# Chapter VI:

Gene expression analysis techniques

Gene expression analysis techniques

# Introduction

- Genes can have specific, differentiated gene expression.
    - over time (specific to a stage of development),
    - in space (specific to a cell, tissue or organ type)
    - or/and characteristic of a given state (normal, pathological or in response to a particular stimulus).

Gene expression analysis techniques

## Introduction

- The study of gene expression consists of characterizing and quantifying the expression products of DNA (messenger RNAs: the transcriptome) in order to identify, in a tissue, in a state and at a given time of development, the active sequences and thus reveal the level of expression of the genes from which they originate.

# Gene expression analysis techniques

- Quantitative PCR (or real-time PCR)
- RT-PCR
- Southern blot
- Northern blot
- Reporter gene
- .....

# Real-time PCR or qPCR

# Real-time PCR or qPCR

- **Introduction of qPCR applied to food:**
- qPCR or also called Real Time PCR is used in food analysis to detect and quantify DNA from various sources, including pathogens, probiotics, and contaminants. It offers faster, more reliable, and more sensitive results than traditional methods for applications like food safety testing, authenticity verification, and labeling accuracy.
- For example In food science, qPCR is a powerful tool for detecting, identifying, and quantifying microorganisms, allergens, genetically modified organisms (GMOs), and adulterants in food products

# qPCR

- In real-time PCR, the amount of DNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated.
- Data collected in the exponential phase of the reaction yield quantitative information on the starting quantity of the amplification target.
- 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 product during amplification.

# qPCR

- The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability.
- By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction

# The advantages of real-time PCR include:

- The advantages of real-time PCR include:
- Ability to monitor the progress of the PCR reaction as it occurs 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 occurs in a single tube, eliminating post-PCR manipulations

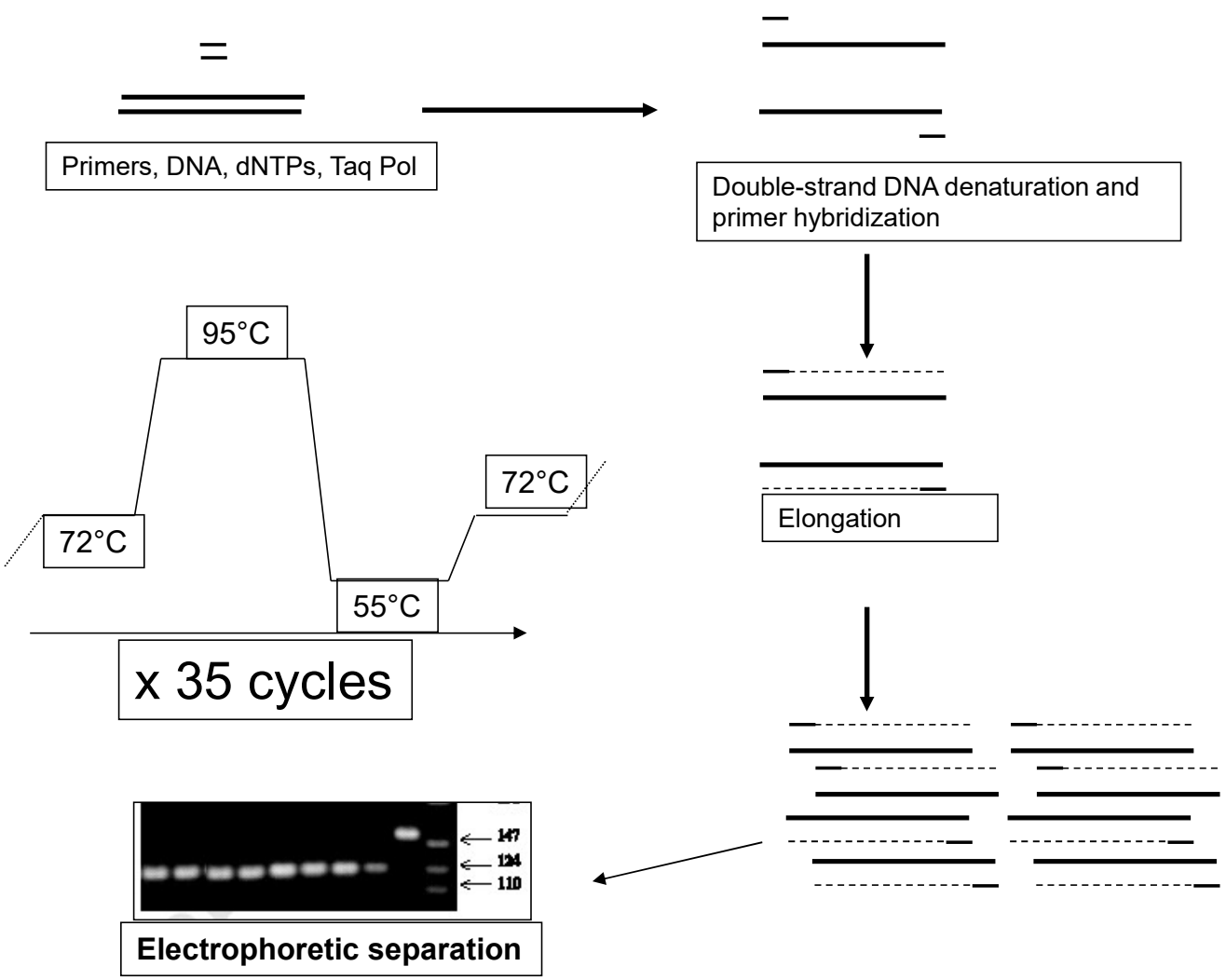
# Advantages of using qPCR

- **Speed:** Provides results much faster than conventional, culture-based methods.
- **Sensitivity and specificity:** Can detect very low levels of target DNA.
- **Quantification:** Enables not just detection but also quantification of the amount of DNA present.
- **Multiplexing:** Allows for the simultaneous detection of multiple targets in a single reaction.
- **Automation:** Can be semi-automated, which streamlines workflow and improves efficiency.
- **Safety:** Reduces the risk of cross-contamination after the initial reaction is complete.

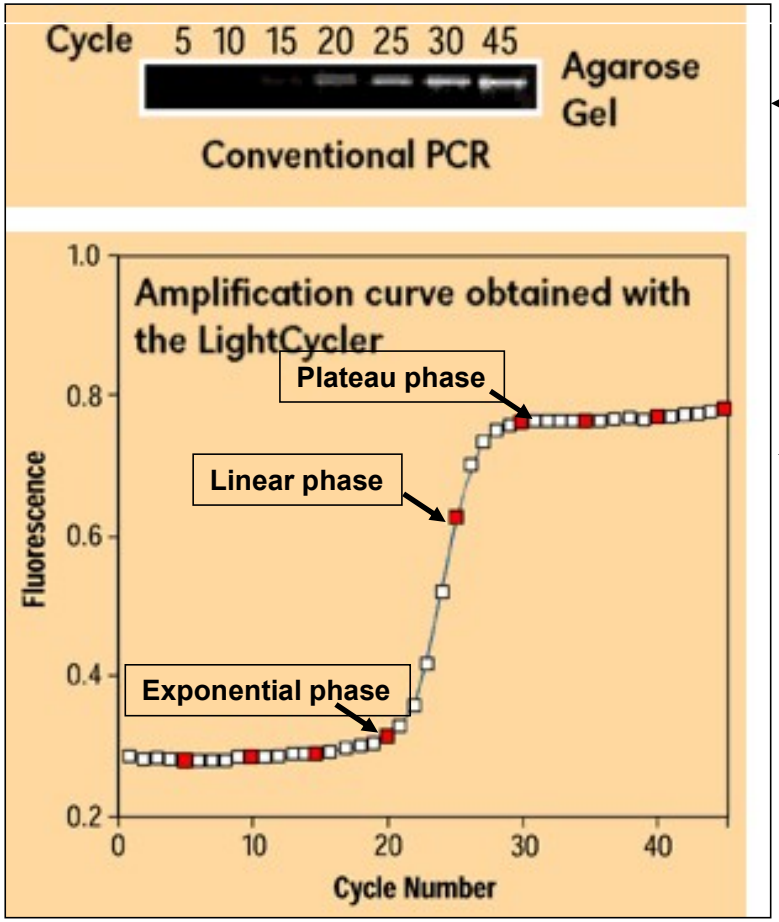
# Principle of qPCR

- qPCR combines traditional PCR amplification with **fluorescent detection** of DNA in real time.
- qPCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore.
- The PCR process generally consists of a series of temperature changes that are repeated 30–40 times or more.
- These cycles normally consist of three stages: the first, at around 95 °C, allows the separation of the nucleic acid's double chain; the second, at a temperature of around 50–60 °C, allows the binding of the primers with the DNA template; the third, at 72 °C, facilitates the polymerization carried out by the DNA polymerase.

# Principle of conventional PCR



# Conventional PCR *versus* quantitative PCR



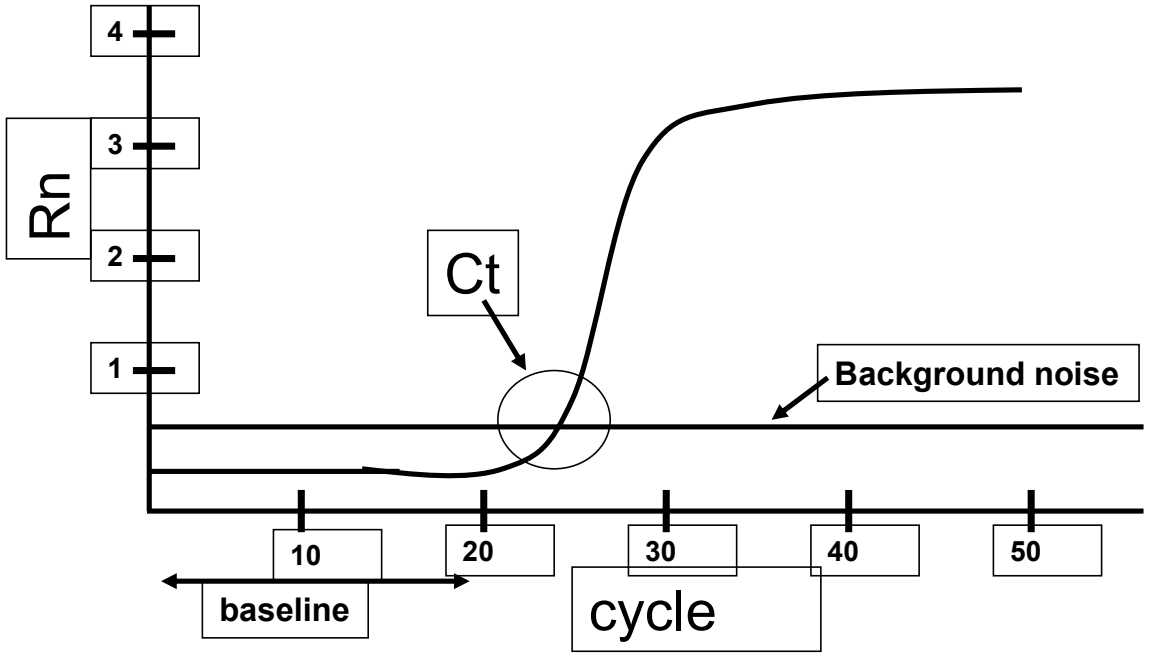

Final point analysis on agarose gel

Dynamic analysis of quantitative PCR

-high precision during the exponential phase

-Significant variability during the plateau phase

# Determination of Ct (cycle threshold)



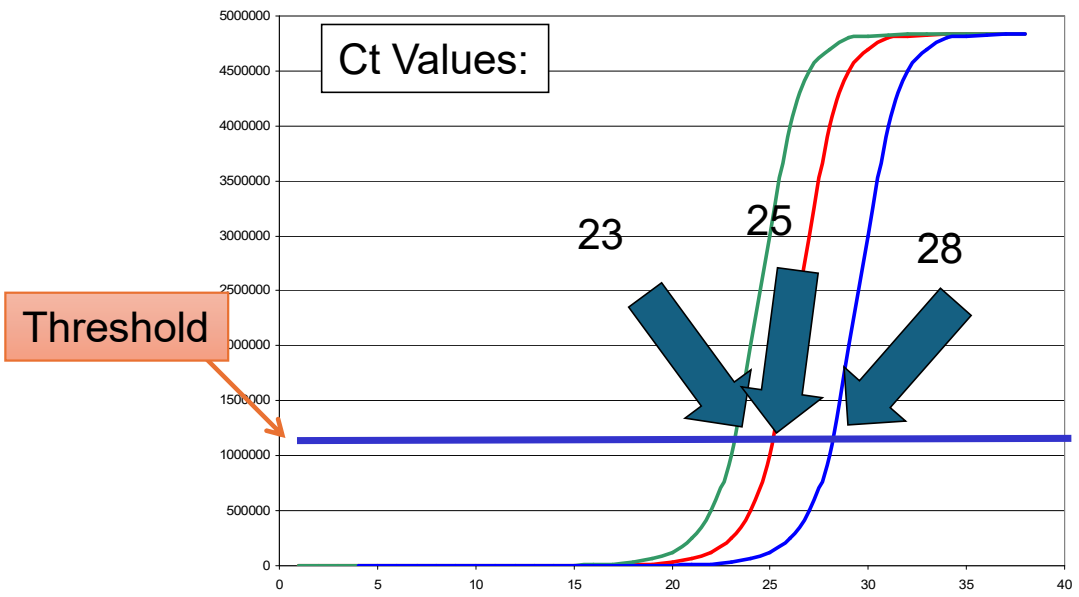

The value of Ct is determined in the exponential phase, at the intersection of the background noise line with the fluorescence curve.

# Imagining Real-Time PCR Measuring Quantities

## The *"ct value"*

- **The value that represents the cycle number where the amplification curve crosses an arbitrary threshold.**
- **Ct or Cq or Cp values are directly related to the starting quantity of DNA, by way of the formula:**

**Quantity = $2^{Ct}$**



| CYCLE NUMBER | AMOUNT OF DNA |
|---|---|
| 0 | 1 |
| 1 | 2 |
| 2 | 4 |
| 3 | 8 |
| 4 | 16 |
| 5 | 32 |
| 6 | 64 |
| 7 | 128 |
| 8 | 256 |
| 9 | 512 |
| 10 | 1,024 |
| 11 | 2,048 |
| 12 | 4,096 |
| 13 | 8,192 |
| 14 | 16,384 |
| 15 | 32,768 |
| 16 | 65,536 |
| 17 | 131,072 |
| 18 | 262,144 |
| 19 | 524,288 |
| 20 | 1,048,576 |
| 21 | 2,097,152 |
| 22 | 4,194,304 |
| 23 | 8,388,608 |
| 24 | 16,777,216 |
| 25 | 33,554,432 |
| 26 | 67,108,864 |
| 27 | 134,217,728 |
| 28 | 268,435,456 |
| 29 | 536,870,912 |
| 30 | 1,073,741,824 |

# Importance of controls

- negative control
  - checks reagents for contamination

# Fluorescence detection methods:

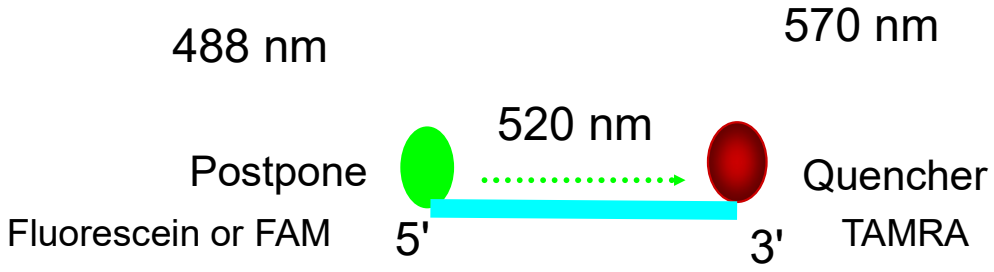
- Fluorescence detection methods:
- (1) **SYBR Green**: binds to double-stranded DNA; simple but less specific.
- (2) **TaqMan probes**: sequence-specific fluorescent probes; high specificity.

# Quantitative PCR with a TaqMan® probe

Principle: During PCR, 3 single-stranded oligonucleotides are added to the reaction medium

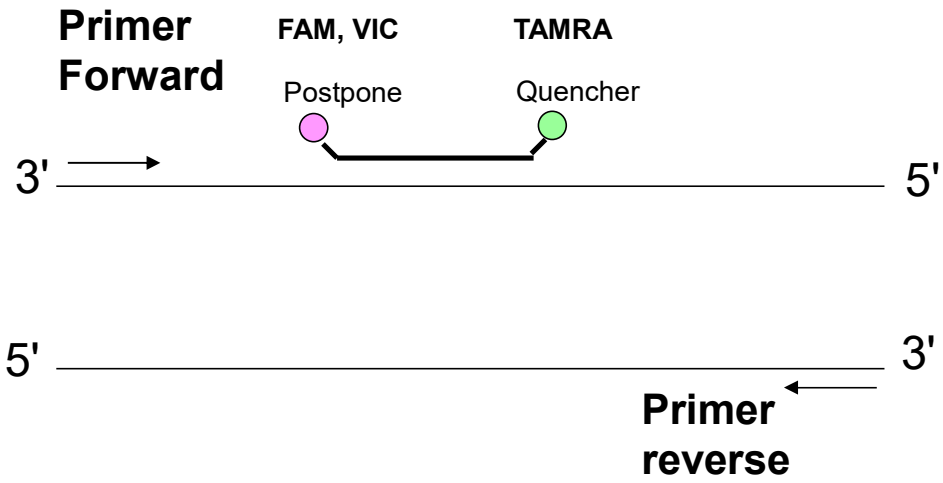
- The two primers that allow amplification (sense primer and antisense primer)
- A probe that can hybridize to a sequence corresponding to the amplified PCR product (specificity!!)

The probe contains two fluorophores: a reporter that is excited by the laser, and a quencher whose excitation wavelength matches the emission wavelength of the reporter. When the two fluorophores are close together, the quencher absorbs the fluorescence of the reporter.



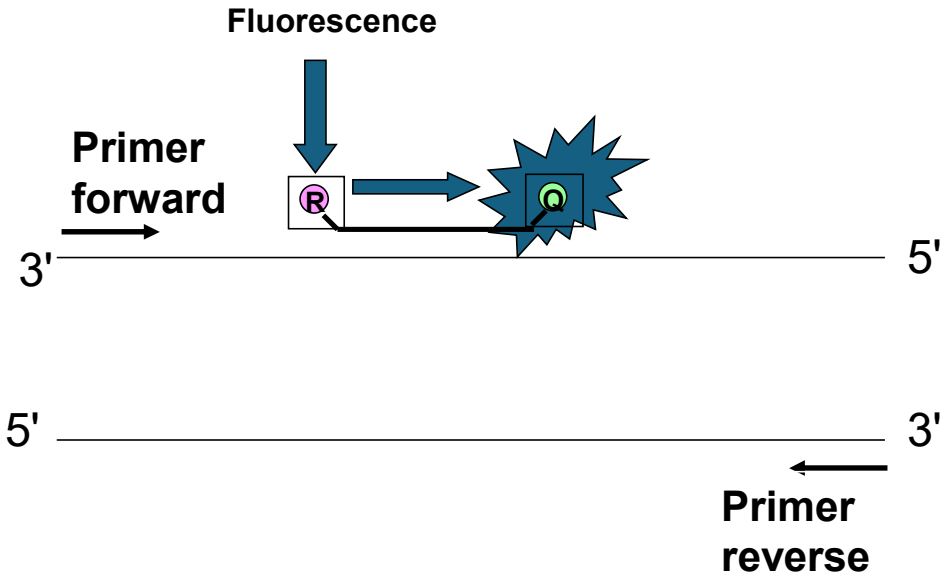
# Quantitative PCR with a TaqMan® probe

This method is based on two principles:
-FRET technology (fluorescence resonance energy transfer)
-the 5'-exonuclease activity of Taq Pol




-Specificity of the primer for PCR
-Specificity of the TaqMan probe hybridization

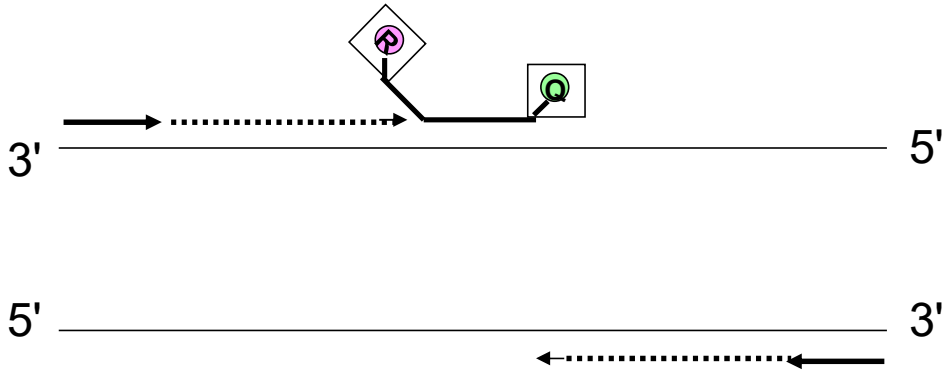
# 5' exonuclease with the TaqMan® probe



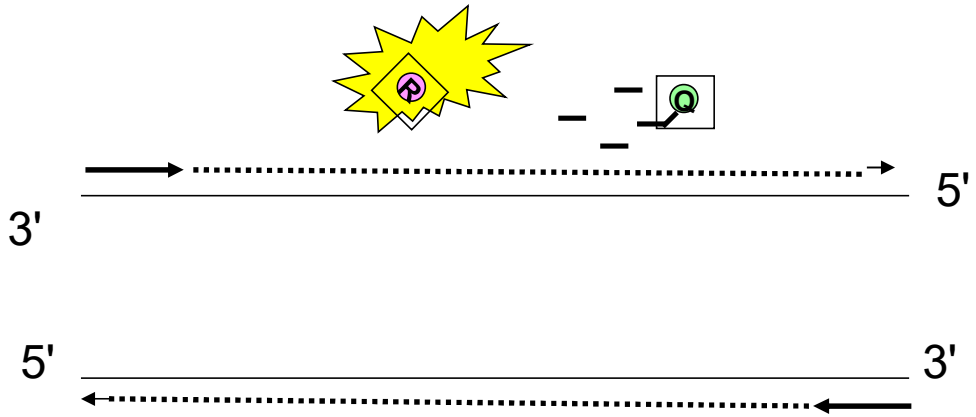

-FRET (fluorescence resonance energy transfer) from the reporter (high energy) to the quencher (low energy),

➔No fluorescent signal emitted by the transmitter when the probe is intact

# 5' exonuclease with the TaqMan® probe

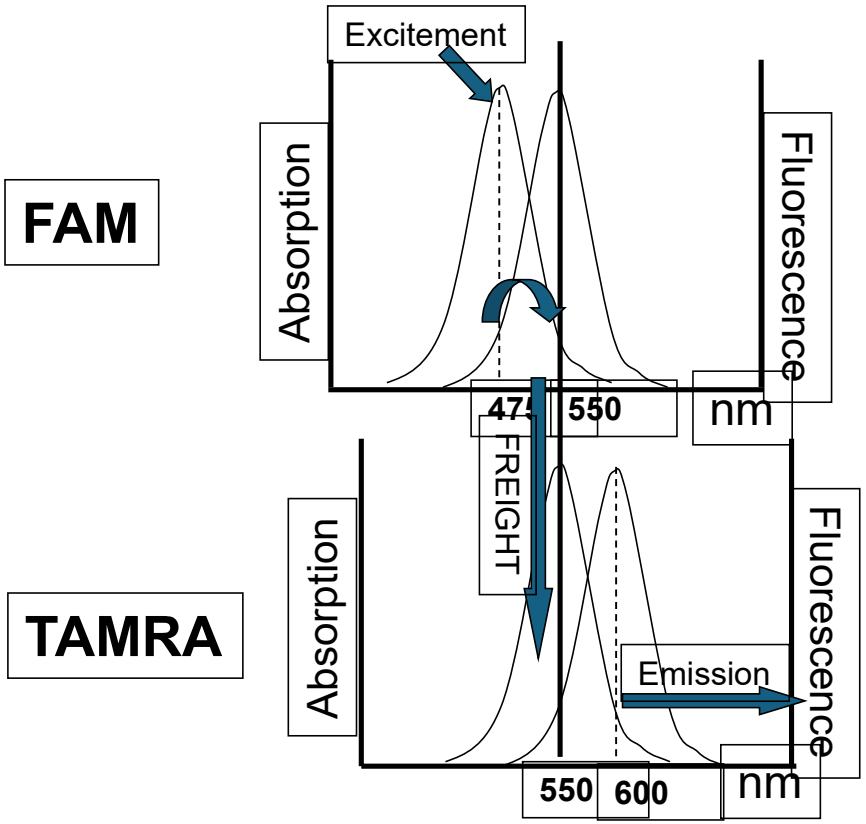



-during Taq Pol-catalyzed elongation, the 5' nuclease activity displaces the probe




-when polymerization is complete, for each DNA molecule synthesized a reporter emits fluorescence.

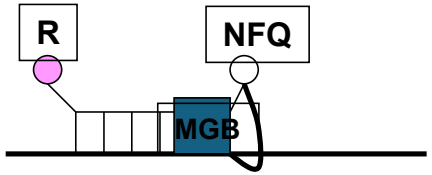
# Quenching mechanism




-The Reporter's emission spectrum overlaps with the Quencher's absorption spectrum

-FREIGHT

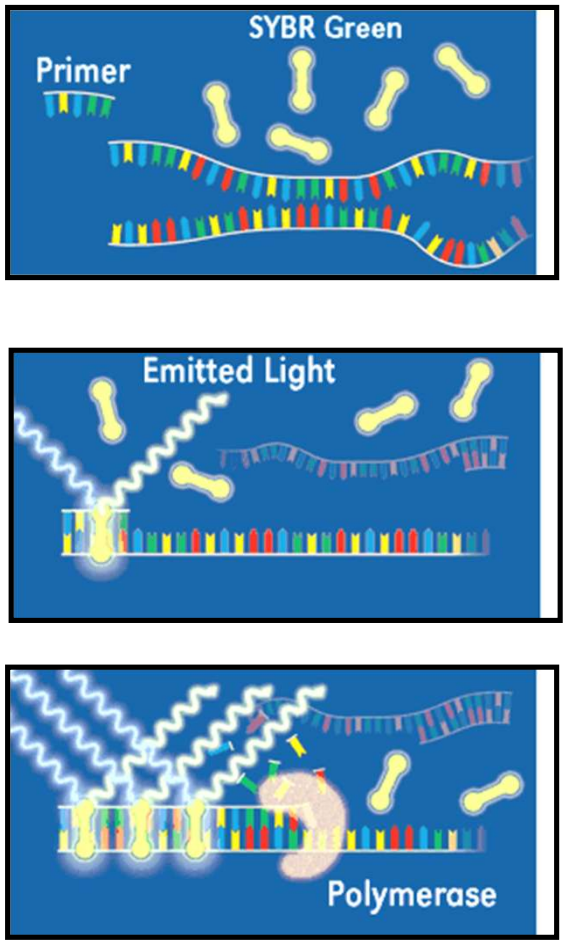
# TaqMan® MGB probes



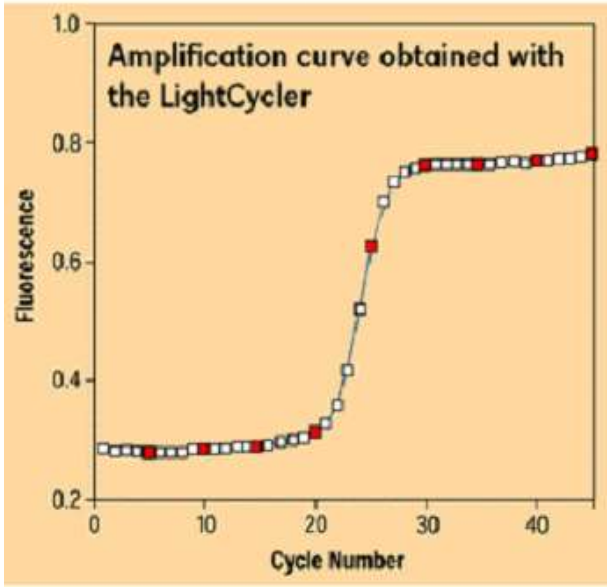

**NFQ non-fluorescent quencher**
➔New innovation

**MGB minor groove binder**
➔ stabilizes the last 5 to 6 bases of the probe (at its 3' end)
➔ shorter and more specific probe (for a given Tm)

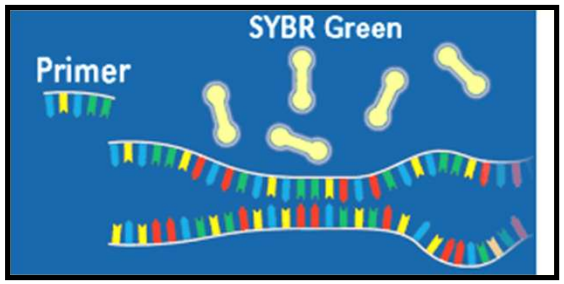
# Quantitative PCR SYBR® Green

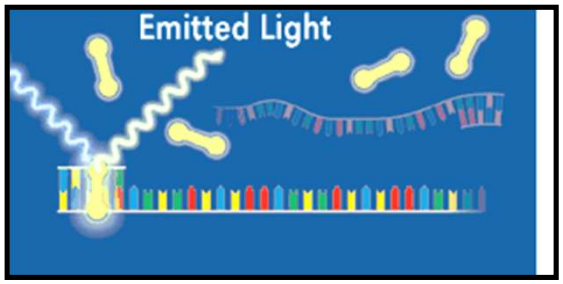


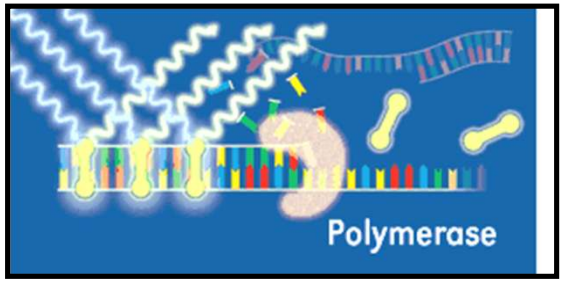

Fluorescence measurement at the end of elongation

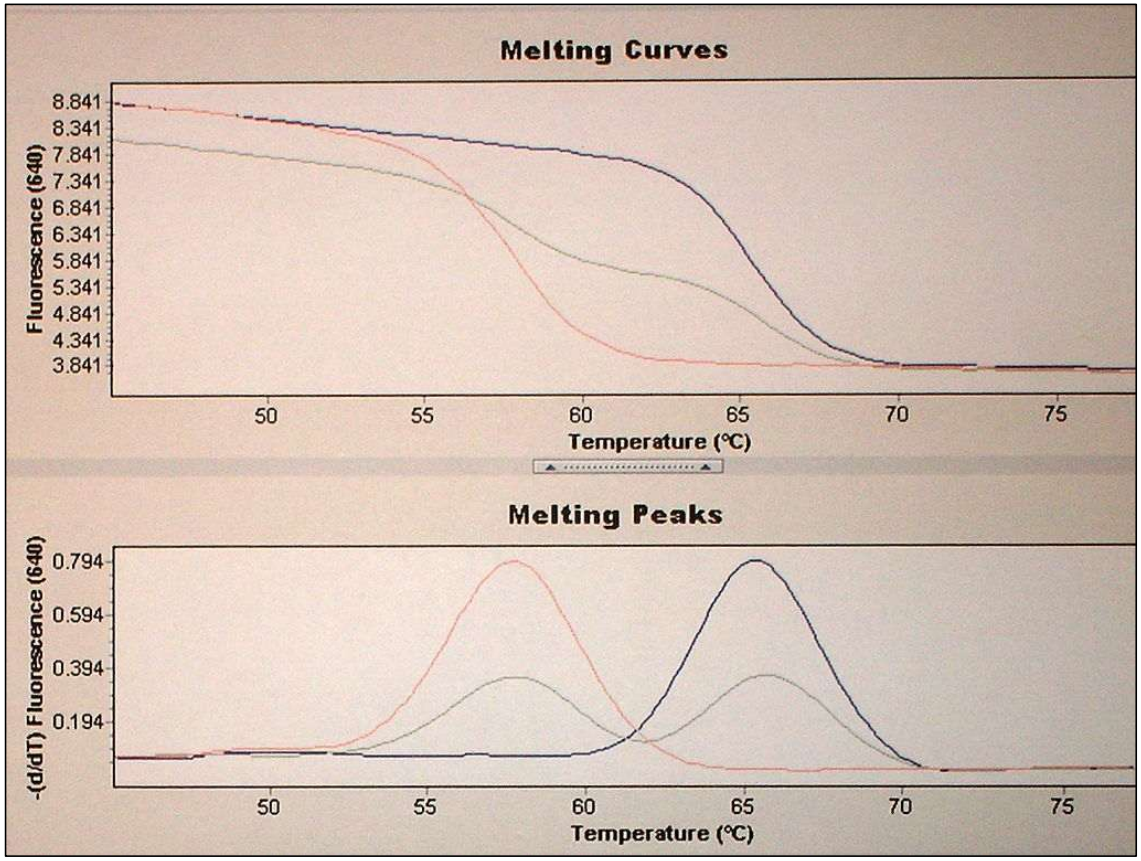


# SYBR® Green : melting curves

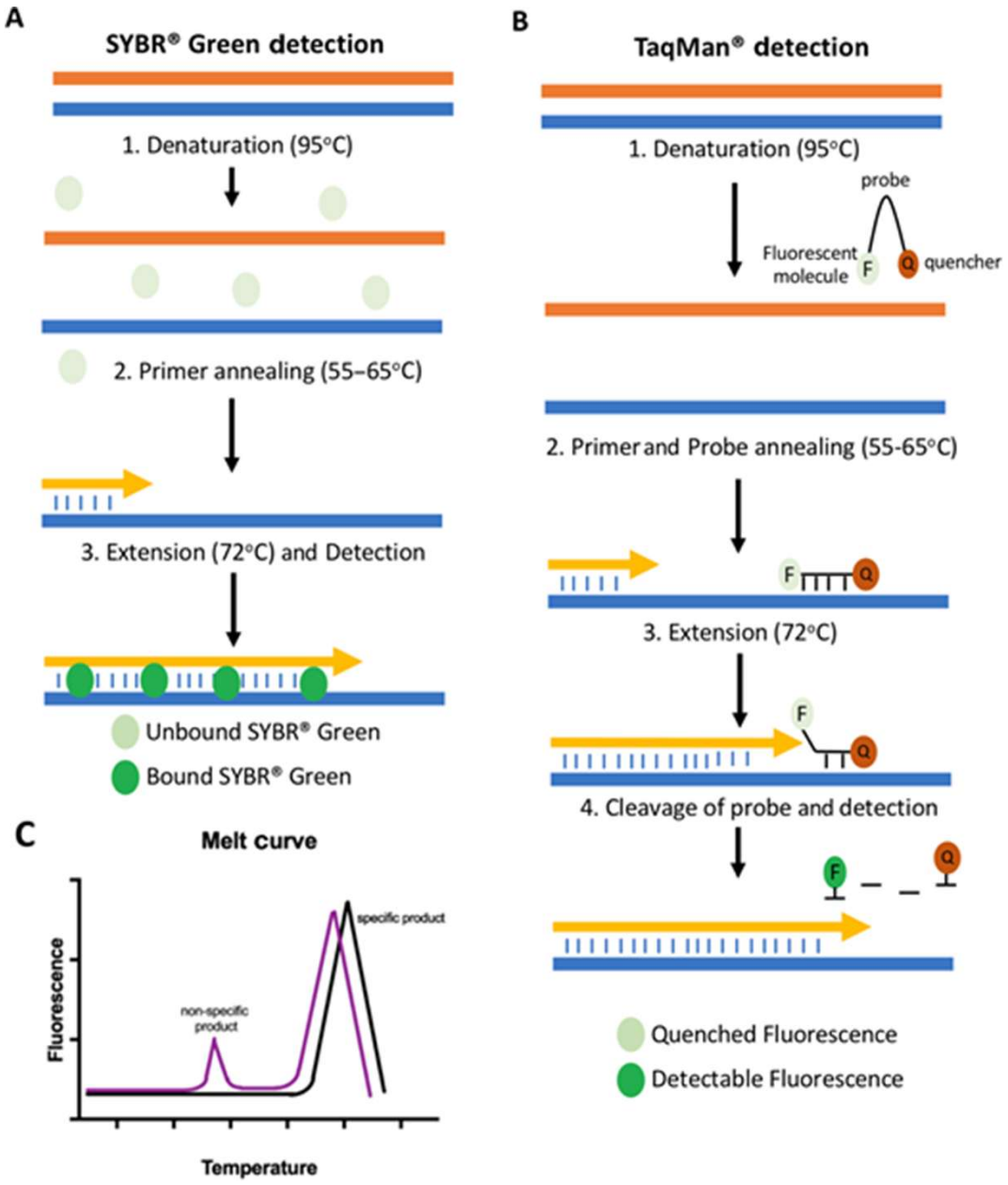


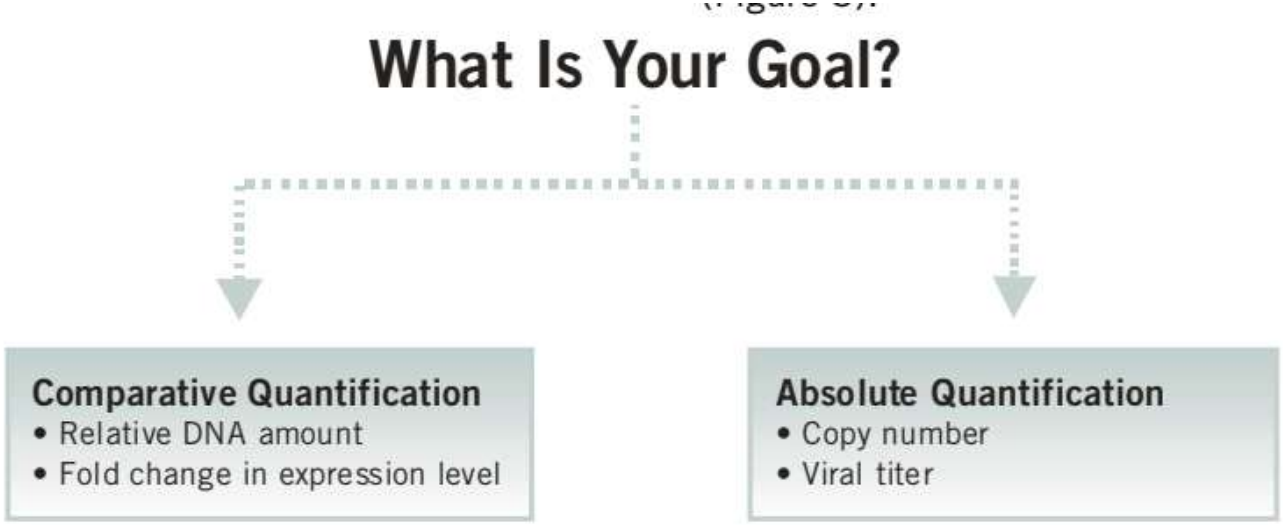



# SYBR® Green : melting curves

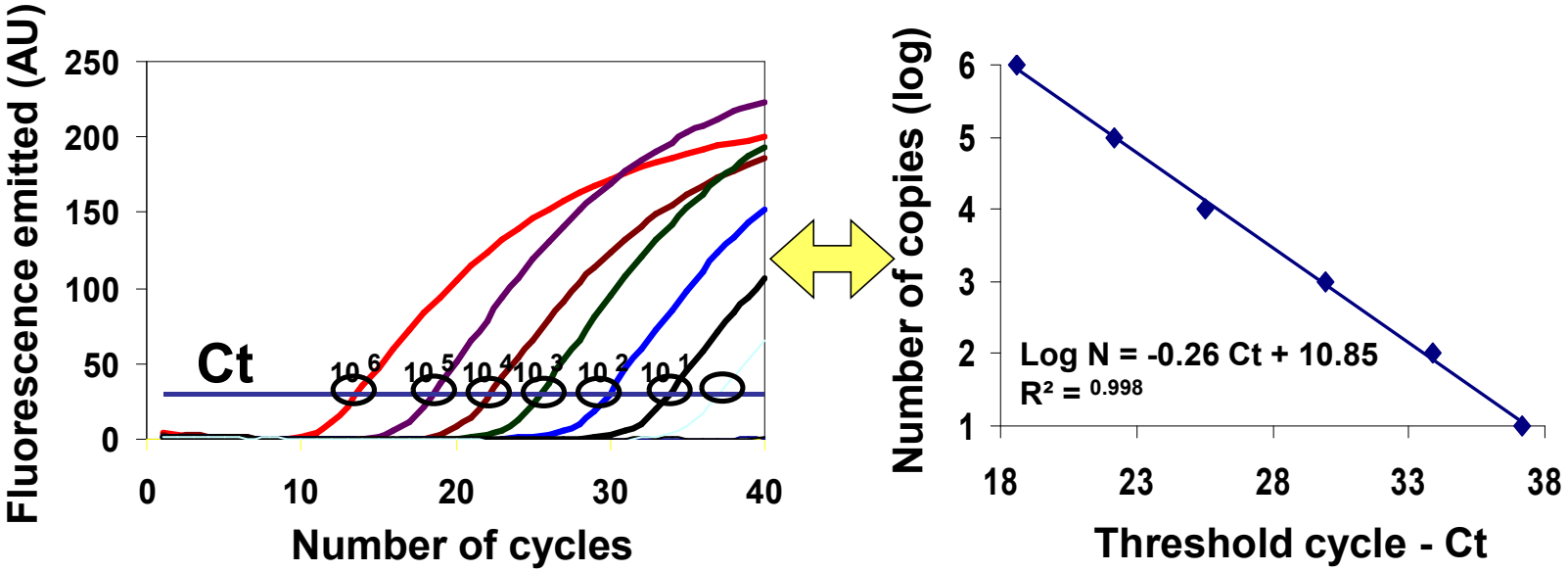


# QUANTIFICATION



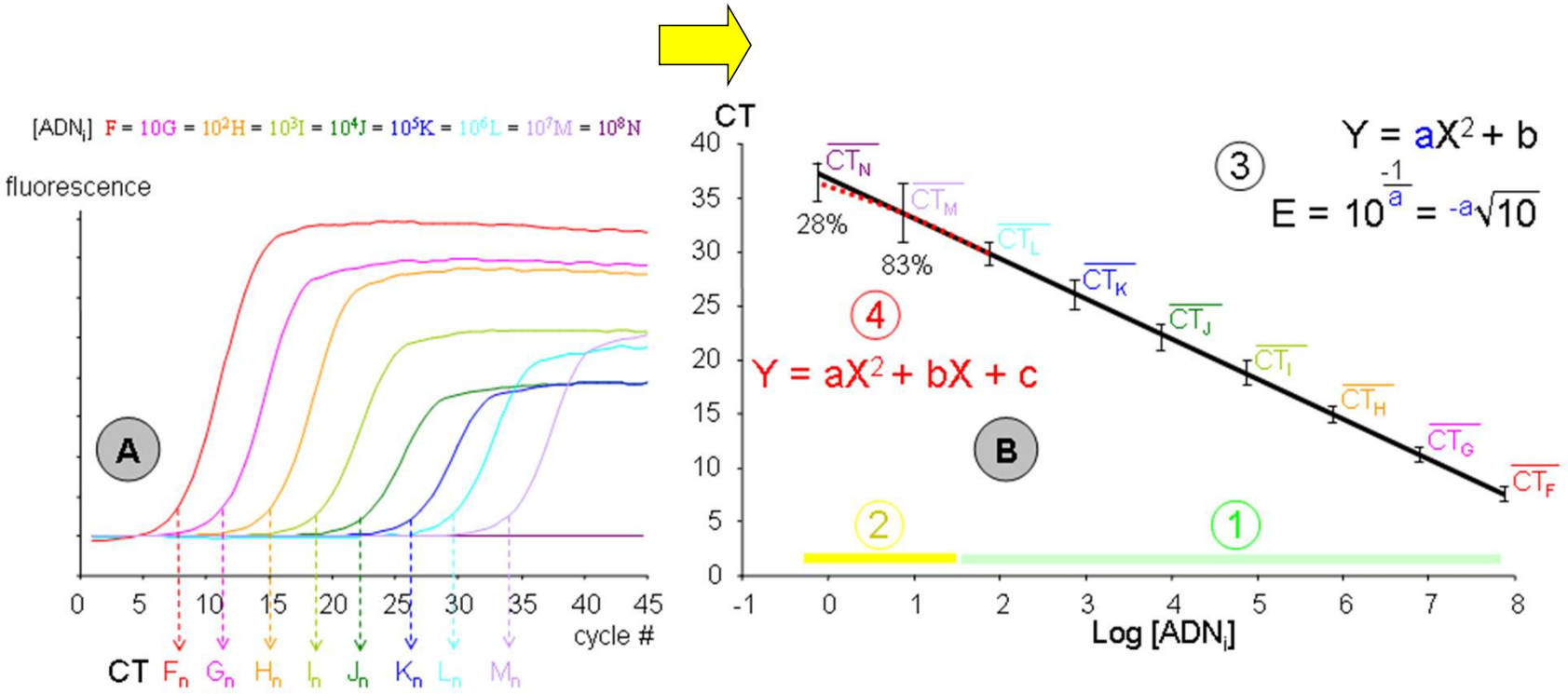
# Calibration curve: compare the Ct values

**Absolute quantification: to determine the exact quantity of target DNA (e.g., viral load)**



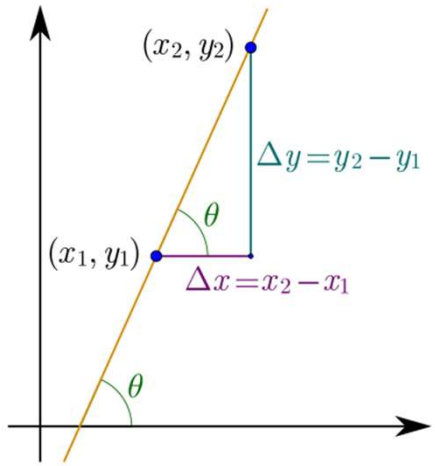

**The number of cycles required to achieve a given fluorescence (log-linear phase) is a function of the number of target DNAs initially present**

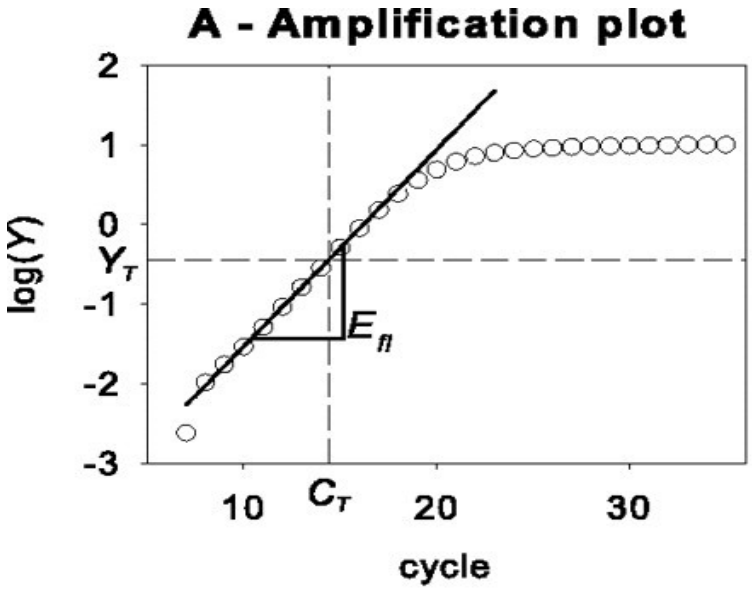
# Absolute quantification

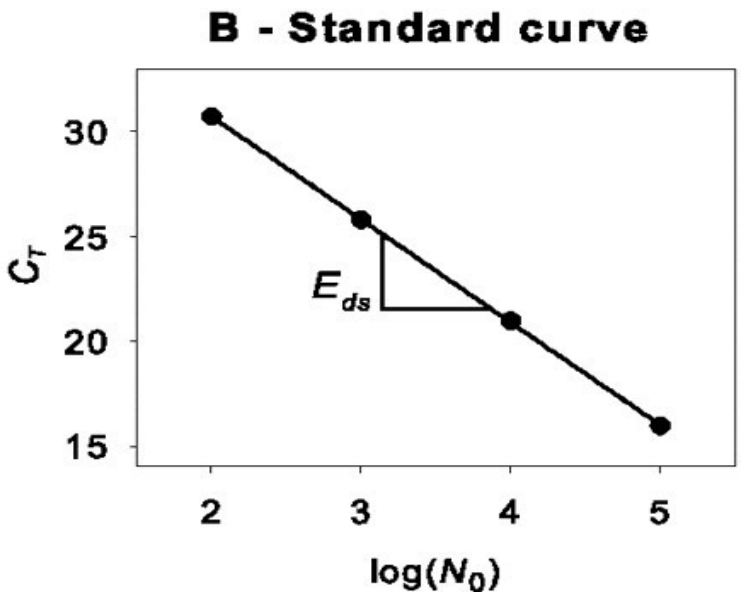


# The slope

$$\frac{\Delta y}{\Delta x} = \tan\theta$$

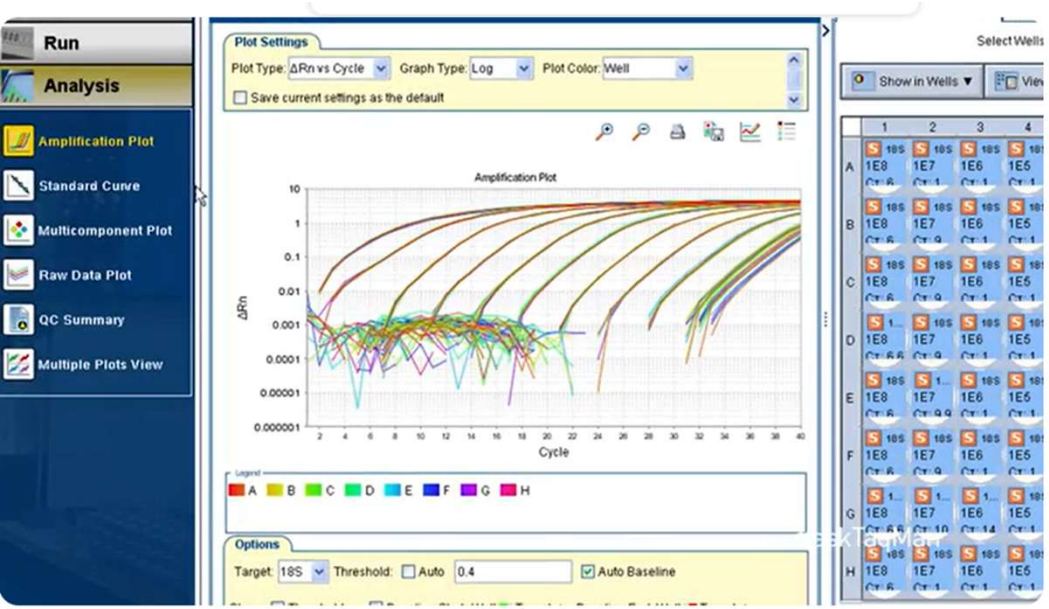


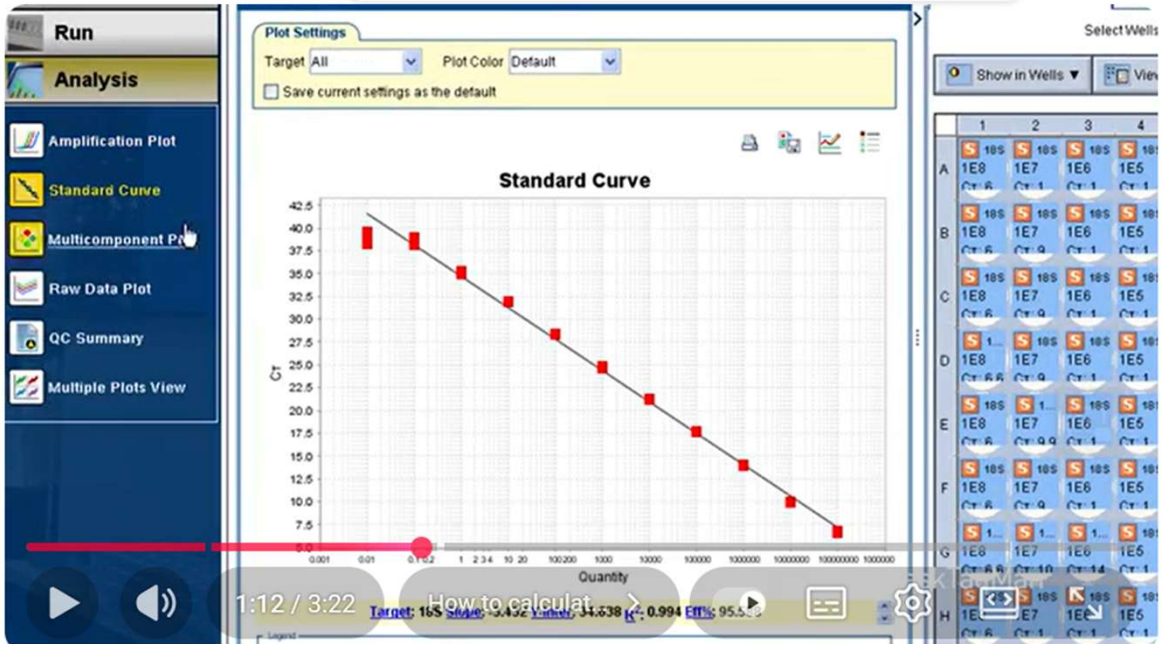




**Abbreviations**

$Y_T$ - threshold fluorescence
$C_T$ - threshold cycle
$N_0$ - starting template concentration
$E$ - Efficiency
$E_{fl}$ - Efficiency estimated from fluorescence increase
$E_{ds}$ - Efficiency estimated from the slope of a dilution series


Run
Analysis
Amplification Plot
Standard Curve
Multicomponent Plot
Raw Data Plot
QC Summary
Multiple Plots View
Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Log
Plot Color: Well
Save current settings as the default
Amplification Plot
ΔRn
Cycle
Legend
A B C D E F G H
Options
Target: 18S
Threshold:
Auto
0.4
Auto Baseline
Select Wells
Show in Wells


Run
Analysis
Amplification Plot
Standard Curve
Multicomponent Plot
Raw Data Plot
QC Summary
Multiple Plots View
Plot Settings
Target All
Plot Color Default
Save current settings as the default
Standard Curve
Ct
Quantity
Select Wells
Show in Wells
1:12 / 3:22
How to calculat

**Efficiency of quantitative PCR**

$$\text{Efficiency} = 10^{(-\text{slope})} - 1$$

Example

slope = -0.26

$E = 10^{(0.26)} - 1$

$= 1.82 - 1$

$= 0.82$ or 82%

# 100% effective PCR

$$C = C_0 (1 + E)^n$$

**specific case E = 1 then**

$$C = C_o\, 2^n$$

**When E = 1 then:**

**$\Delta$ Ct = + 1 ➔ increase x2**

**$\Delta$ Ct= + 3.32 ➔ increase x10**

**C = number of copies of the target gene**

**C = initial copy number**

**E=efficiency**

**n = number of PCR cycles**

# Relative quantification RQ

no need for a standard curve

calculation of results not comparisons of Ct

need to define:

-a target gene serving as endogenous control,

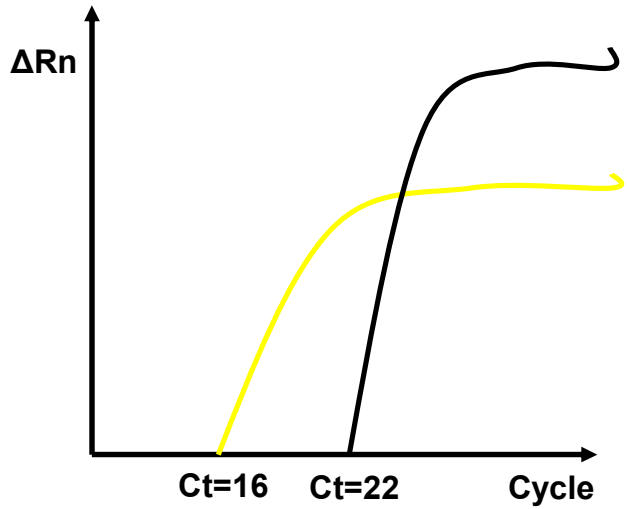
-a target gene serving as a standard.

✓**<u>endogenous control:</u>**

- provides an image of the amount of template supplied (DNA or cDNA),
- is present in constant quantity in all samples,
- normalizes:
- extraction biases and contamination by inhibitors (DNA)
- variations in reverse transcription efficiency

# Comparison of the target gene with the endogenous control

**Relative quantification: to determine the change in gene expression**




Endogenous control

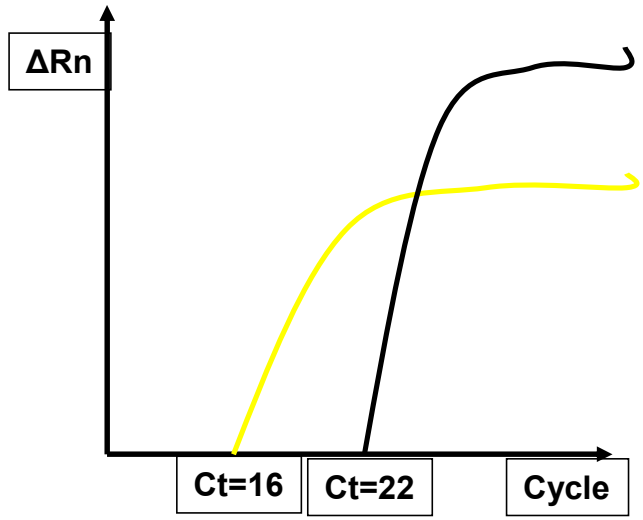
Target gene

**ΔCt = 22 – 16 = 6**

# Standard


-t0 addition of atrazine to soil mesocosms,
-t0 will be used as the ***standard value***

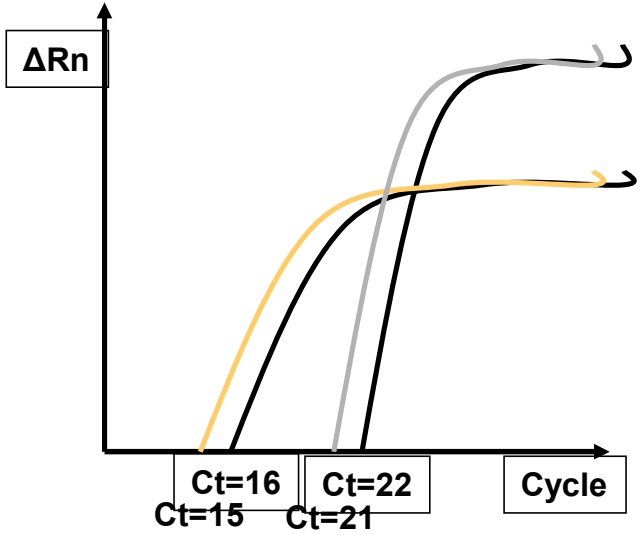
## Comparison of the target gene with the endogenous control




Endogenous control

Target gene

**ΔCt = 22 – 16 = 6**



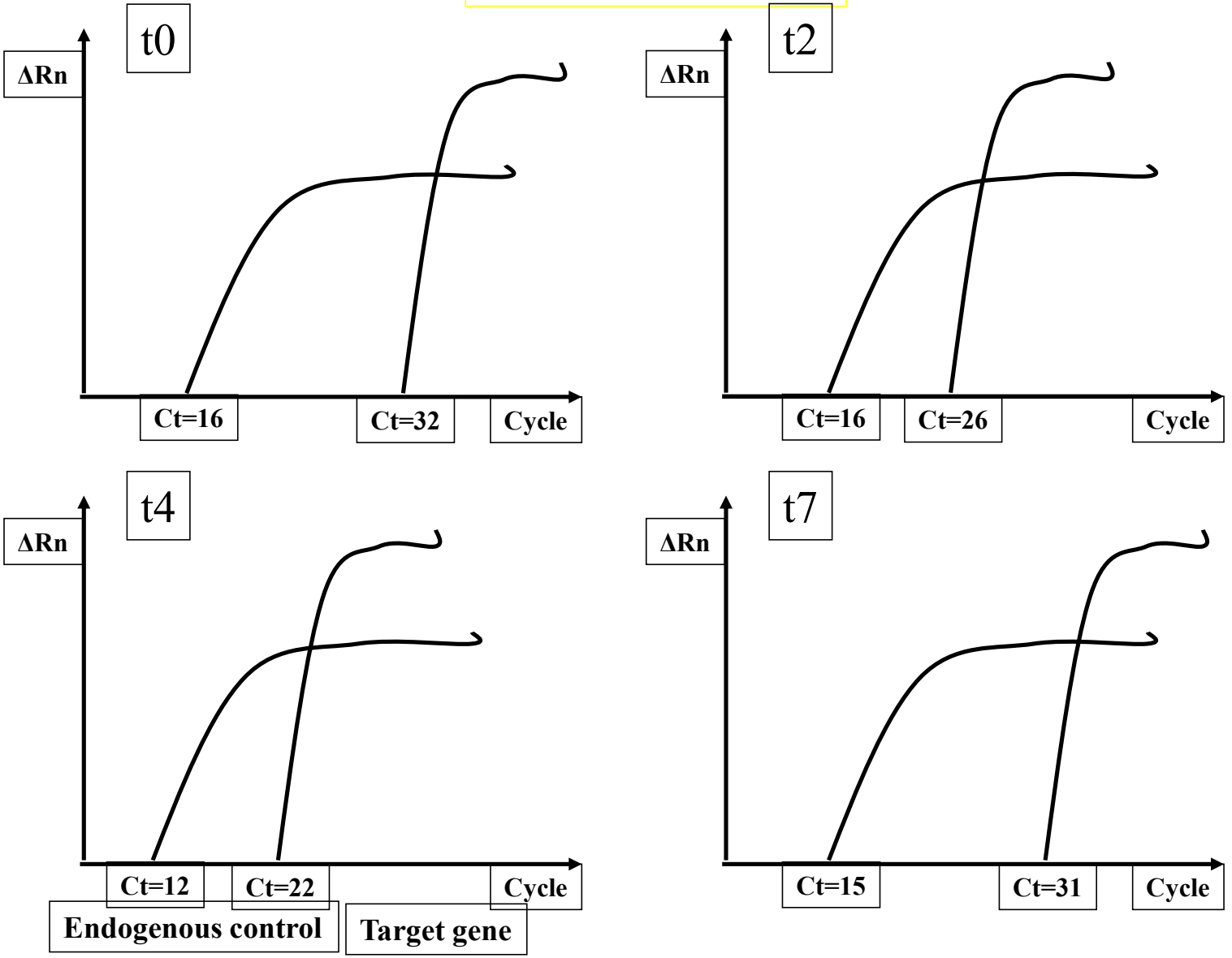

What if there's twice as many matrices?

Endogenous control

Target gene

**ΔCt = 21 – 15 = 6**

# Comparison of Ct



## Comparison of Ct

**Step 1:** Normalization with respect to endogenous control

$$Ct_{\text{Target gene}} - Ct_{\text{Endogenous control}} = \mathbf{\Delta Ct}$$

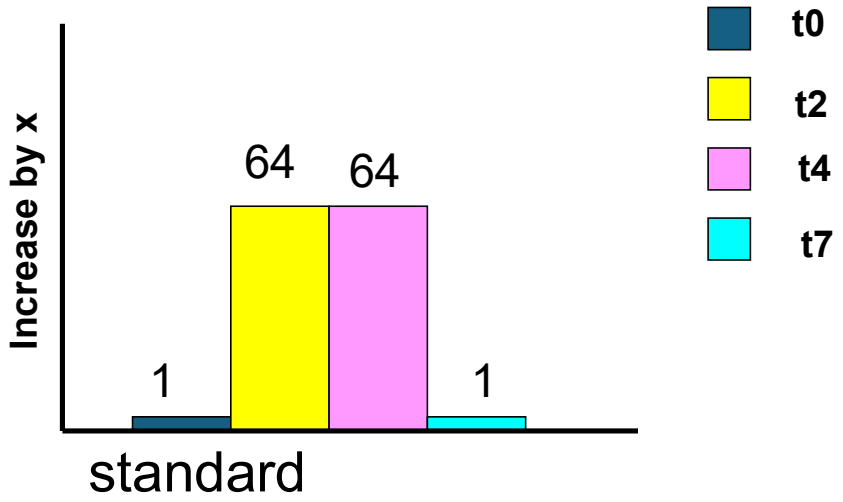
**Step 2:** Standardization against the standard

$$\mathbf{\Delta Ct}_{\text{sample}} - \mathbf{\Delta Ct}_{\text{standard}} = \mathbf{\Delta\Delta Ct}$$

**Step 3:** Determining the variation in the copy number of the target gene

$$\mathbf{2^{-\Delta\Delta Ct}}$$

# Relative quantification of results



# Exercise (1/4)

**Control**
**16S rRNA Ct 14**
***atzA* Ct 32**

**Atrazine**
**16S rRNA Ct 16**
***atzA* Ct29**

**Which DNA sample is the most concentrated and by how many times?**

**16S rRNA is used as an endogenous control….**

## Exercise (2/4)

**Control**

**16S rRNA Ct 14**

***atzA* Ct 32**

$$\Delta Ct_{\ 16SrRNA} = 16 - 14 = 2$$

**Atrazine**

**16S rRNA Ct 16**

***atzA* Ct29**

$$2^{\ \Delta Ct\ 16SrRNA} = 2^{\ 2} = 4$$

**The DNA concentration of the control sample is 4 times higher than that of the atrazine sample.**

# Exercise (3/4)

**Control**
**16S rRNA Ct 14**
***atzA* Ct 32**

**Atrazine**
**16S rRNA Ct 16**
***atzA* Ct29**

**Which DNA sample contains the greatest number of copies of the *atzA sequence* ?**

# Exercise (4/4)

**Control**

**16S rRNA Ct 14**

***atzA* Ct 32**

$$\Delta Ct_{control} = 32 - 14 = 18$$

$$\Delta Ct_{atrazine} = 29 - 16 = 13$$

**Atrazine**

**16S rRNA Ct 16**

***atzA* Ct29**

$$\Delta\Delta Ct = 18 - 13 = 5$$

$$2^{\Delta\Delta Ct} = 2^{5} = 32$$

**The copy number of the *atzA gene* is 32 times higher in the soil sample treated with atrazine than in the control**

# RT-PCR

- A **complementary DNA (cDNA)** library is made by by reverse transcription of all the mRNA produced by a particular cell
- A **cDNA library** represents only part of the genome—only the subset of genes transcribed into mRNA in the original cells

# RT-PCR

- Reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique combining reverse transcription of RNA into DNA (in this context called complementary DNA or cDNA) and amplification of specific DNA targets using polymerase chain reaction (PCR).
- It is primarily used to measure the amount of a specific RNA. This is achieved by monitoring the amplification reaction using fluorescence, a technique called real-time PCR or quantitative PCR (qPCR).
- Combined RT-PCR and qPCR are routinely used for analysis of gene expression and quantification of viral RNA in research and clinical settings.

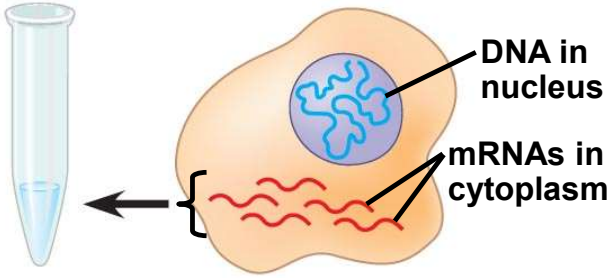
# RT-PCR

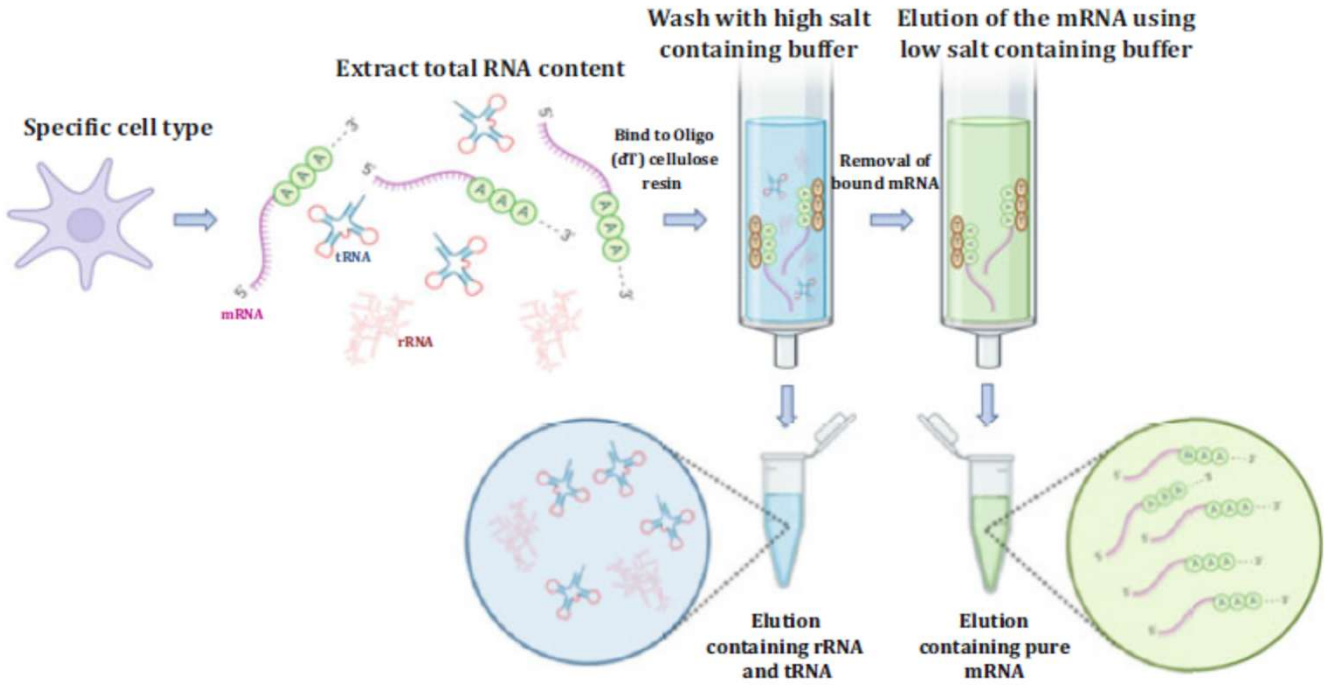
| Technique | Abbreviation |
| --- | --- |
| Polymerase chain reaction | PCR |
| Reverse transcription polymerase chain reaction | RT-PCR |
| Real-time polymerase chain reaction | qPCR |
| RT-PCR / qPCR combined technique | qRT-PCR |

# RT-PCR : Purification of mRNA from total RNA

- Eukaryotic mRNA consists of 50 to 250 adenylate residues (poly-A tail) at the 30 end, which facilitates simple separation of mRNA by affinity chromatography using oligo(dT).
- Chromatographic columns or magnetic beads coupled to oligo(dT) are commonly used to purify mRNA from the much more prevalent rRNA and tRNA in a cell lysate. The poly-A tail at the 30 end of the mRNA allows for its efficient binding to the oligo(dT) beads.
- After providing sufficient washes to remove impurities, these mRNAs can then be eluted using strong magnetic force or a low-salt buffer; the bound mRNA is isolated from the total RNA content.

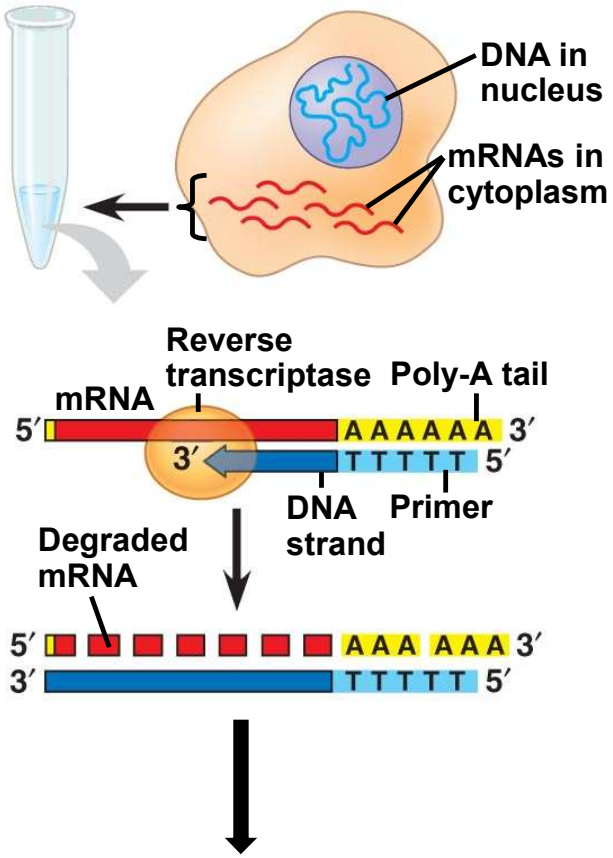
# Purification of mRNA from total RNA



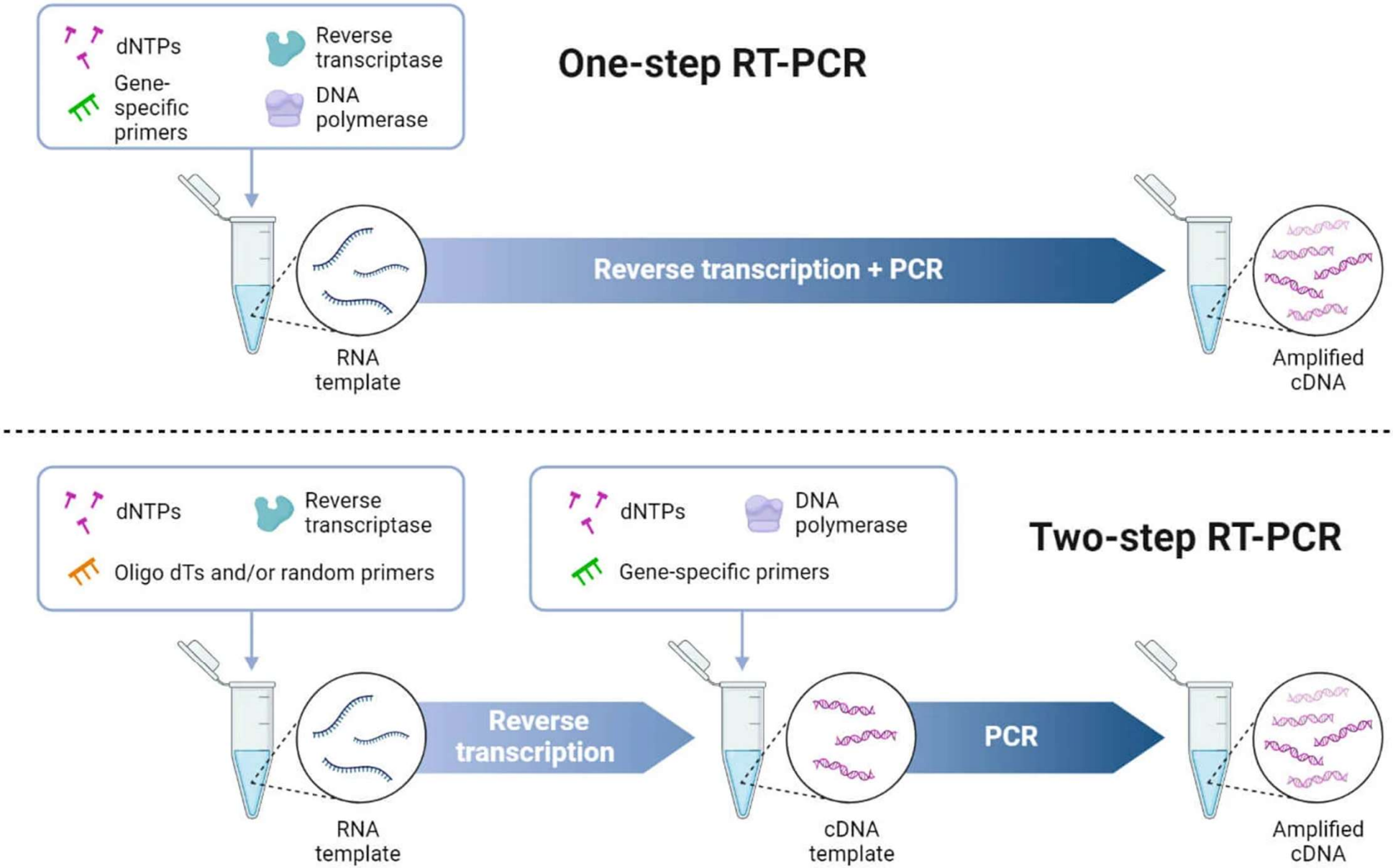



# RT-PCR : cDNA synthesis

- The synthesis of cDNA requires numerous steps. The principle is based on:
- (i) the extraction of RNA and sometimes the purification of polyadenylated mRNAs from the organ (for example, by affinity chromatography on a polyT column);
- (ii) the copying of these mRNAs into single-stranded complementary DNA by the action of a reverse transcriptase;
- (iii) the specific removal of mRNAs by RNase H.




# PCR or qPCR


dNTPs
Reverse transcriptase
Gene-specific primers
DNA polymerase
One-step RT-PCR
Reverse transcription + PCR
RNA template
Amplified cDNA
dNTPs
Reverse transcriptase
Oligo dTs and/or random primers
dNTPs
DNA polymerase
Gene-specific primers
Two-step RT-PCR
Reverse transcription
PCR
RNA template
cDNA template
Amplified cDNA

# Southern blot

- A technique called **Southern blotting** combines gel electrophoresis of DNA fragments with nucleic acid hybridization
- Specific DNA fragments can be identified by Southern blotting, using labeled probes that hybridize to the DNA immobilized on a “blot” of gel

Fig. 20-11

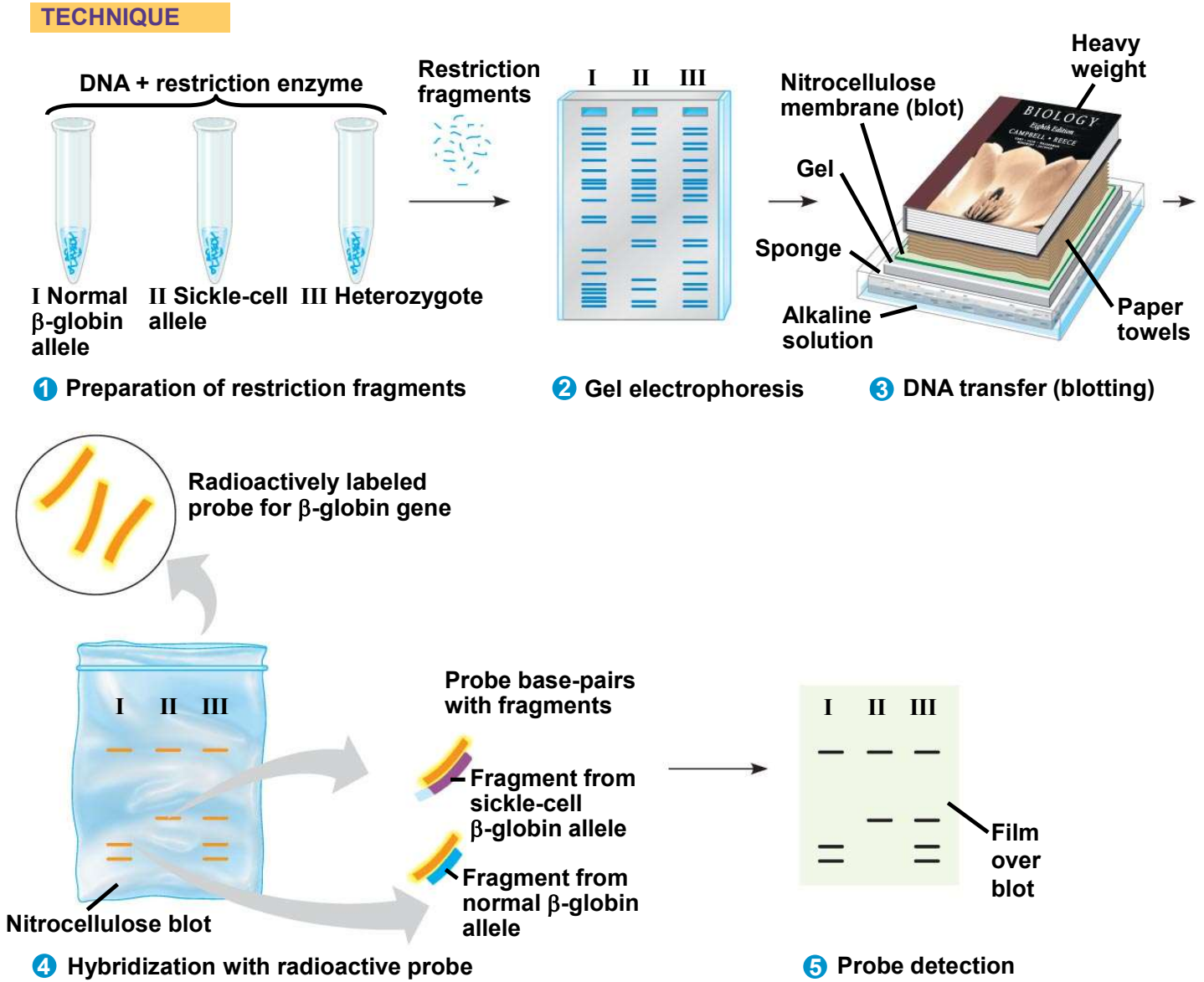


Fig. 20-11a

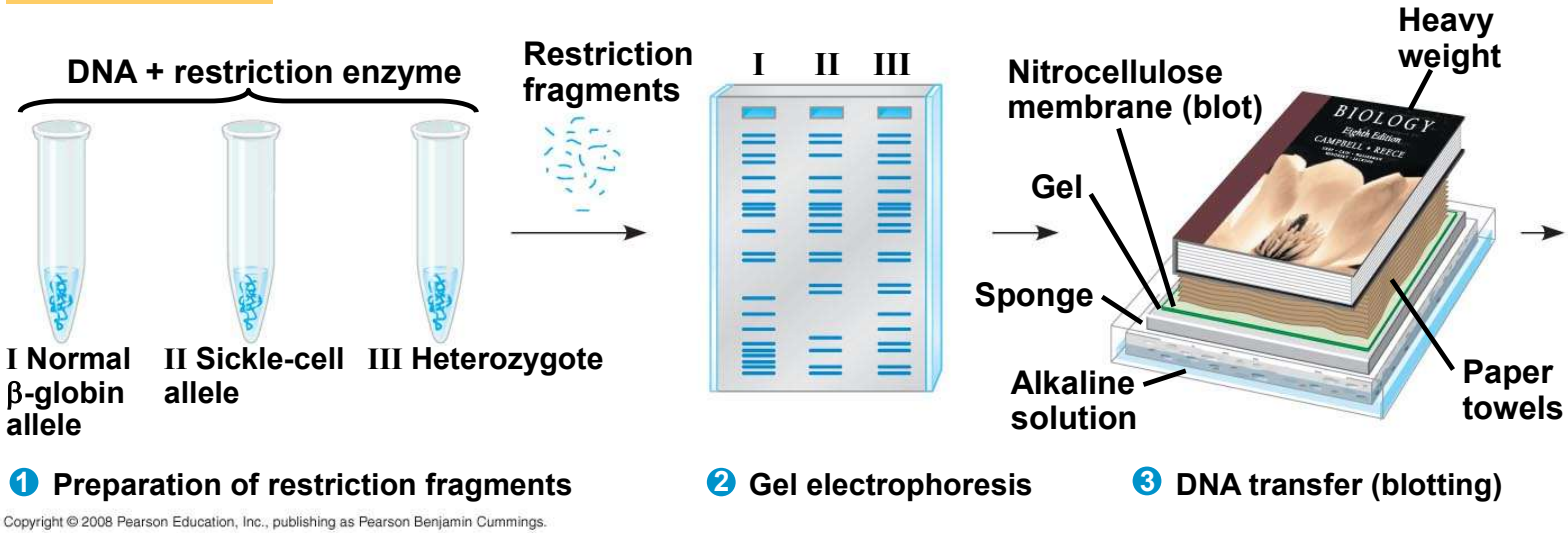
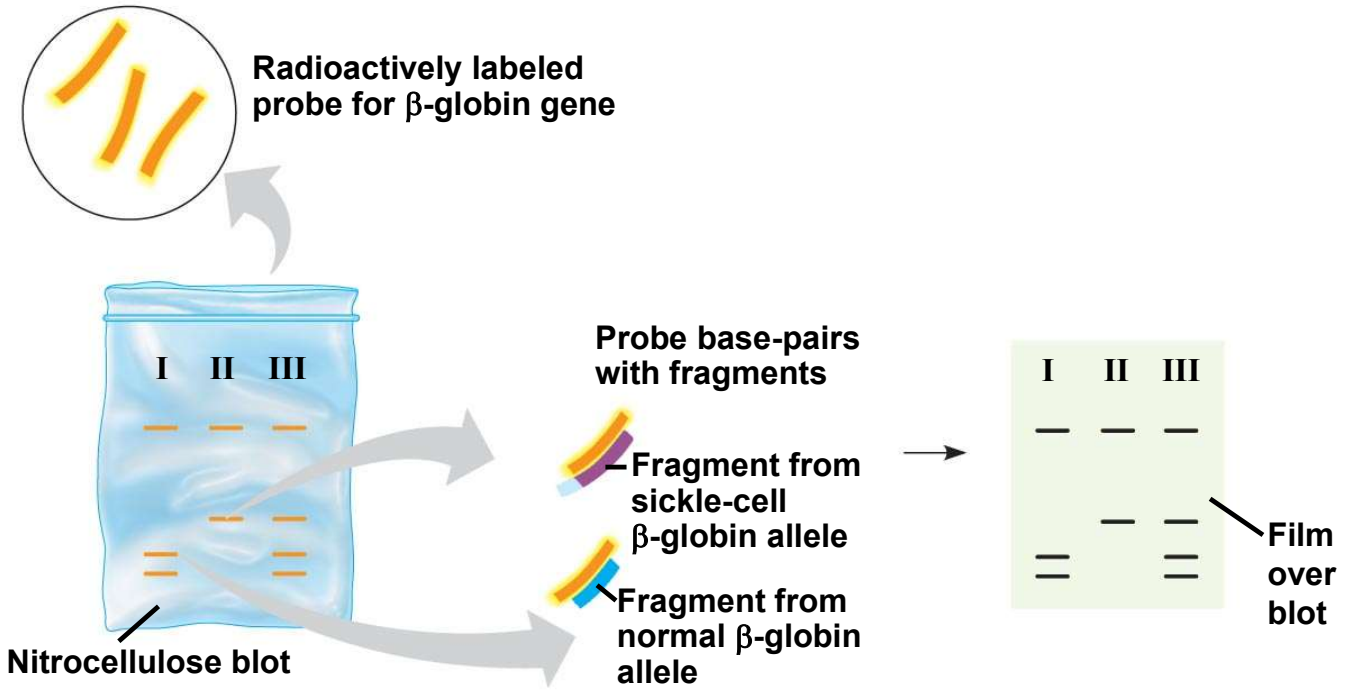


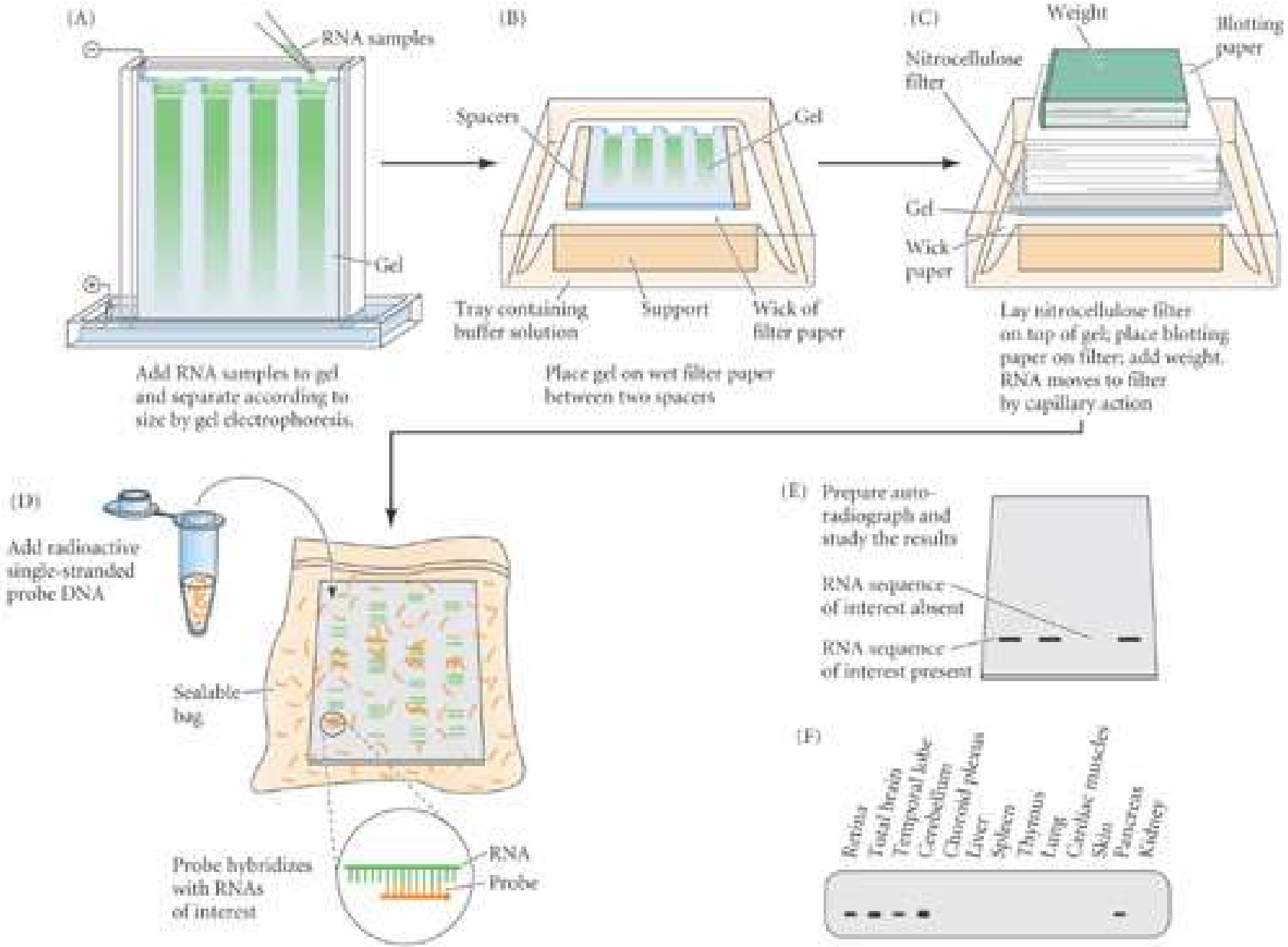
Fig. 20-11b



# Northern blot

- It is derived from the Southern blot except that instead of studying DNA, RNA is studied.
- It allows us to appreciate the distribution of RNA in tissues and to study their relative abundance in order to deduce the greater or lesser expression of certain genes.
- This technique is used to detect maturation intermediates and different forms of RNA splicing.

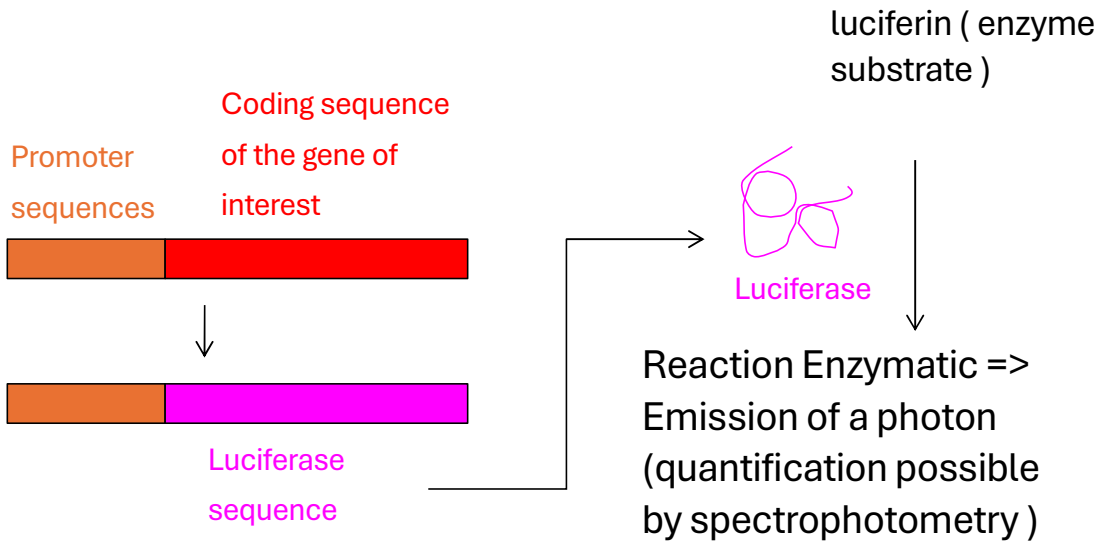
Nc


(A)
RNA samples
Gel
Add RNA samples to gel and separate according to size by gel electrophoresis.
(B)
Spacers
Gel
Tray containing buffer solution
Support
Wick of filter paper
Place gel on wet filter paper between two spacers
(C)
Weight
Blotting paper
Nitrocellulose filter
Gel
Wick paper
Lay nitrocellulose filter on top of gel; place blotting paper on filter; add weight. RNA moves to filter by capillary action
(D)
Add radioactive single-stranded probe DNA
Sealable bag
Probe hybridizes with RNAs of interest
RNA
Probe
(E) Prepare autoradiograph and study the results
RNA sequence of interest absent
RNA sequence of interest present
(F)
Retina
Fetal brain
Temporal lobe
Cerebellum
Choroid plexus
Liver
Spleen
Thymus
Lung
Cardiac muscle
Skin
Pancreas
Kidney

# Reporter genes

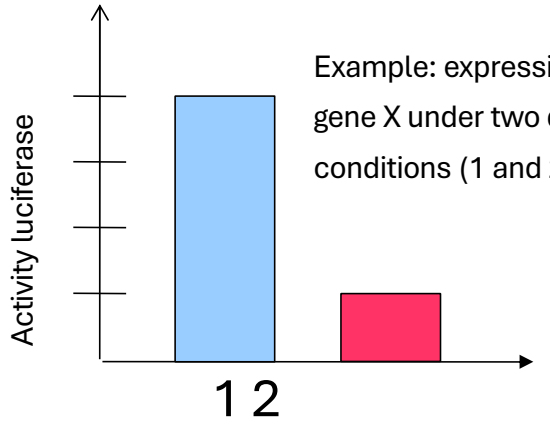
The level gene expression can be quantified using **reporter genes** : replacement of the coding portion of the gene considered by a gene rapporteur :
- embarrassed of an enzyme ( measurement enzymatic )
- embarrassed of a protein fluorescent ( fluorescence measurement )

Example : Replacing the sequence codingstudied gene by the sequence coding of an enzyme: the **luciferase .**




Activity measurement enzymatic *in vitro* with the addition of the substrate . This are relative data , which must be compared to a reference .

# Reporter genes




Gene X is expressed four times more in condition 1 than in condition 2

We obtain information on the level gene expression In a cell at a given moment, for given conditions ( results quantitative (unlike Western blot)