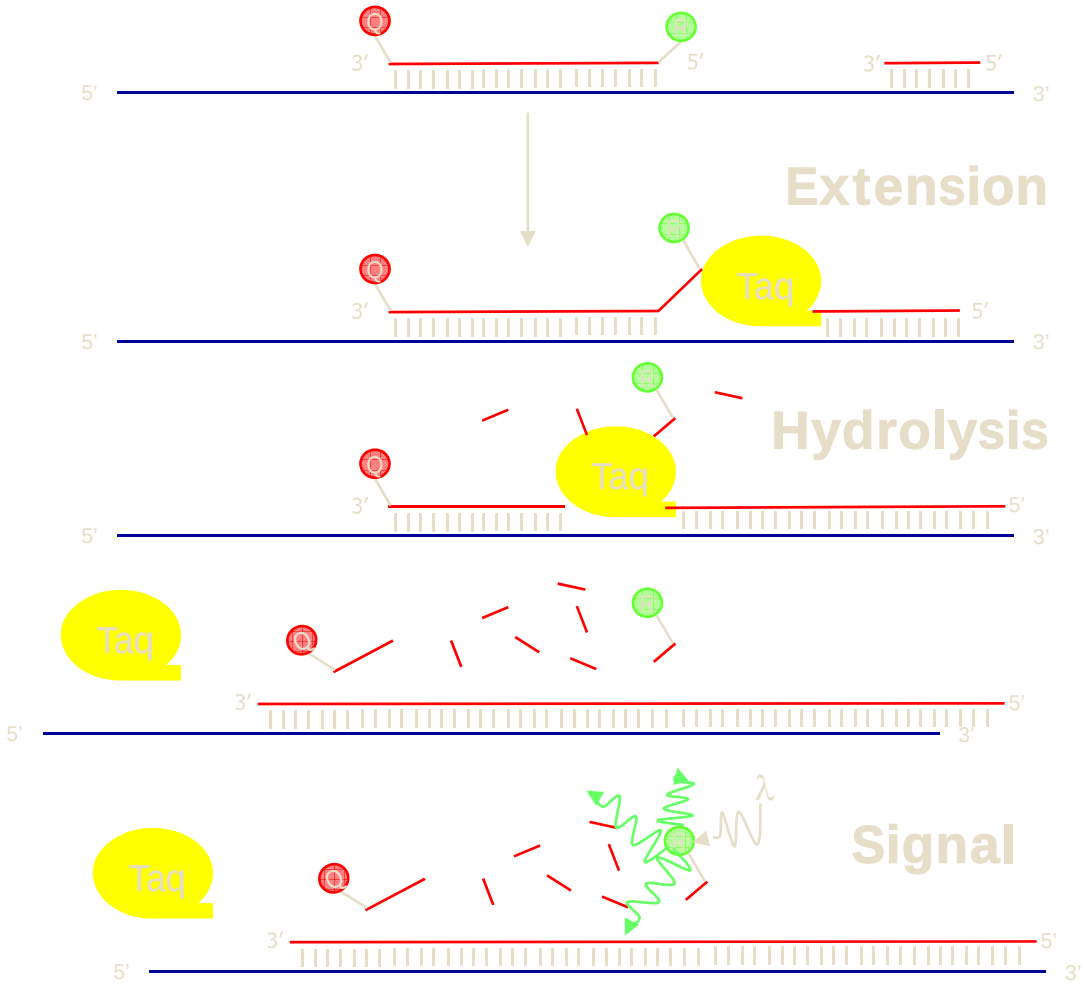
3) Cleavage of probe and fluorescence from the reporter dye.

Fluorescence from reporter dye is directly proportional to the number of amplicons generated

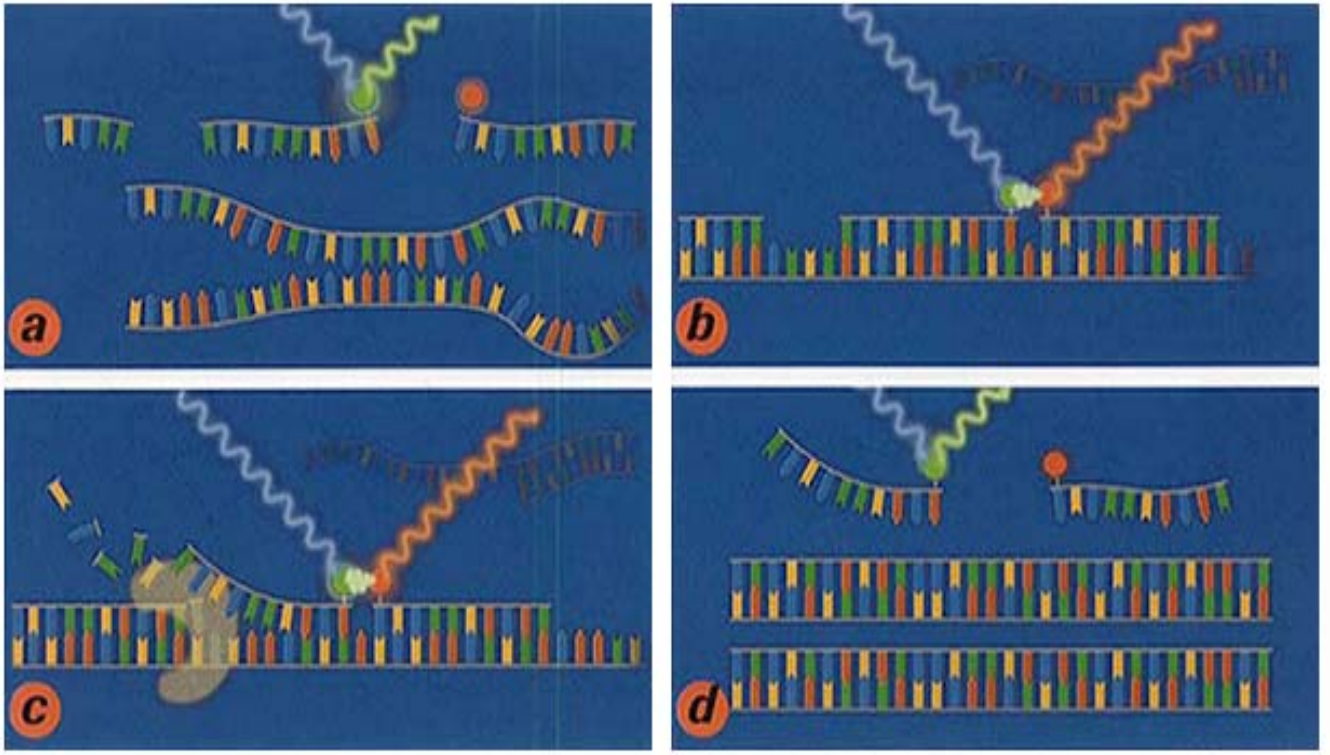


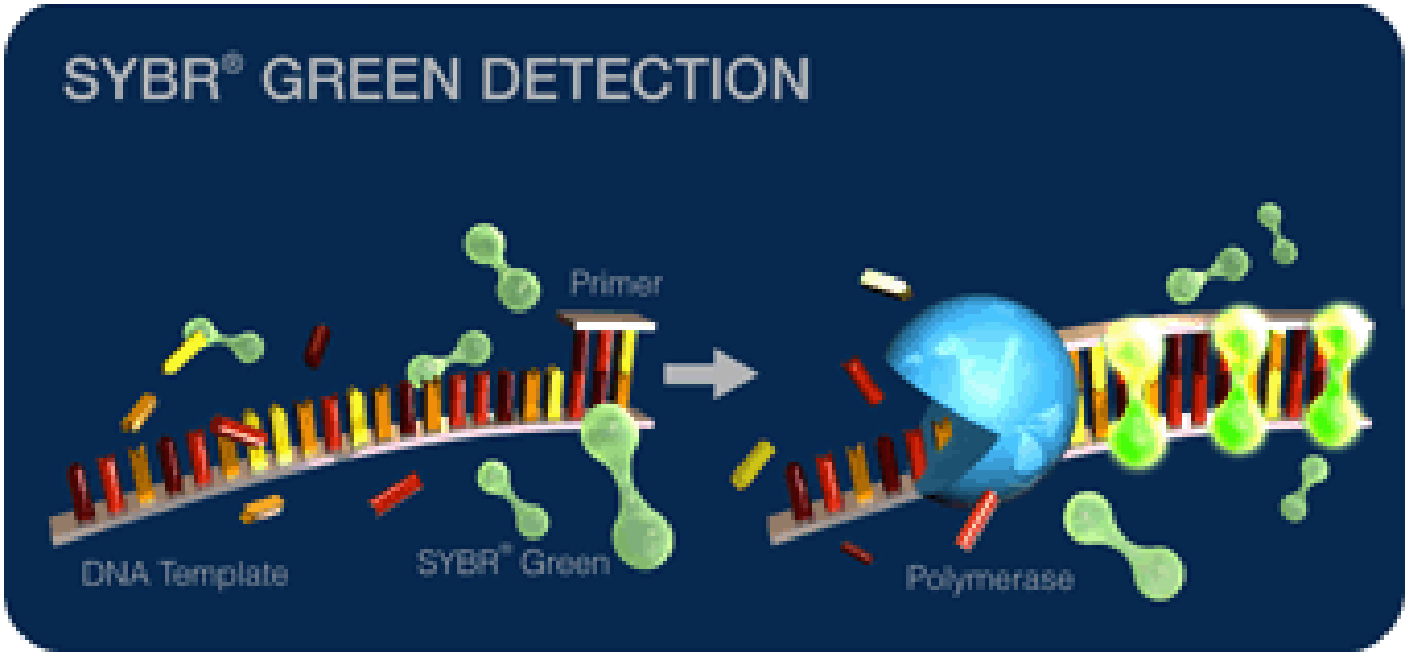
Fluorescent Dyes in PCR

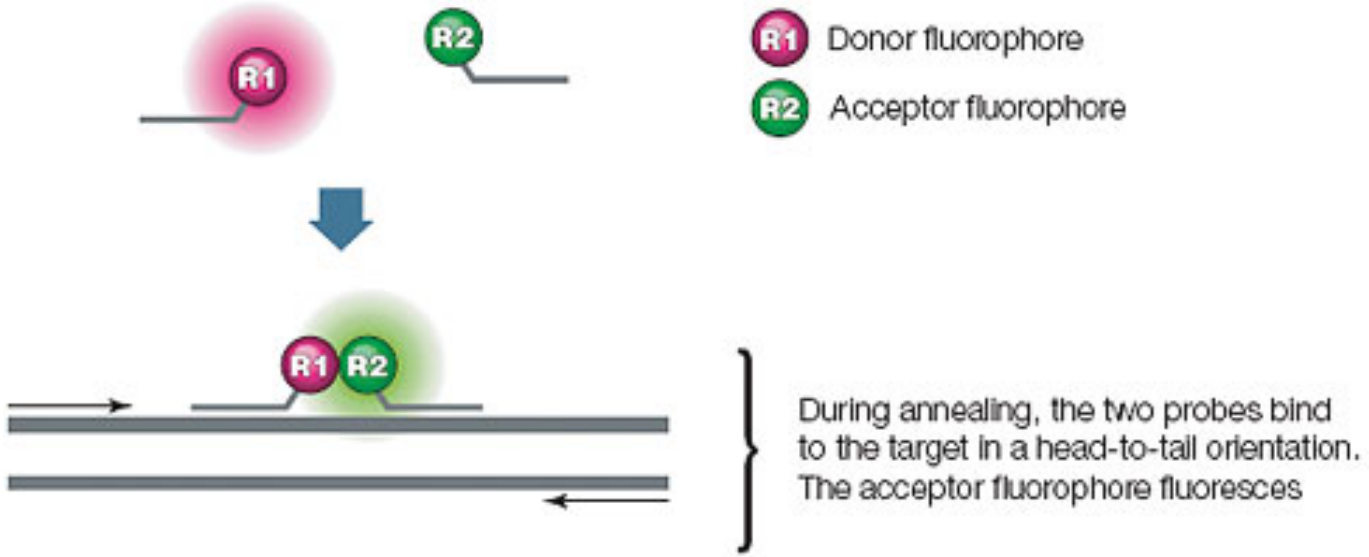
Probes



Hybridization probes technique



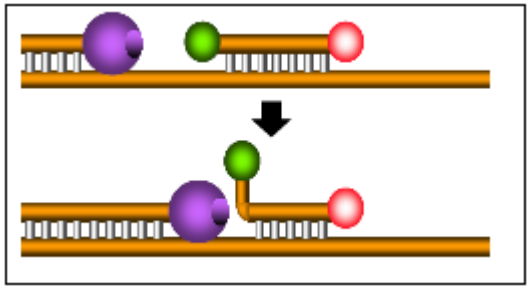

SYBR® GREEN DETECTION
Primer
DNA Template
SYBR® Green
Polymerase

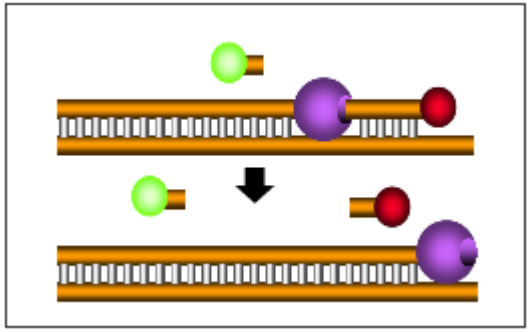

R1
R2
R1 Donor fluorophore
R2 Acceptor fluorophore
R1
R2
During annealing, the two probes bind
to the target in a head-to-tail orientation.
The acceptor fluorophore fluoresces

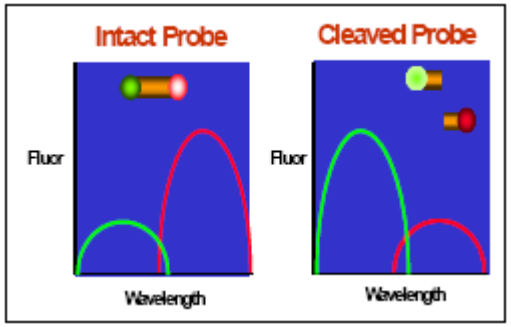
RT detection methods

Fluorescence quenching : the fluorescence of a fluorescent molecule can be quenched by close proximity to a quenching agent upon removal of the quencher, the fluorescence siganl returns

Exonuclease activity: DNA polymerase ability to synthesize new DNA strand ability to remove nucleotides from a second strand of DNA as it's moving on the template





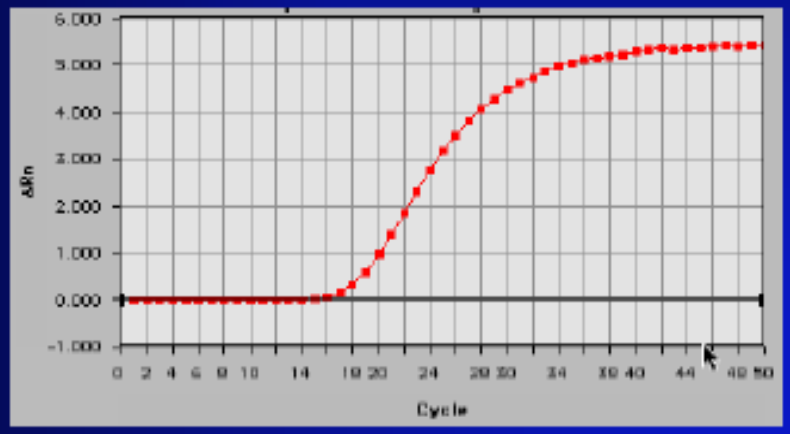


Real-time PCR is kinetic

- Detection of "amplification-associated fluorescence" at each cycle during PCR
- No gel-based analysis at the end of the PCR reaction
- Computer based analysis of the cycle-fluorescence time course

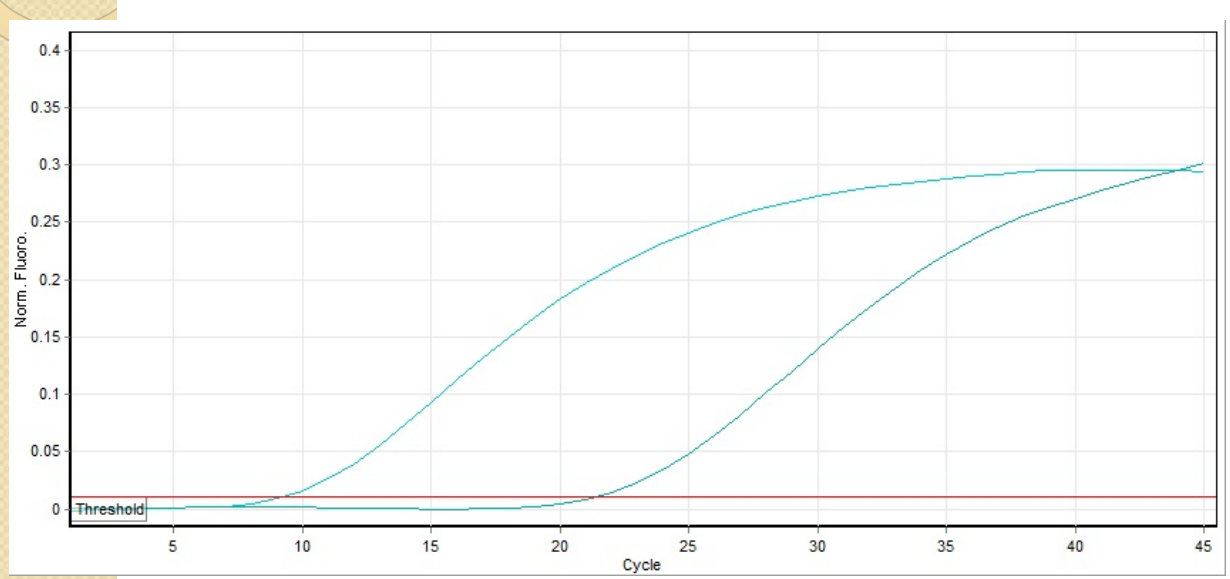
Increasing fluorescence

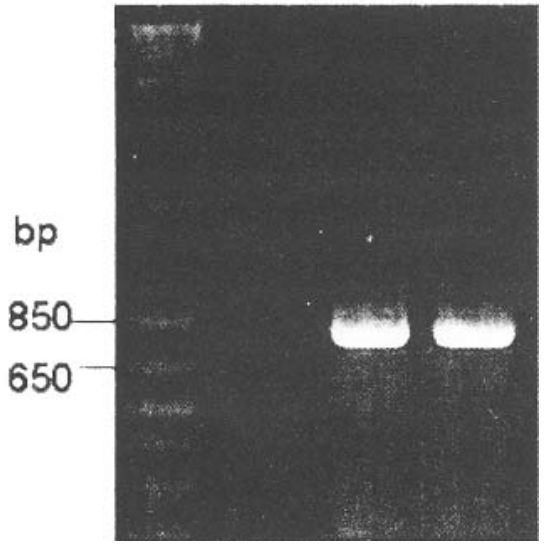
Linear plot

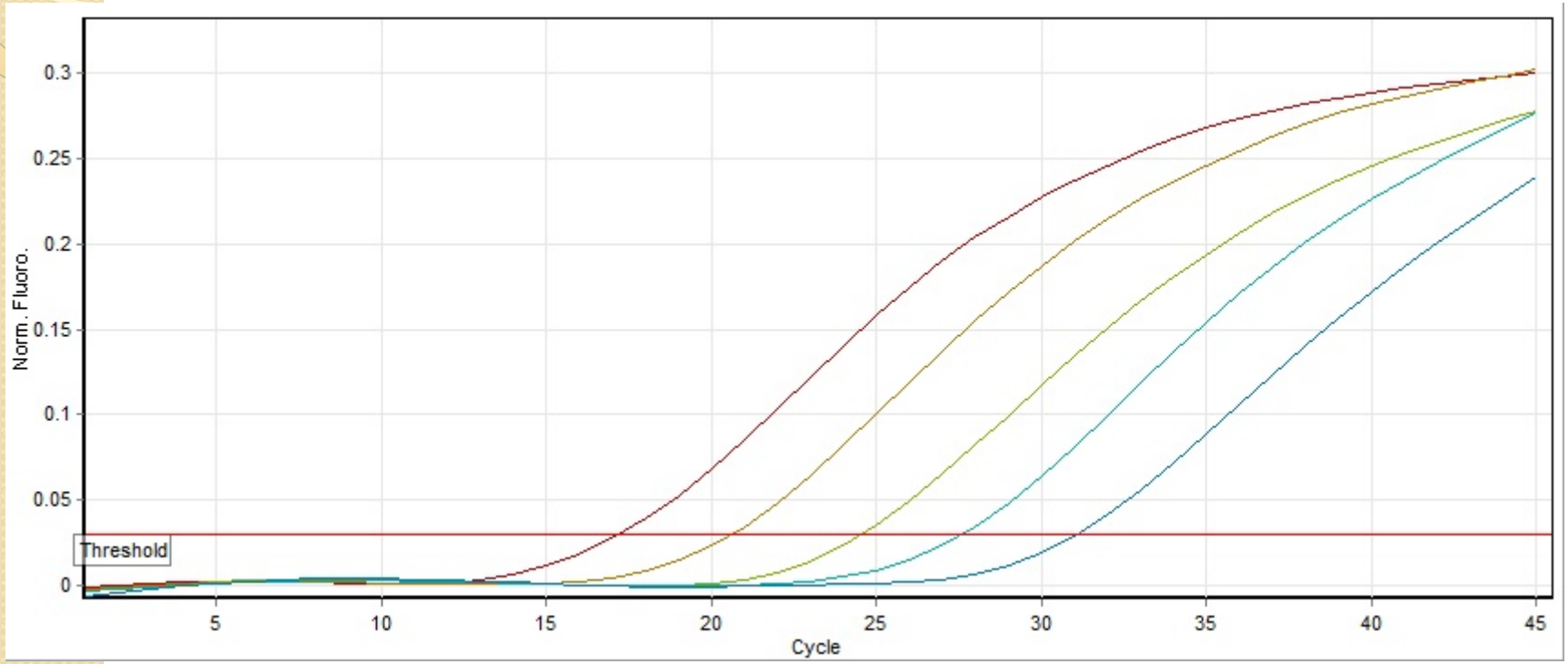


PCR cycle

Nigel Walker, NIEHS (www)


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Norm. Fluoro.
Threshold
5
10
15
20
25
30
35
40
45
Cycle


bp
850
650


0.3
0.25
0.2
0.15
0.1
0.05
0
Norm. Fluoro.
Threshold
5
10
15
20
25
30
35
40
45
Cycle

External Controls

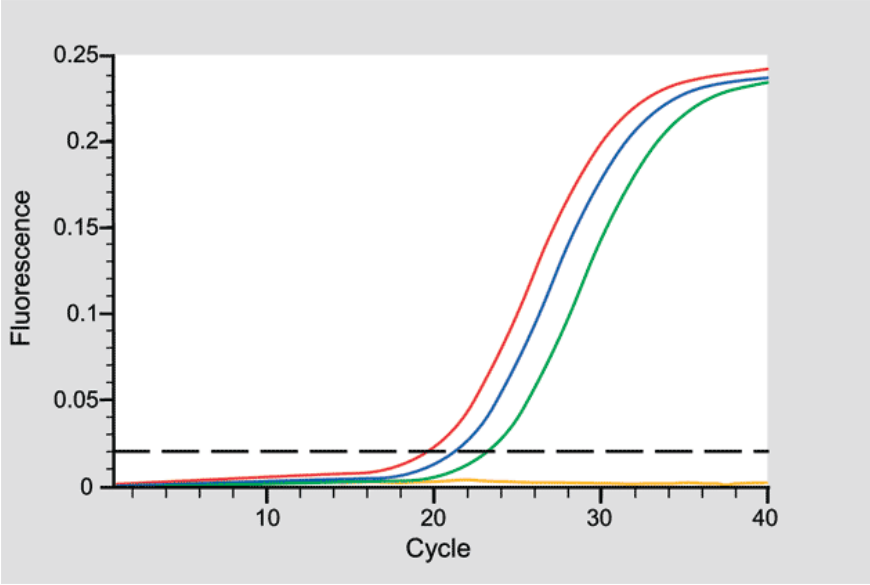
- **Reagent Blanks** (Nontemplate Controls): Applicable reagent controls should be interspersed within each amplification batch run. These controls contain all of the necessary components of the reaction without the addition of template nucleic acid.
- **Negative Controls** Negative controls should contain known nontarget nucleic acid rather than only water or buffer. Always dispense and transfer reagents to negative controls last so that they reflect cumulative effects during manipulations.

External Controls

Positive Controls: A positive control that has a low concentration of target nucleic acid and amplifies weakly, but consistently should be selected.

Internal Control

An advantage of using an internal calibrator is that it allows for, but generally does not distinguish between, detection of inhibitors and recognition of nucleic loss during extraction. When added **prior to specimen extraction**, internal calibrators will undergo the same assay process as the specimen itself.



- temperature is raised slowly, the two strands of the amplicon melt apart
- and the amount of fluorescence decreases. The data are transformed and
- analyzed by plotting the first derivative of fluorescence on the y-axis and
- temperature on the x-axis. The specific amplified product will have a
- characteristic melting peak at its predicted melting temperature (Tm),

- characteristic melting peak at its predicted melting temperature (Tm),
- whereas the primer dimers and other nonspecific products should have a
- different Tm or give broader peaks

Fluorescent reporters used in real-time PCR include double-stranded DNA (dsDNA)–binding dyes, or dye molecules attached to PCR primers or probes that hybridize with PCR products during amplification.

The advantages of real-time PCR include:

- Ability to monitor the progress of individual PCR reactions as they occur in real time
- Ability to precisely measure the amount of amplicon at each cycle, which allows highly accurate quantification of the amount of starting material in samples
- An increased dynamic range of detection
- Amplification and detection occur in a single tube, eliminating post-PCR manipulations