

BCH 462- Biotechnology & Genetic Engineering [Practical]

Lab (8) Quantitative Reverse Transcription PCR (RT-qPCR) “ Real-time PCR”



Polymerase chain reaction (PCR)

- One of the most powerful technologies in molecular biology.
- PCR is used in molecular biology to make many copies of (amplify) small sections of DNA or a gene
- In such traditional PCR (endpoint), detection of the amplicon is performed at the end of the reaction after the last PCR cycle, using gel electrophoresis. Why RT-PCR ?



RT-PCR is identical to standard PCR except that the progress of the reaction is monitored during each PCR cycle.

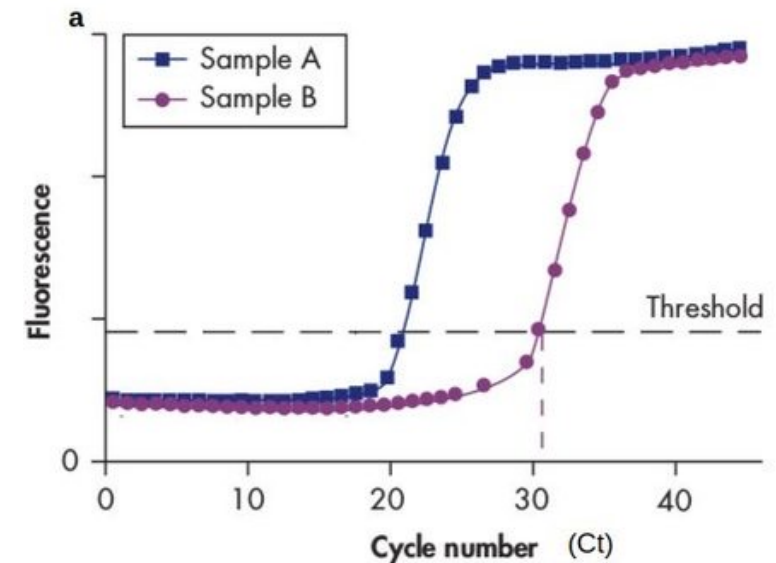
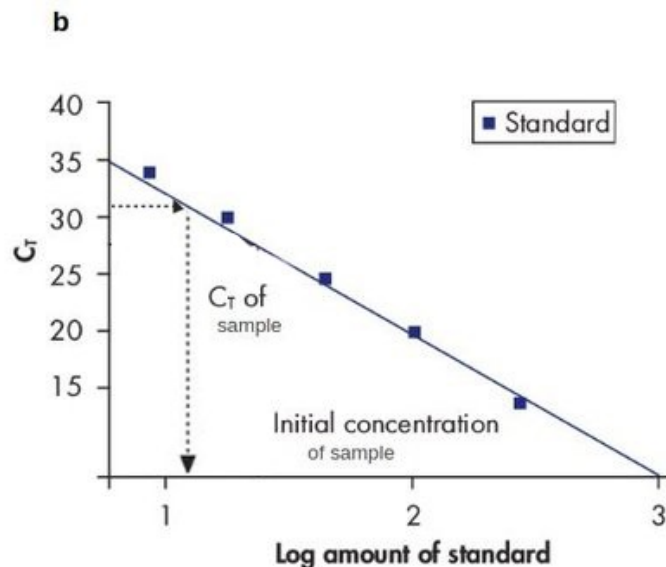
Real-time quantitative (RT-qPCR)

- In real-time quantitative PCR **fluorescent reporter dyes** allow a PCR reaction to **be visualized “in real time”** as the reaction progresses by combine the **amplification and detection steps** in the PCR reaction.
- The assay relies on measuring the increase in fluorescent signal.
 - Is **proportional** to the amount of DNA produced during each PCR cycle.
 - Gives a **quantitative** information on the starting quantity of the amplified target.
- **Applications:**
 1. Gene expression (most common application).
 2. Microbial load testing (type and # of microorganism)



RT-qPCR quantification methods

- 1. Absolute quantification:** the exact concentration of sample can be determined, in which samples (typically a plasmid, oligonucleotide, or purified PCR product) of known quantity are serially diluted and then amplified to **generate a standard curve**.
 - **Unknown samples** are then quantified by comparison with this curve.
- 2. Relative quantification (Comparative):** analyze changes in gene expression in a given sample **relative to the same gene** in another reference sample (disease vs normal control sample). The results are expressed as fold change (increase or decrease).
 - **A normalizer gene** (such as β -actin) is used as a control for experimental variability in this type of quantification.

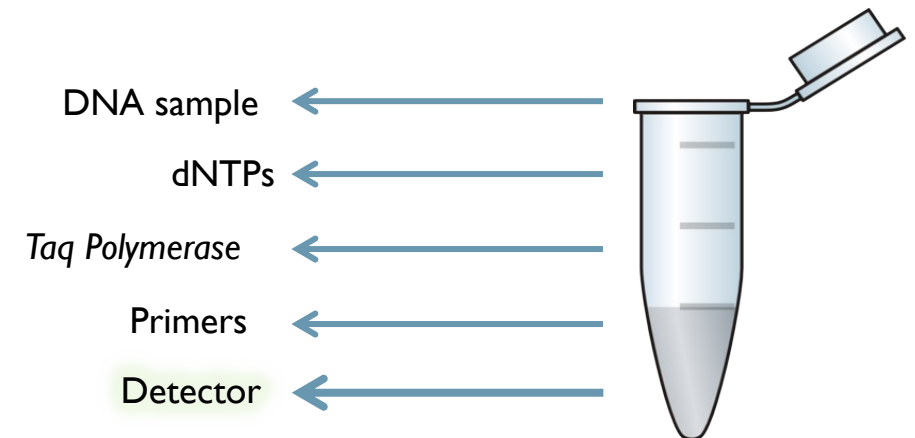


RT-qPCR chemistries (detection systems)

I. SYBR-green based assay:

Principle:

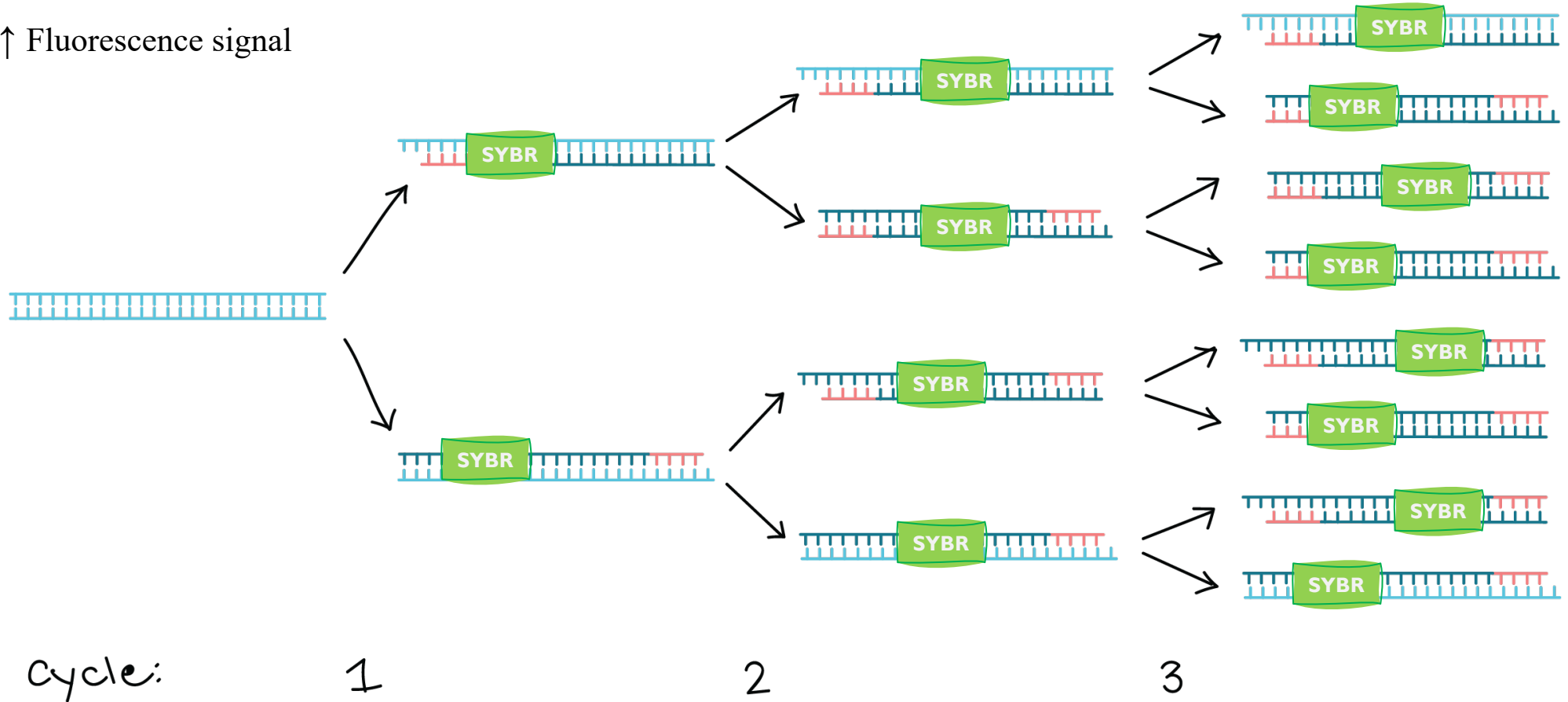
- The double-strands **DNA-intercalation** agent (DNA-binding dyes) such as **SYBR Green**.
- The **SYBR Green 1** is **only** fluorescing when intercalated into dsDNA.
 - The intensity of the fluorescence signal is therefore **dependent** on the quantity of dsDNA present in the reaction.
- The main disadvantage of this method is that it is not specific since the dye binds to all dsDNAs formed during the PCR reaction (i.e., nonspecific PCR products and primer-dimers).



More dsDNA → More binding → More fluorescence

↑ Quantity of dsDNA

↑ Fluorescence signal

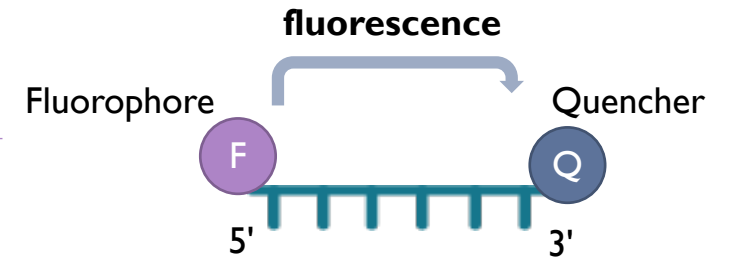


RT-qPCR chemistries (detection systems)

II. Fluorogenic 5' nuclease assay (TaqMan® probe assay):

Principle:

- A probe is used in the real-time quantitative TaqMan assay.
- The **probe** is a specific sequence which has a fluorescent reporter dye linked to its 5' end and a non-fluorescent quencher at its 3' end.
- Whilst the probe is **intact**, the **quencher absorbs the fluorescence emitted by the reporter dye**.
- The TaqMan probe anneals downstream the target sequence from one of the primer sites and is **cleaved by the 5' nuclease activity of the *Taq polymerase*** during the PCR extension phase.
- **Cleavage** of the probe by *Taq polymerase* during PCR will cause the **separation of the reporter and quencher dyes**, thereby allowing the reporter's fluorescent signal to be liberated.
- With each cycle additional reporter dye molecules are cleaved from their respective probes, **leading to an increase in fluorescence intensity proportional to the amount of amplicon produced**.



dsDNA



Denaturation



ssDNA



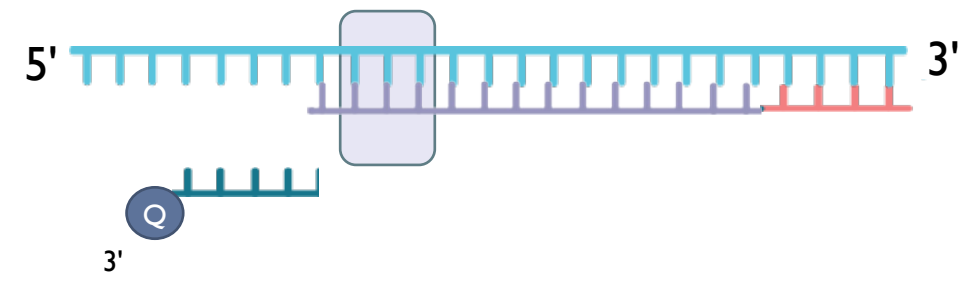
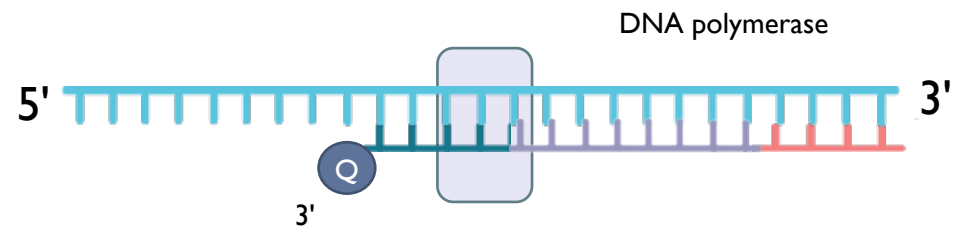
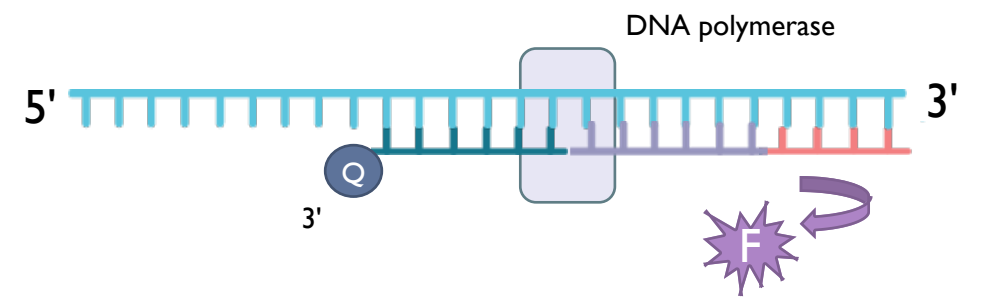
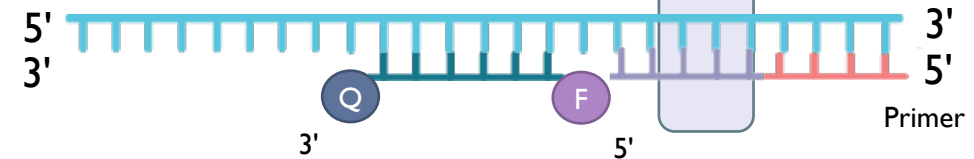
Hybridization



Annealing



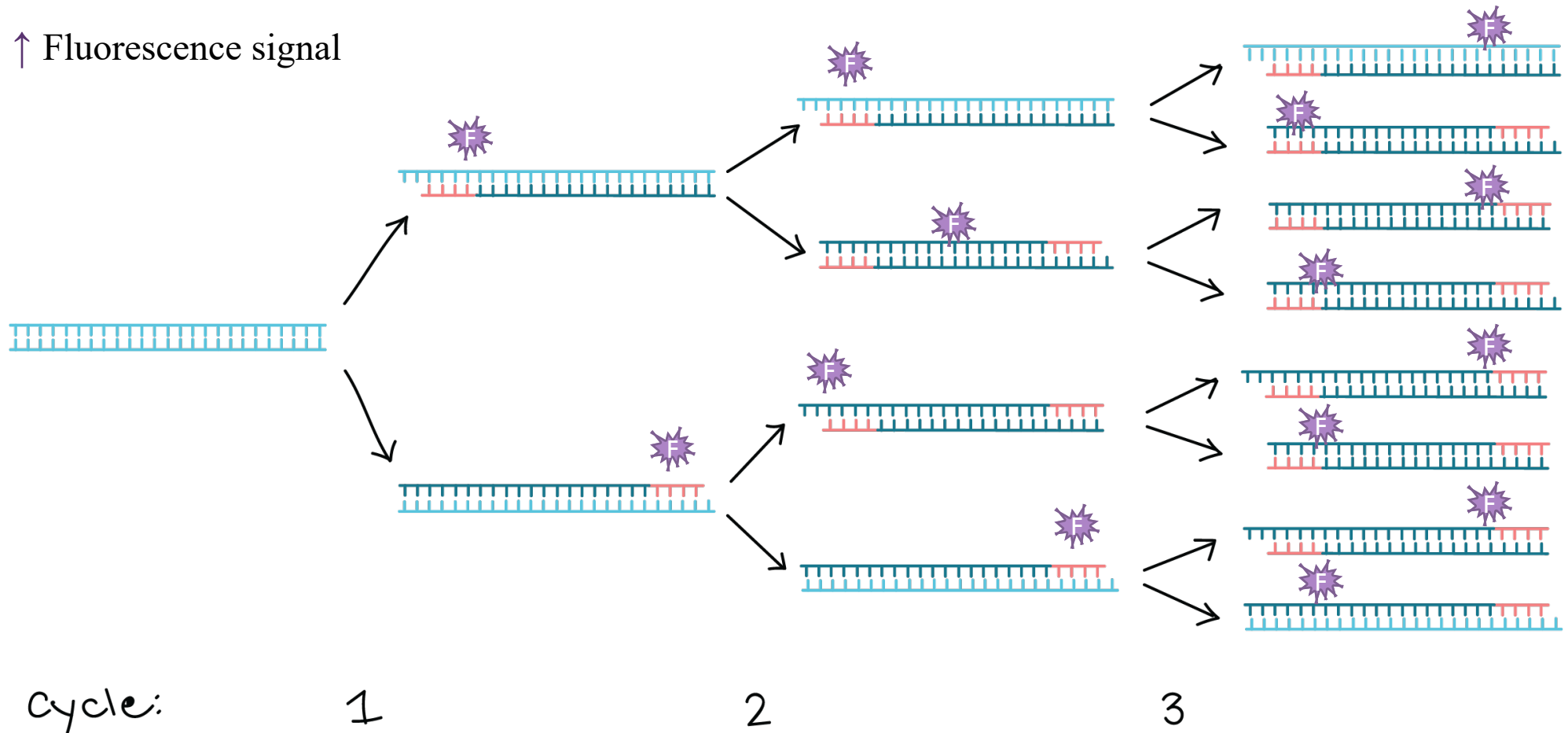
Extension



More dsDNA → More probe displacement and cleavage → More fluorescence

↑ Quantity of dsDNA

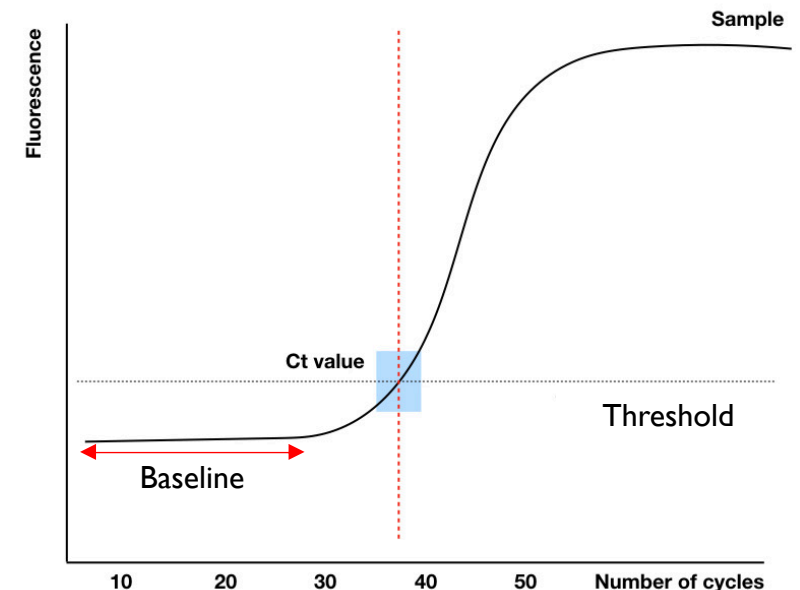
↑ Fluorescence signal



RT-qPCR amplification curve

Amplification plot is the plot of fluorescence signal versus cycle number.

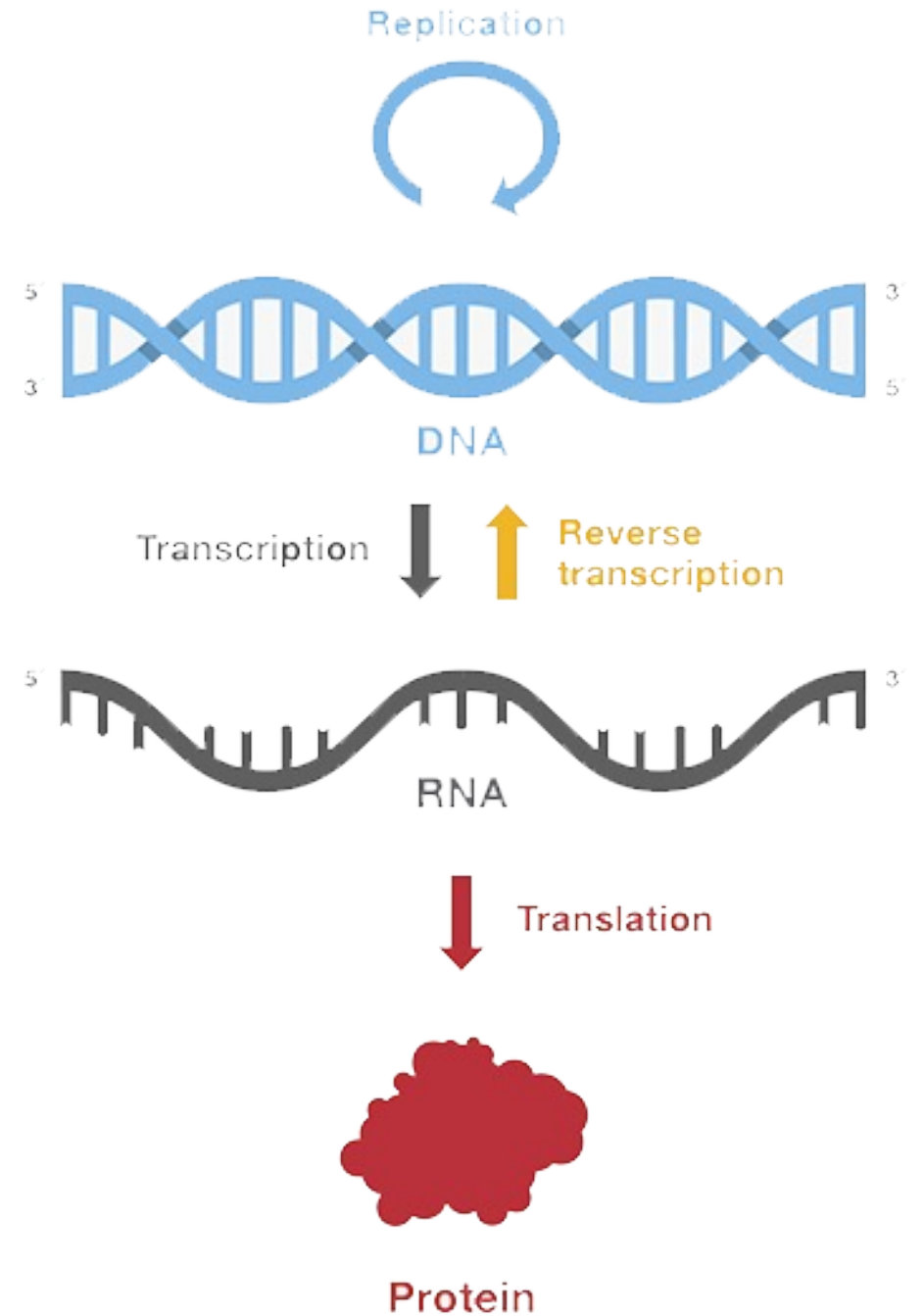
1. **Baseline:** The baseline of the real-time PCR reaction refers to the **little change in fluorescent signal** during the **initial cycles of PCR** (background or the “**noise**” of the reaction).
2. **Threshold:** is the level of detection or the point at which a reaction **reaches a fluorescent intensity above background (baseline) levels**.
3. **C_t (threshold cycle):** The threshold cycle (Ct) is the PCR cycle number at which your **sample’s reaction curve intersects the threshold line**.



Practical Part

RT-qPCR for gene expression analysis

Gene expression is the process by which the heritable information in a **gene** is made into a functional **gene product**, such as **protein** or **RNA**.



Steps of evaluation gene expression by RT-qPCR

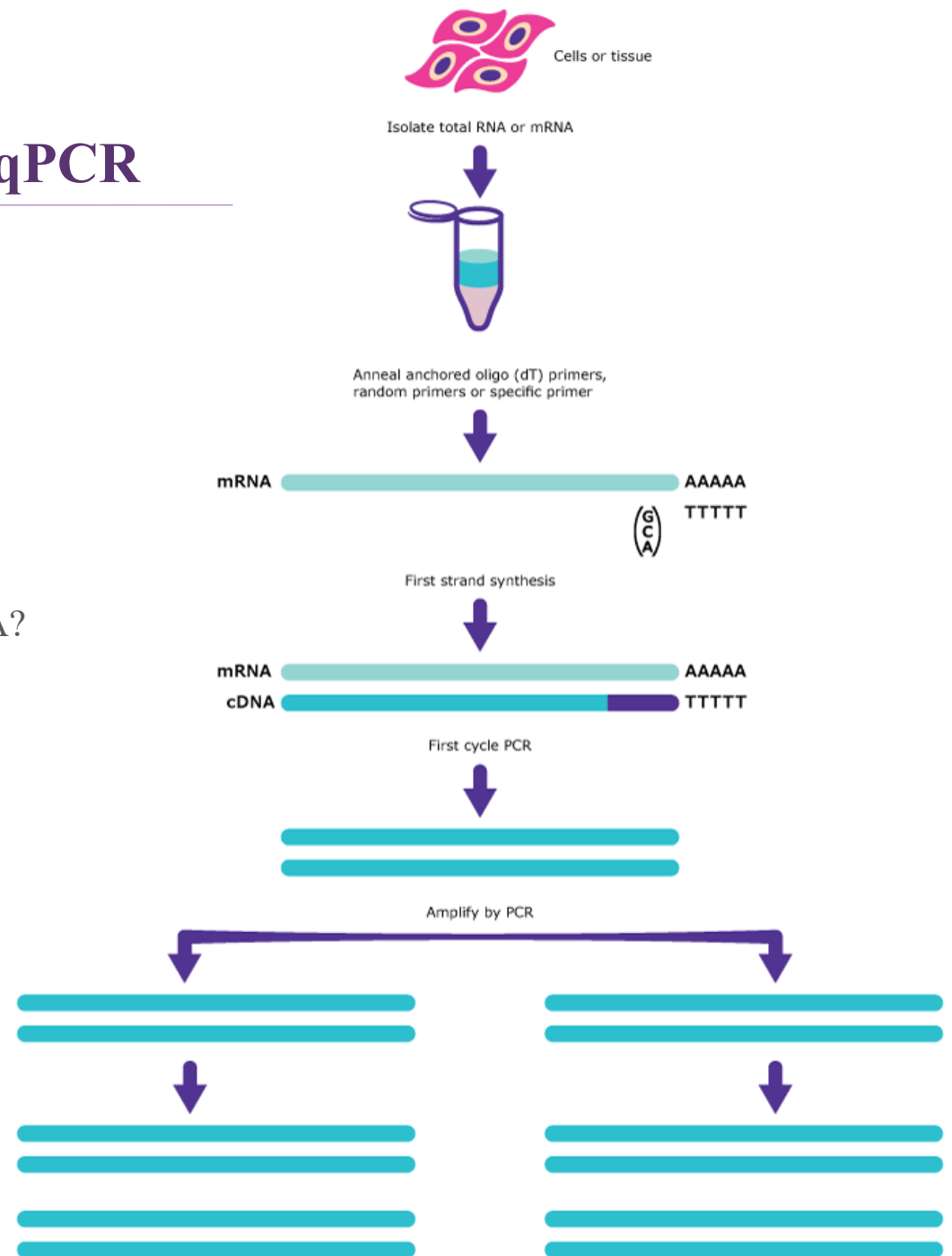
Step 1: RNA Extraction from Tissue/ Cell line or blood

Step 2: Reverse transcription to convert RNA to cDNA

Step 3: Determination of cDNA using real time PCR

Step 4: Data analysis

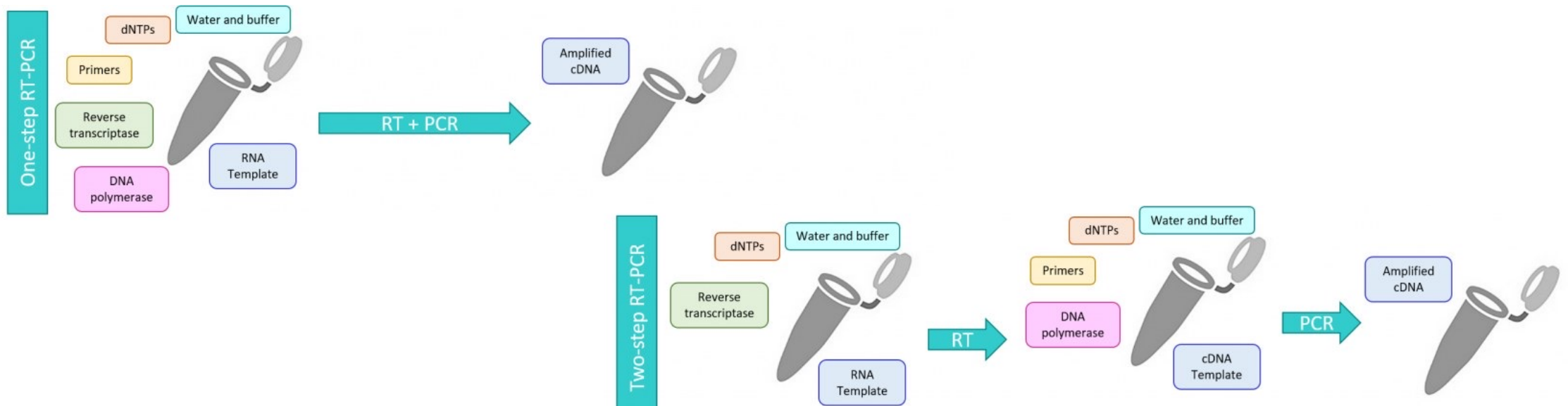
💡 **Pause and Think** why do we use cDNA instead of gDNA or RNA?



RT-qPCR types

The RT-qPCR assay can be performed either as a one-tube single RT and PCR enzyme method or a separate RT and PCR enzyme technique using one or a two tubes.

1. **One-step RT-qPCR**, combines reverse transcription and PCR in a **single tube** and buffer, using a **reverse transcriptase** along with a **DNA polymerase**, simplifying reaction setup and reducing the possibility of contamination.
1. **Two-step RT-qPCR**, the reverse transcription and PCR steps are performed in **separate tubes**, with different optimized buffers, reaction conditions, and priming strategies.



RT-qPCR for gene expression analysis

Reaction Components

| Component | Volume per 12.5µl reaction |
|---------------------|----------------------------|
| 2X Green Master mix | 6.25 |
| Forward primer | 0.25 |
| Reverse primer | 0.25 |
| Nuclease free water | 3.75 |
| cDNA Template | 2 |
| Total: | 12.5 µl |

SYBR® Green PCR Master Mix

Hot-start iTaq DNA polymerase

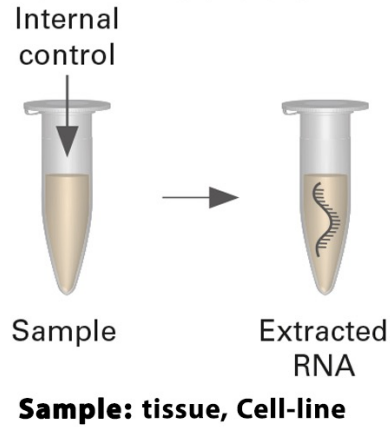
dNTPs

MgCl₂

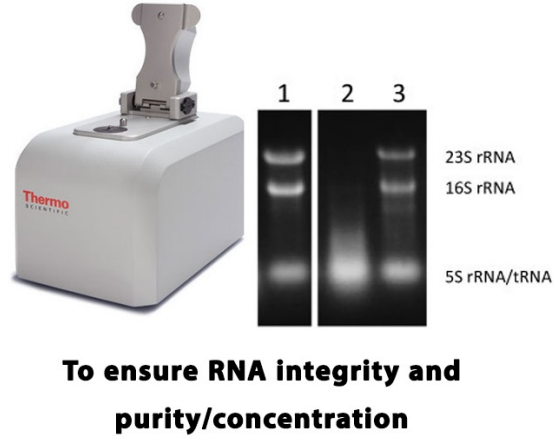
SYBR® Green I dye



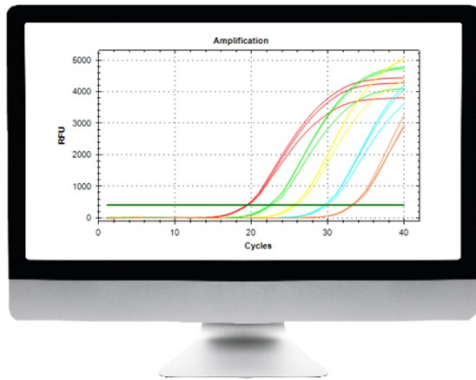
1. RNA isolation



2. RNA assesment



3. Reverse transcription

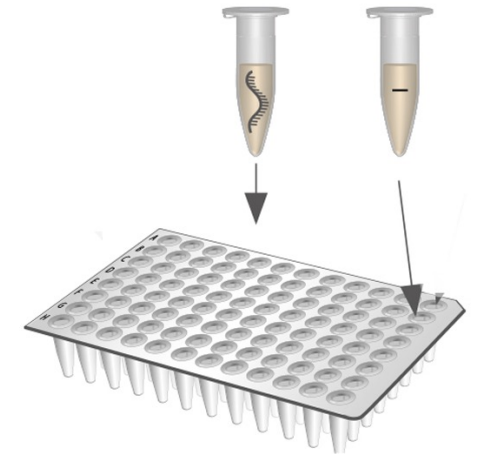


6. Data analysis

5. Real-Time PCR Thermal Cyclers (Thermocycler + Spectrofluorometry)



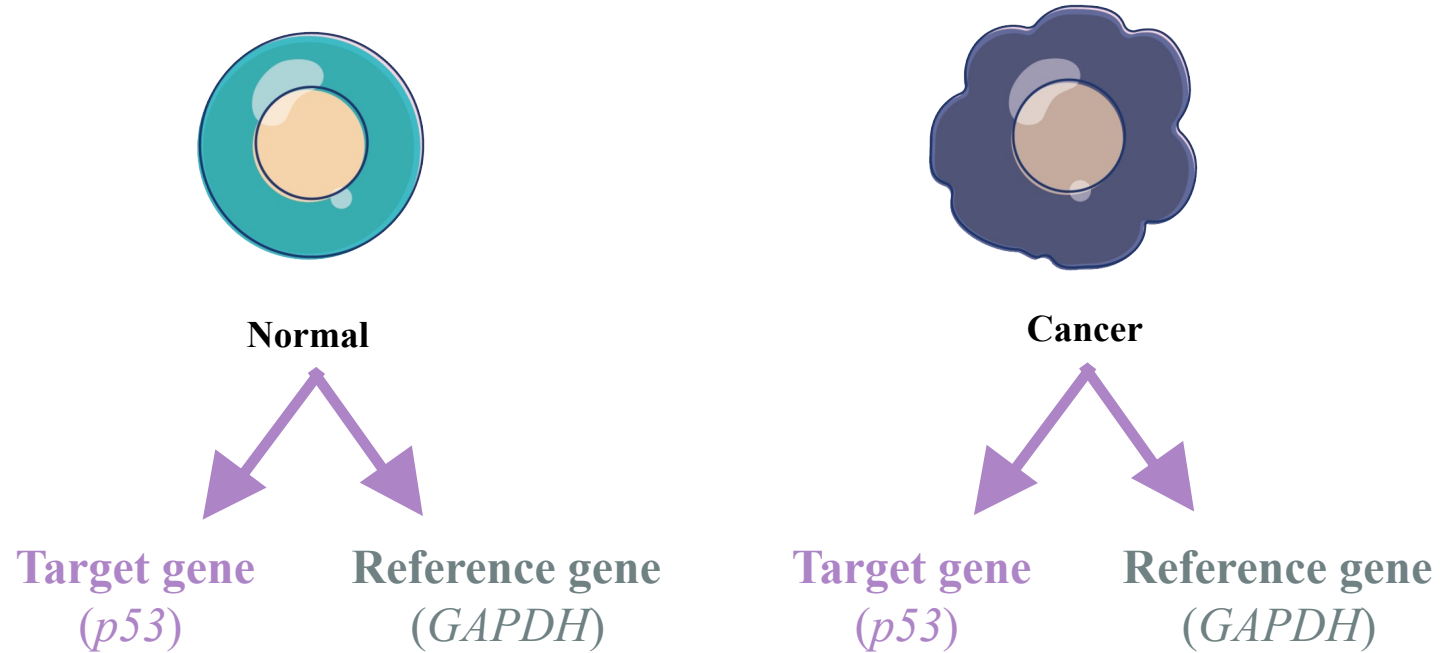
4. Plate load



Quantitative real-time PCR workflow

- Let's assume we want to study p53 expression in colorectal cancer patient using **Relative RT-PCR**.

Patient 1



Each As triplicate

Each As triplicate

Target gene Cancer cell Target gene Normal cell Reference gene Normal cell Reference gene Cancer cell

Each row represents one patient



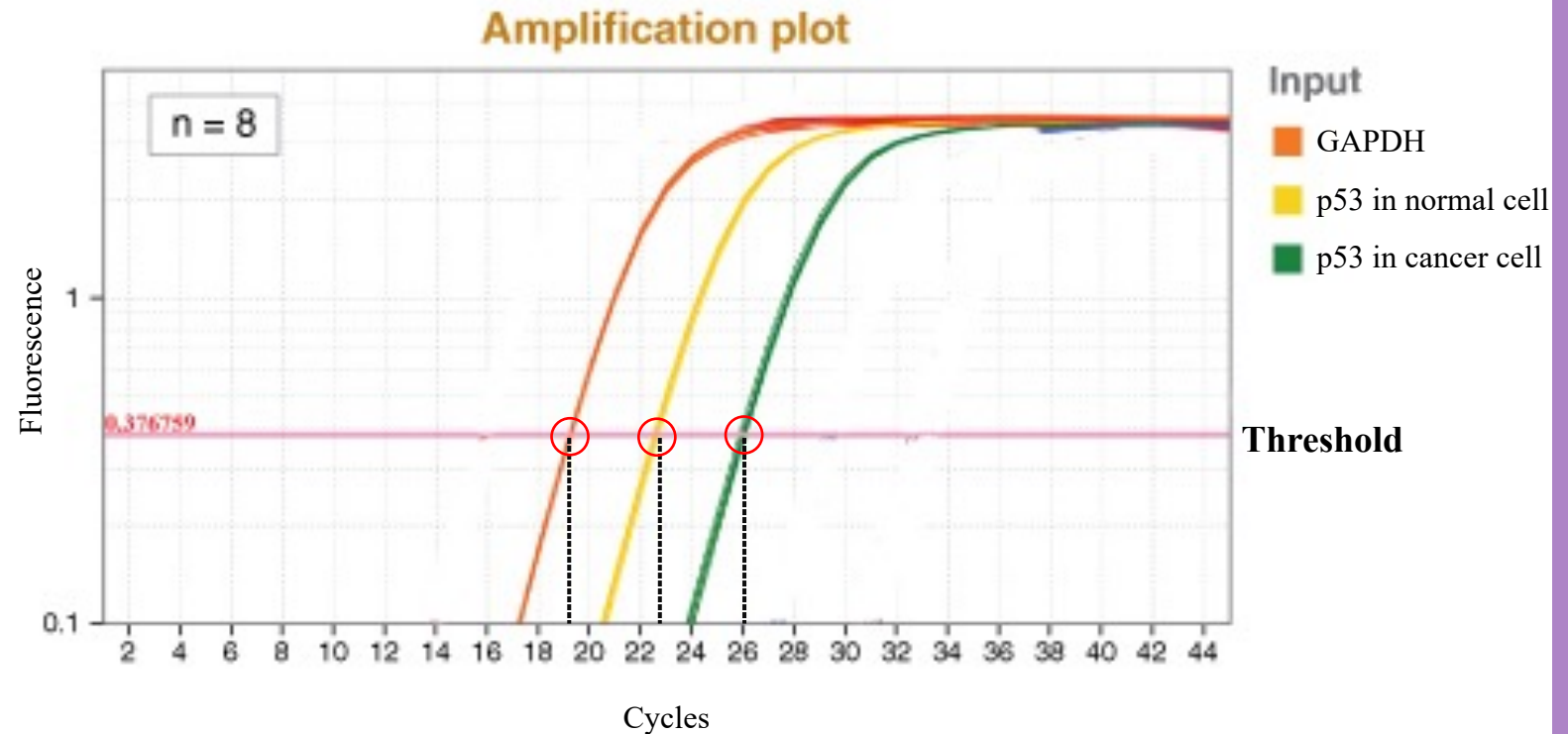
| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|----|----|----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| B | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| C | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| D | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| E | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| F | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| G | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| H | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ● | ● |

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-ve control for each master mix (no template control)

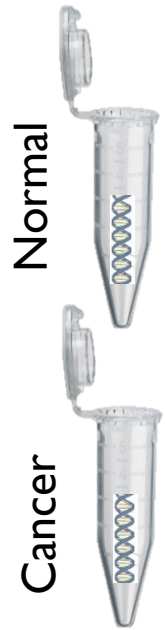
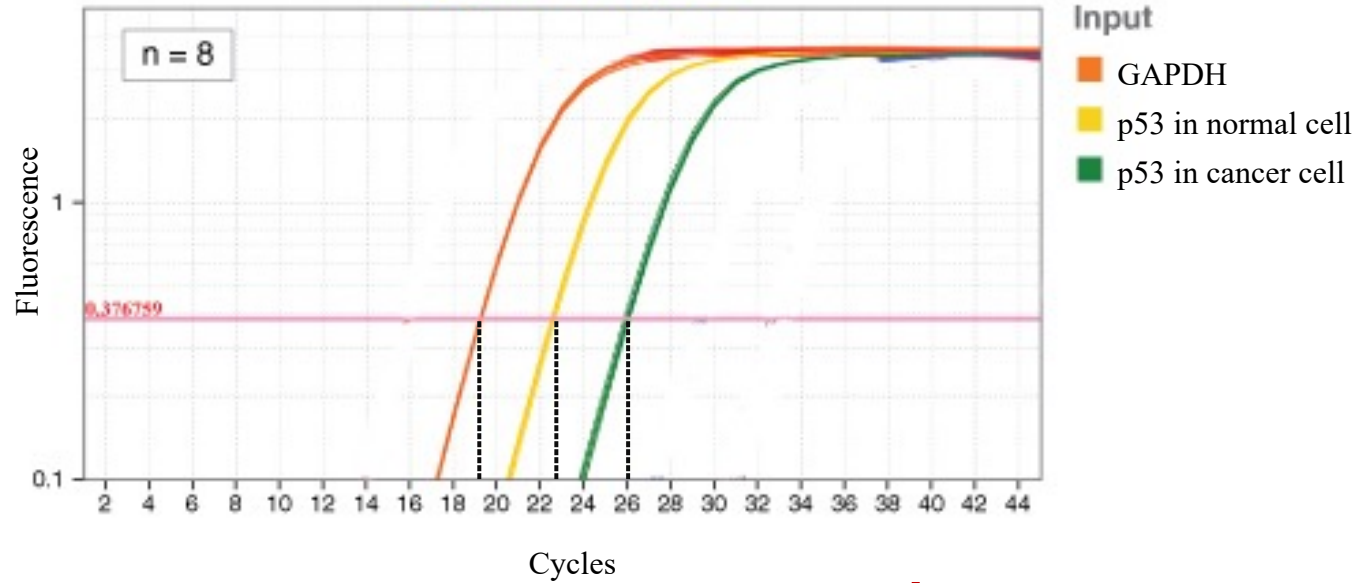
- Ct levels are **inversely proportional** to the **amount of target nucleic acid** expression in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample).

| Samples | Ct value | |
|----------|----------|-------|
| | GAPDH | p53 |
| Normal 1 | 19.90 | 23.00 |
| Normal 2 | 19.80 | 22.74 |
| Normal 3 | 19.85 | 22.80 |
| Tumor 1 | 20.00 | 26.00 |
| Tumor 2 | 19.75 | 26.20 |
| Tumor 3 | 19.72 | 26.12 |

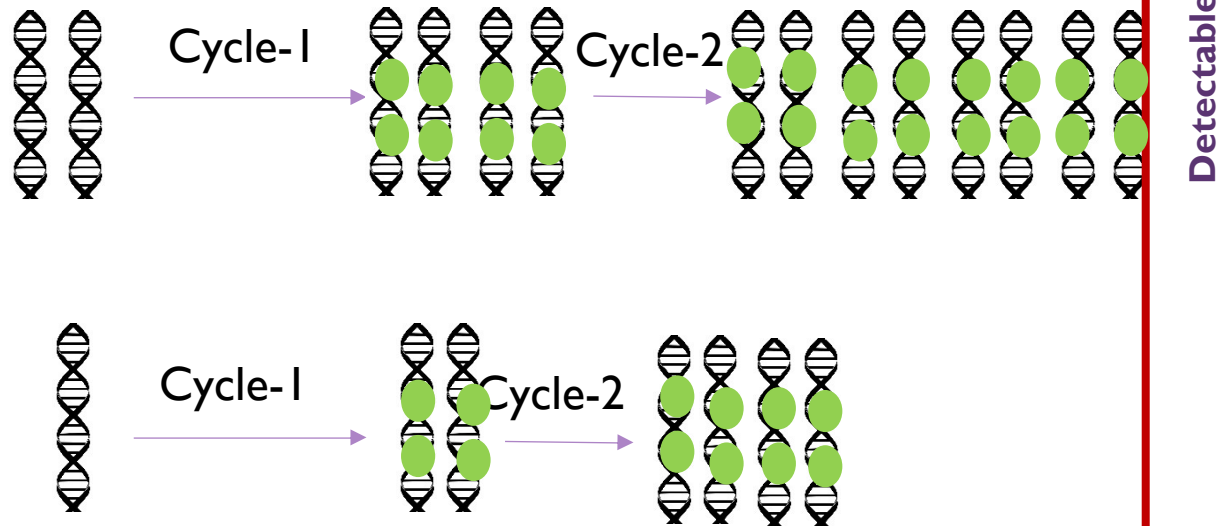


- 1- Did GAPDH expression change in normal and cancer cell? why?
- 2- What happened to p53 expression in cancer? Up/down?

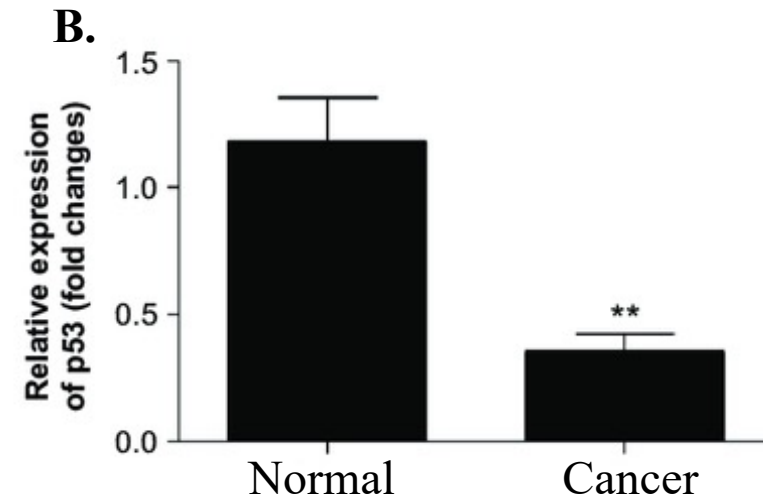
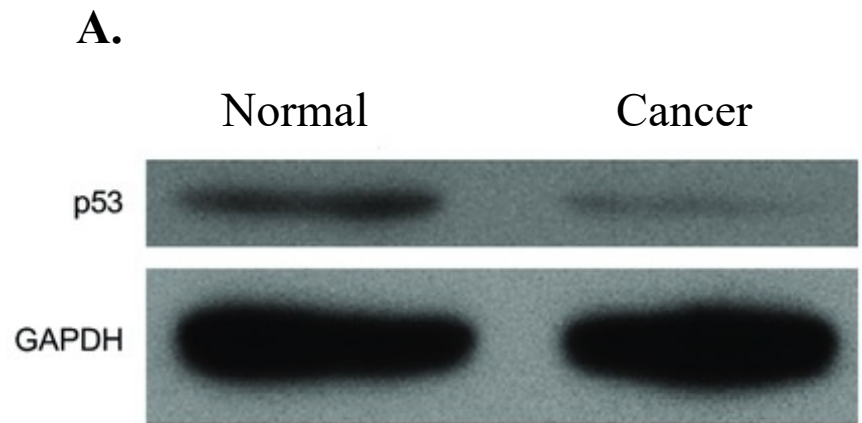
Amplification plot



Original P53 expression



Threshold

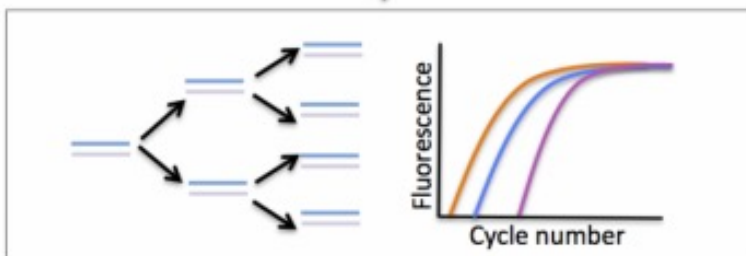
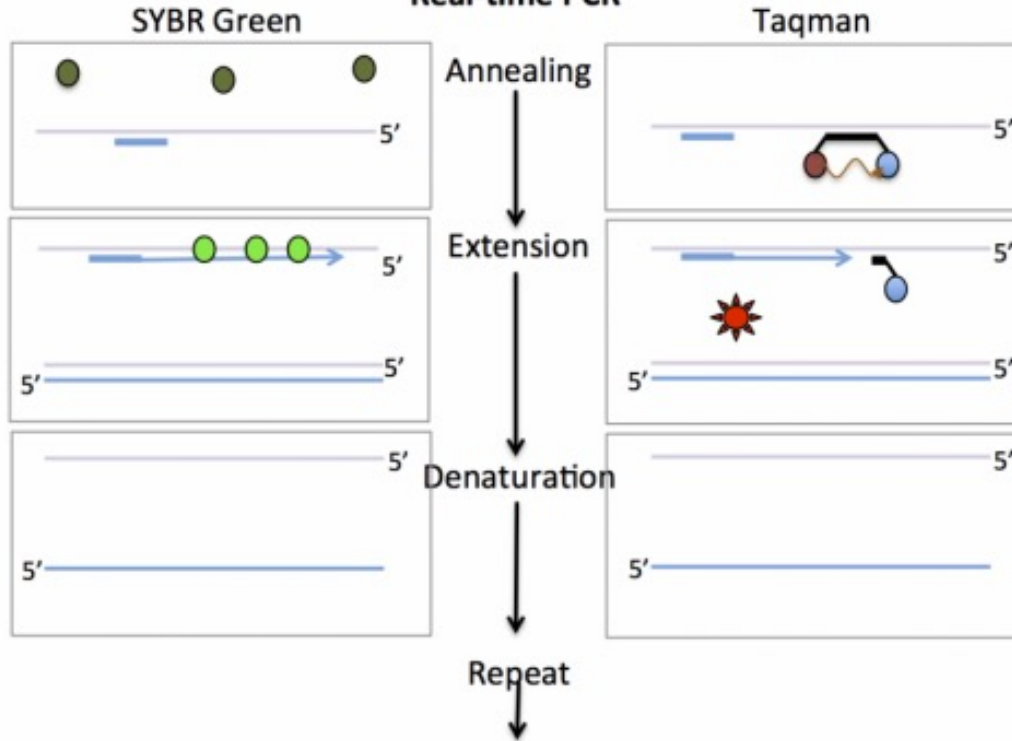


- Based on **western blot** and **RT-PCR** results we can conclude that **p53** is in CRC.
- And that could be explained through its function asgene.

Reverse transcription



Real-time PCR



Overview of RT-PCR technique

Comparison between Conventional PCR and RT-PCR

| Conventional PCR | Real-time PCR |
|---------------------------------|------------------------|
| Qualitative / semi-quantitative | Quantitative |
| Endpoint | Real time |
| Gel-based PCR | Fluorescence-based PCR |

Resources:

- **Gene Regulation Virtual Lab** (*both RT-PCR and Western blot are included*)

<https://www.labster.com/simulations/gene-regulation/>

تم بحمد الله ... 