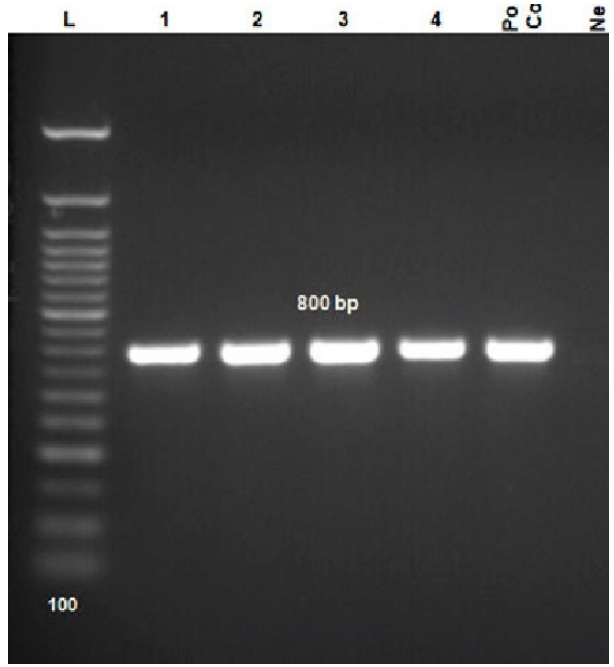
BCH 462- Biotechnology & Genetic Engineering [Practical]

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)

PCR → RT-PCR

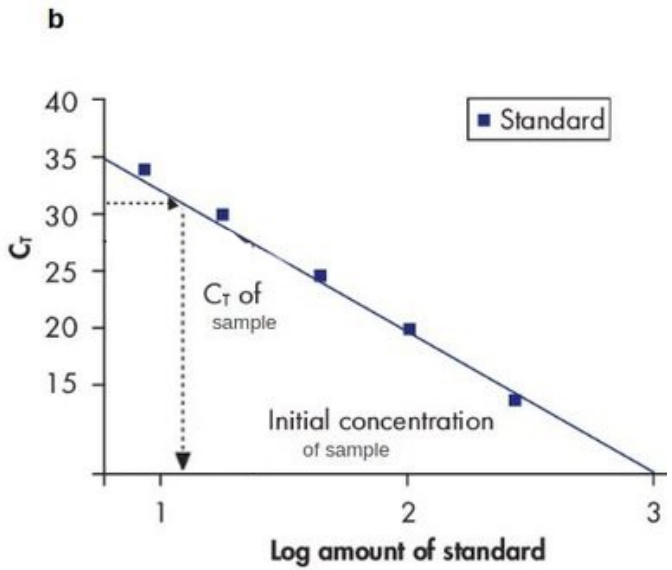
↓

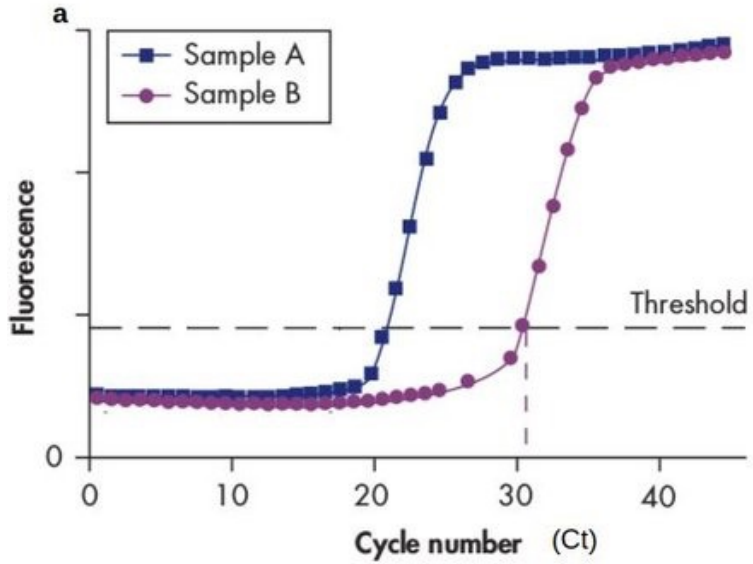
(PCR + Spectrofluorometry)

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.



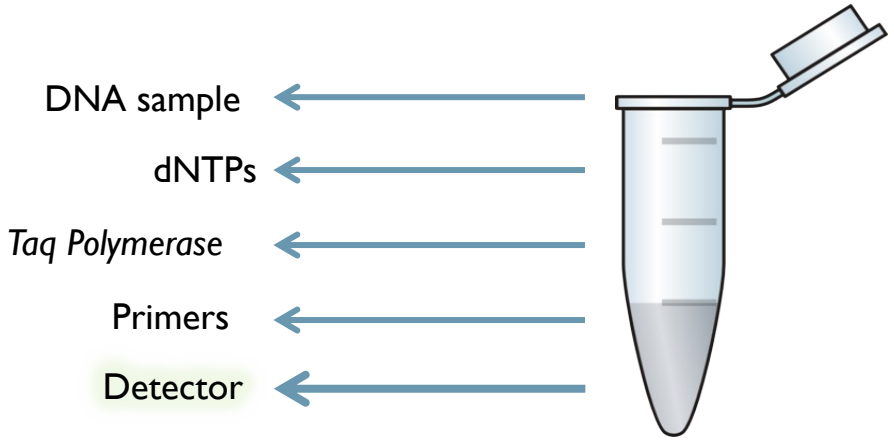


RT-qPCR chemistries (detection systems)

I. SYBR-green based assay:

Principle:

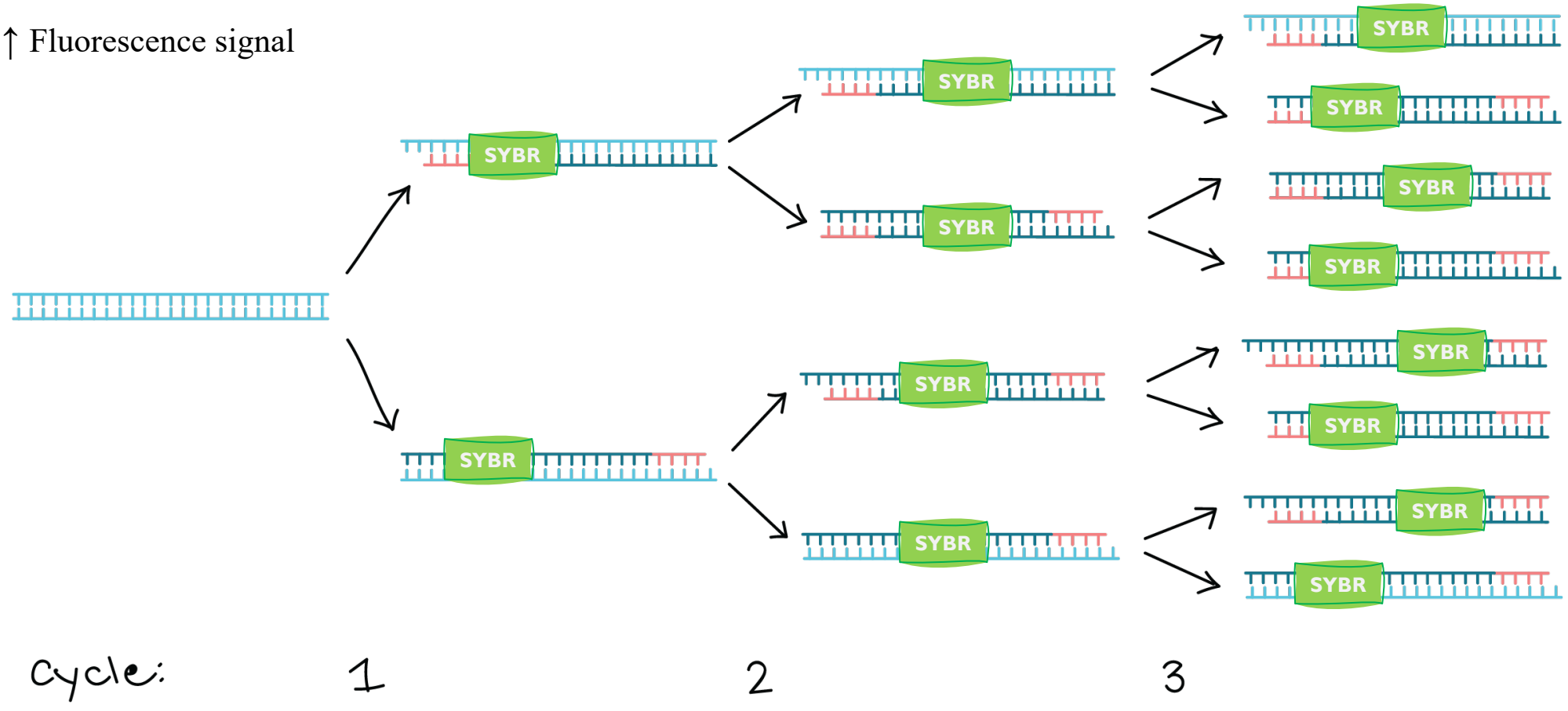
- The double-strands **DNA-intercalation** agent (DNA-binding dyes) such as SYBR Green.
- The SYBR Green 1 is **only** fluorescing when intercalated into dsDNA.
  - The intensity of the fluorescence signal is therefore **dependent** on the quantity of dsDNA present in the reaction.
- The main disadvantage 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).



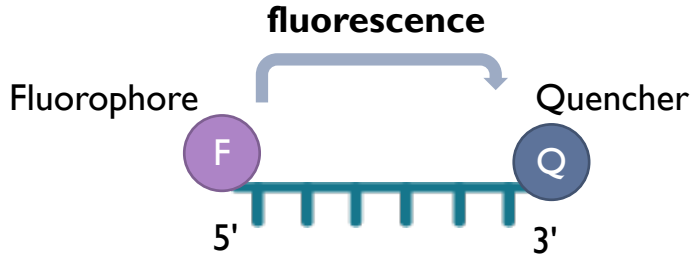
More dsDNA → More binding → More fluorescence

↑ Quantity of dsDNA

↑ Fluorescence signal



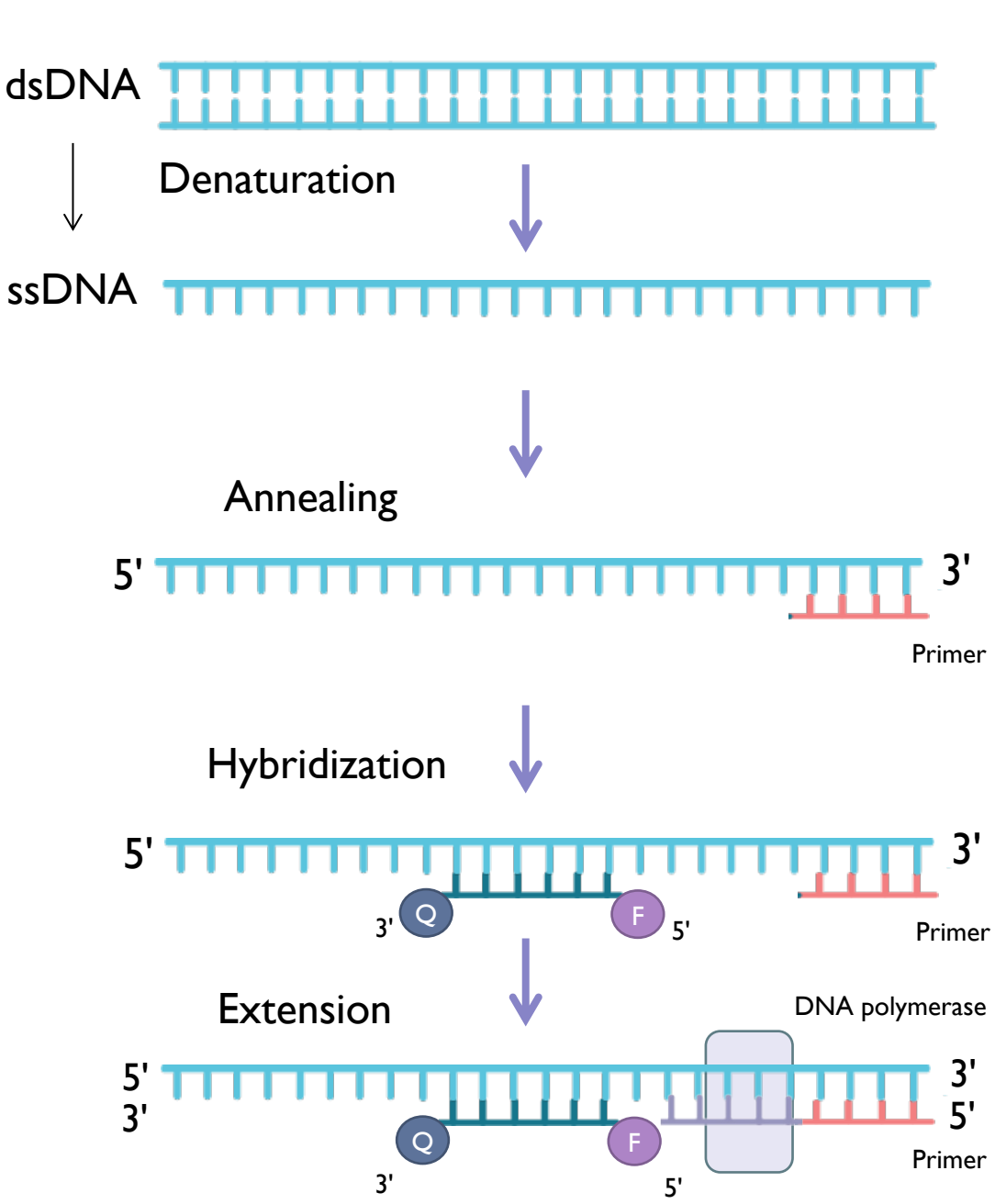
RT-qPCR chemistries (detection systems)

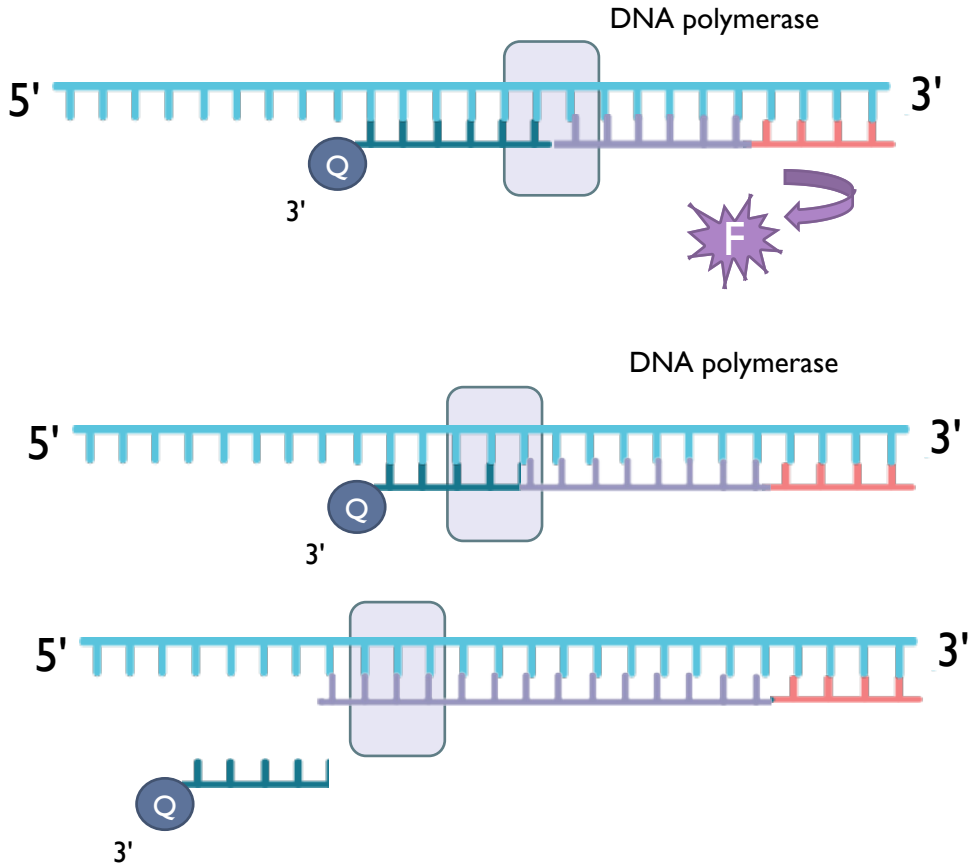


II. Fluorogenic 5' nuclease assay (TaqMan® probe assay):

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.**

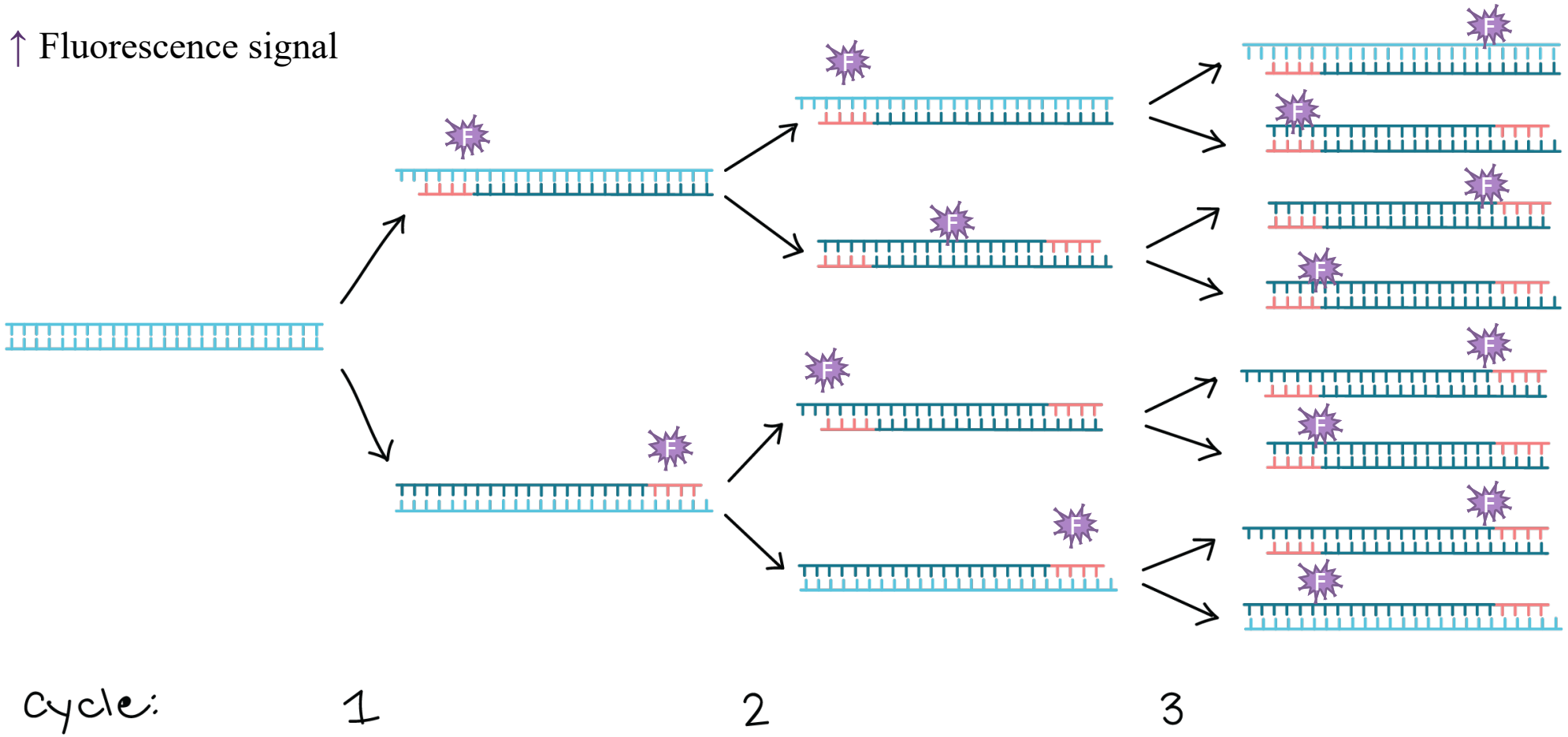

dsDNA
Denaturation
ssDNA
Annealing
5'
3'
Primer
Hybridization
5'
3'
Q
F
3'
5'
Primer
Extension
DNA polymerase
5'
3'
3'
5'
Q
F
3'
5'
Primer


DNA polymerase
5'
3'
Q
3'
F
DNA polymerase
5'
3'
Q
3'
5'
3'
Q
3'

More dsDNA → More probe **displacement** and **cleavage** → More fluorescence

↑ Quantity of dsDNA

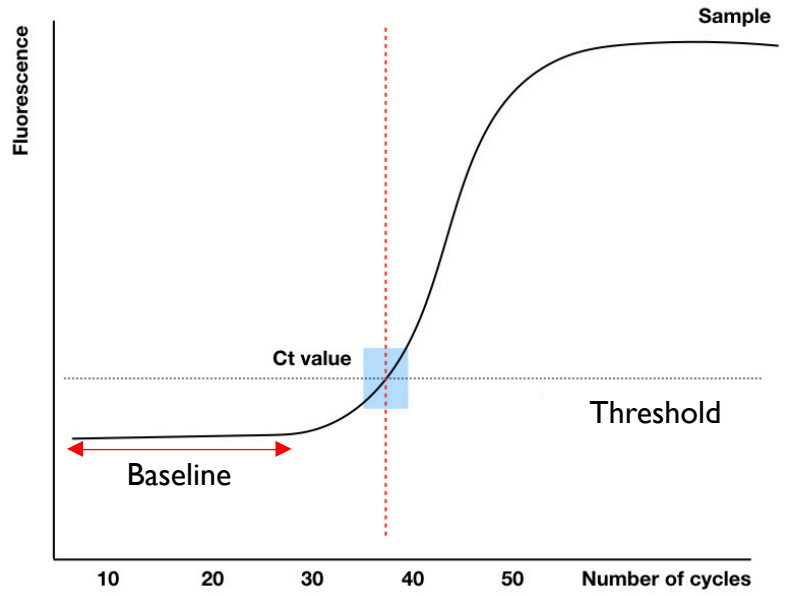
↑ Fluorescence signal



RT-qPCR amplification curve

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

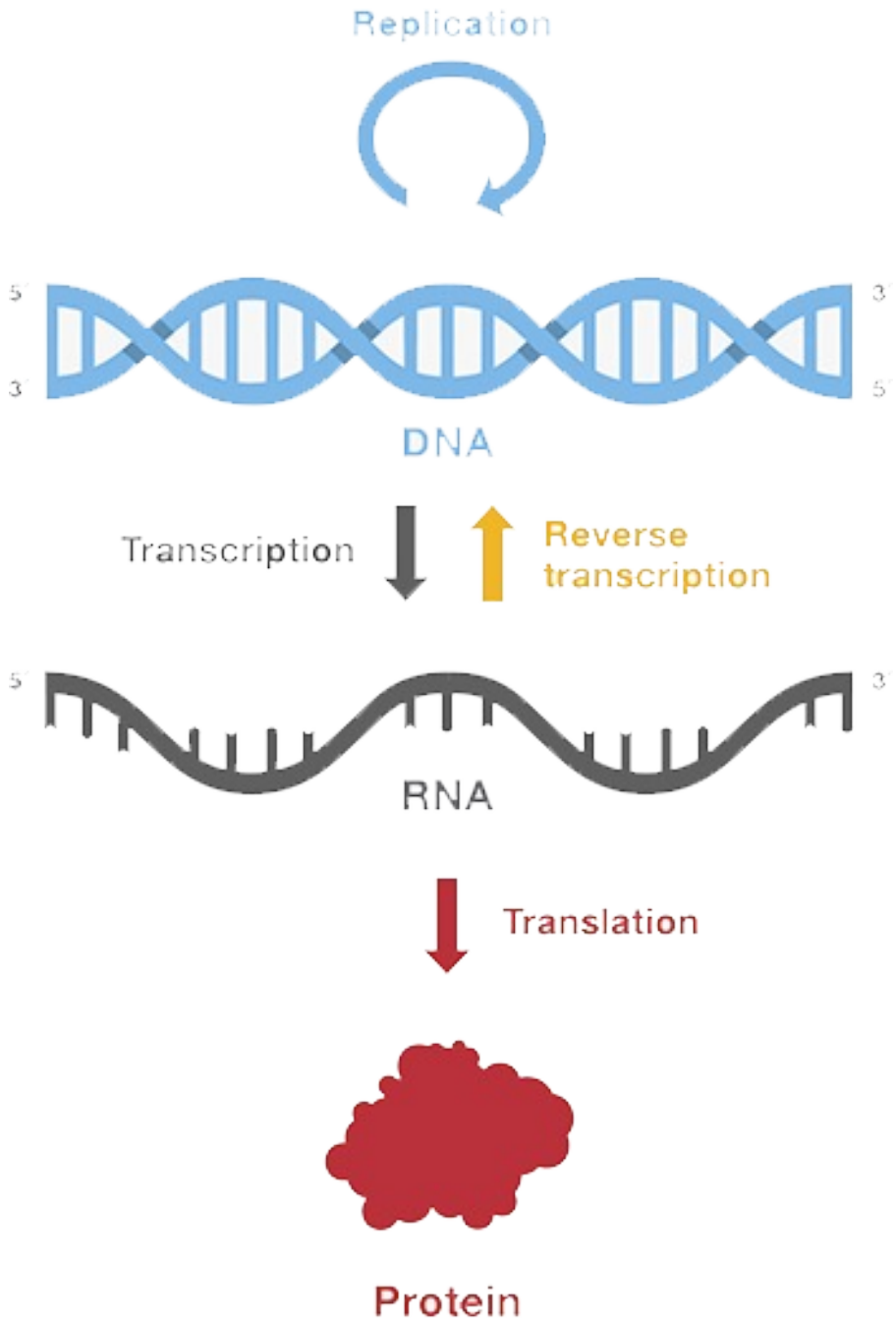
1. **Baseline**: The baseline of the real-time PCR reaction refers to the **little change in fluorescent signal** during the **initial cycles of PCR** (background or the "**noise**" of the reaction).
2. **Threshold:** is the level of detection or the point at which a reaction **reaches a fluorescent 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

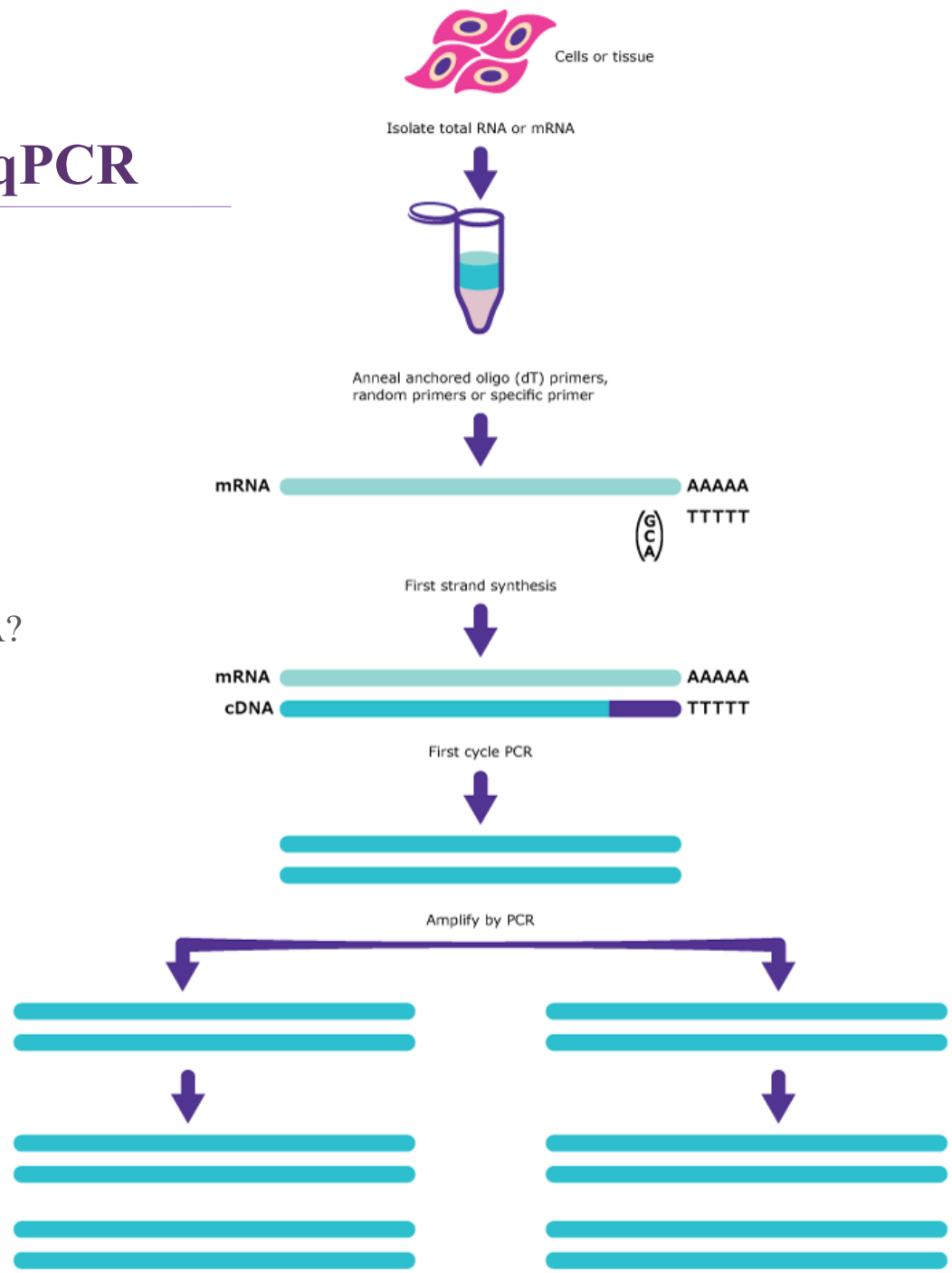
Step 1: **RNA Extraction from Tissue/ Cell line or blood**

Step 2: **Reverse transcription to convert RNA to cDNA**

Step 3: **Determination of cDNA using real time PCR**

Step 4: **Data analysis**

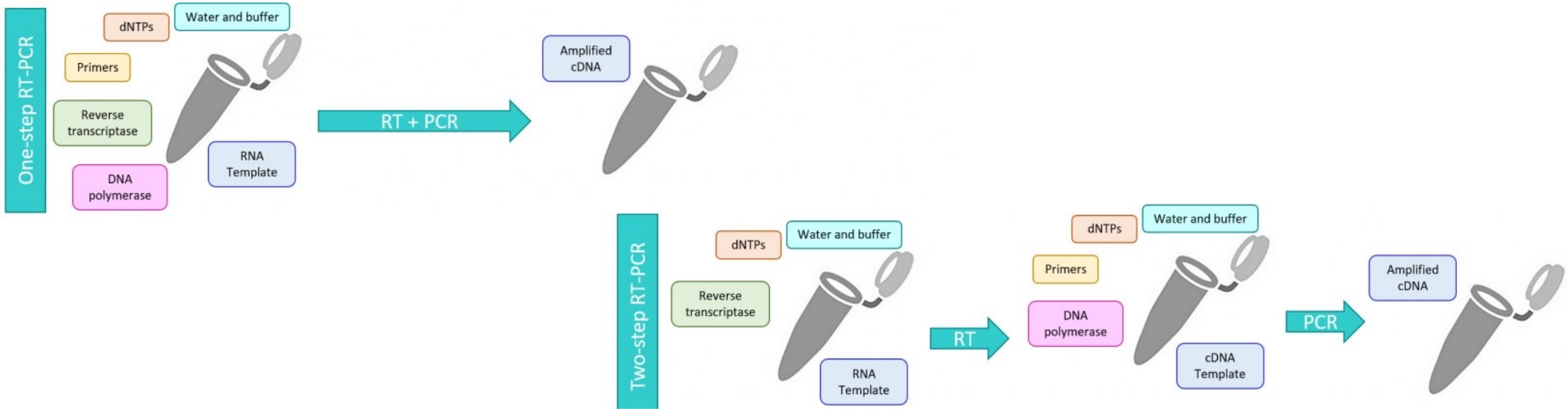
💡 Pause and Think why do we use cDNA instead of gDNA or RNA?



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.

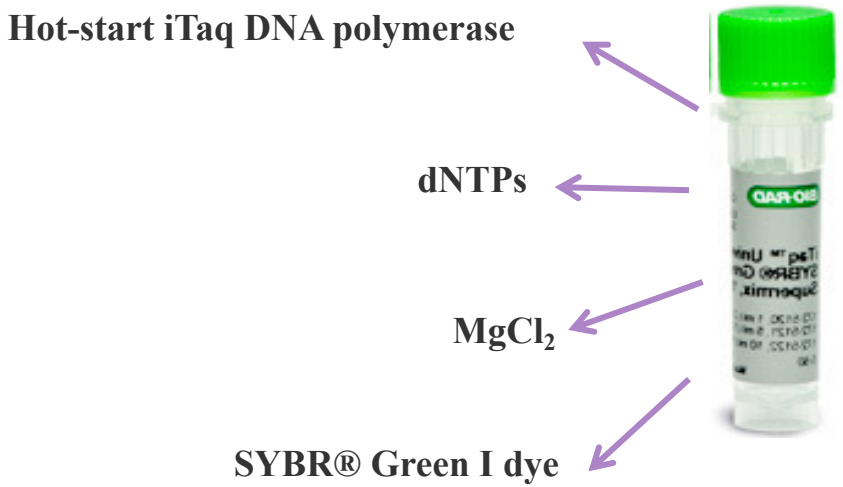


RT-qPCR for gene expression analysis

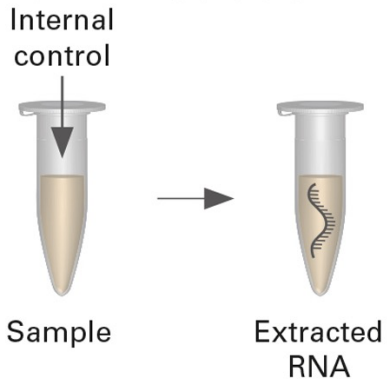
Reaction Components

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

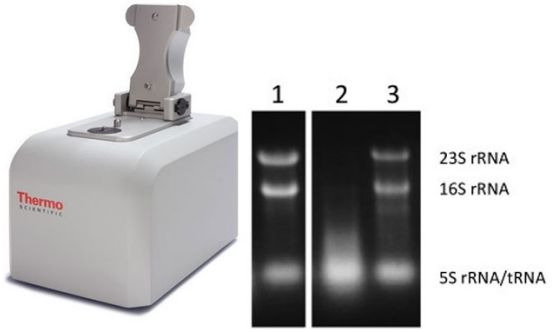


1. RNA isolation



Sample: tissue, Cell-line

2. RNA assesment

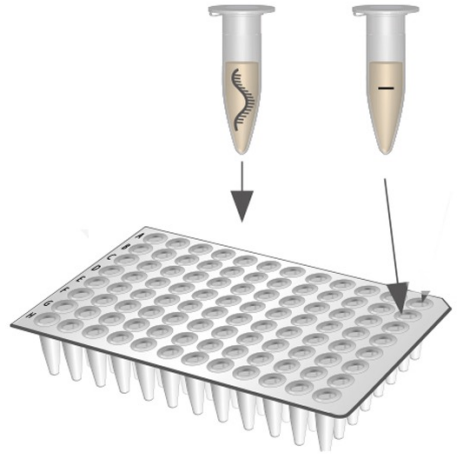


To ensure RNA integrity and purity/concentration

3. Reverse transcription

cDNA

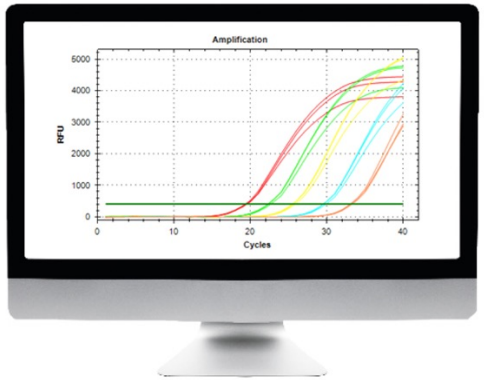
RNA as the starting material



4. Plate load

5. Real-Time PCR Thermal Cyclers

(Thermocycler + Spectrofluorometry)

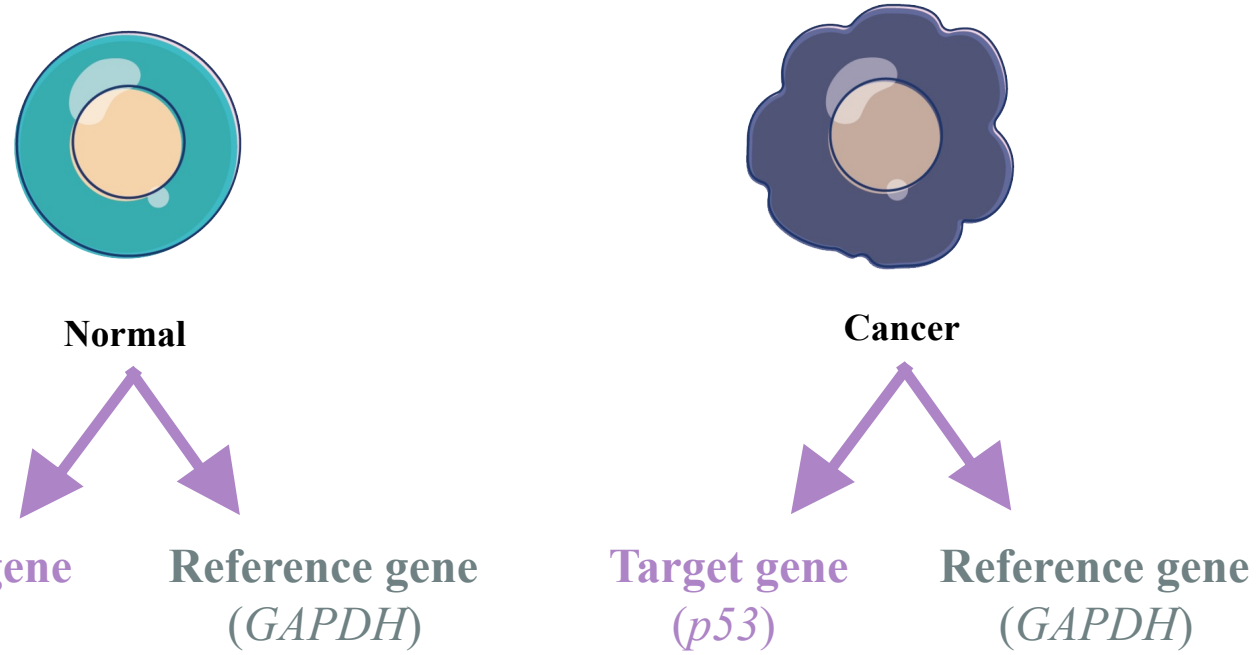


6. Data analysis

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

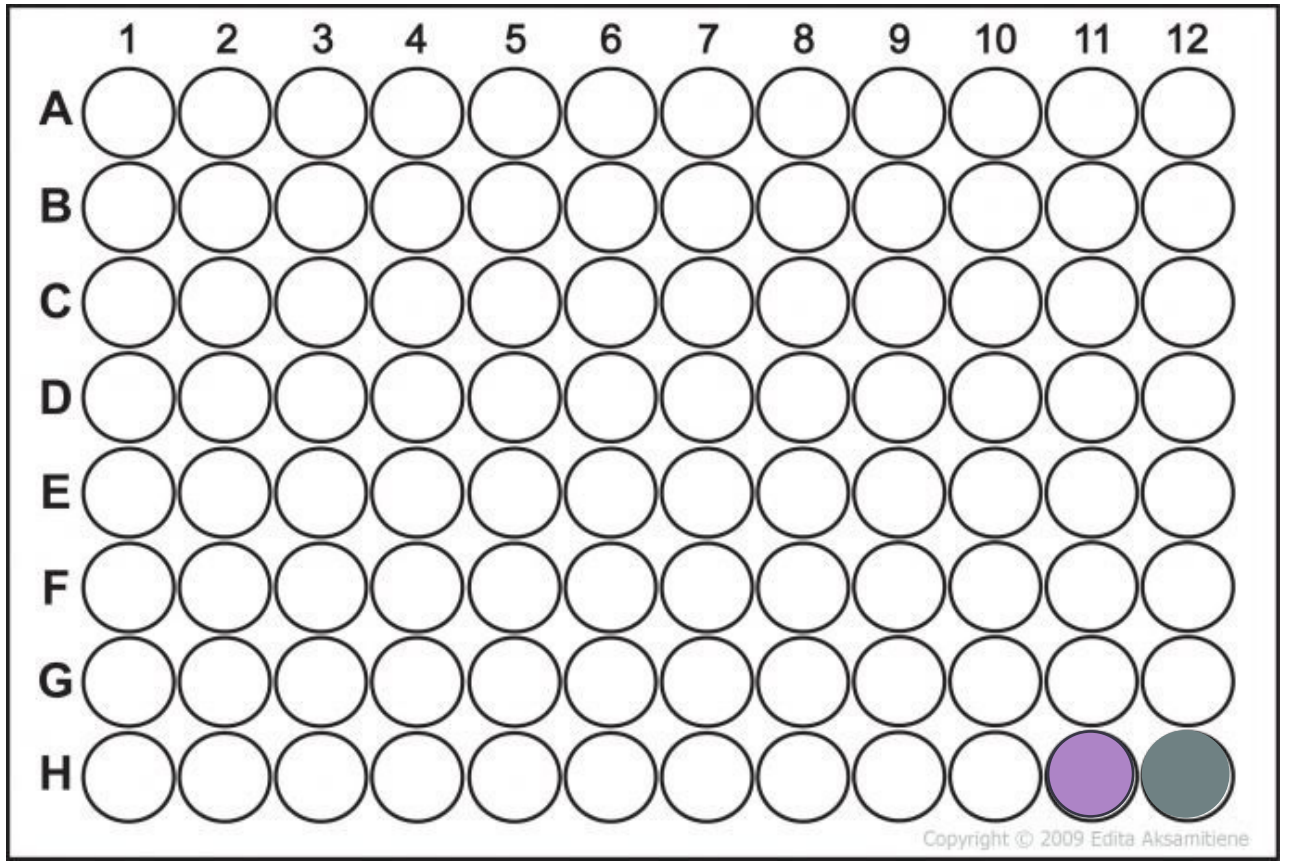
Target gene
Cancer cell

Target gene
Normal cell

Reference gene
Normal cell

Reference gene
Cancer cell

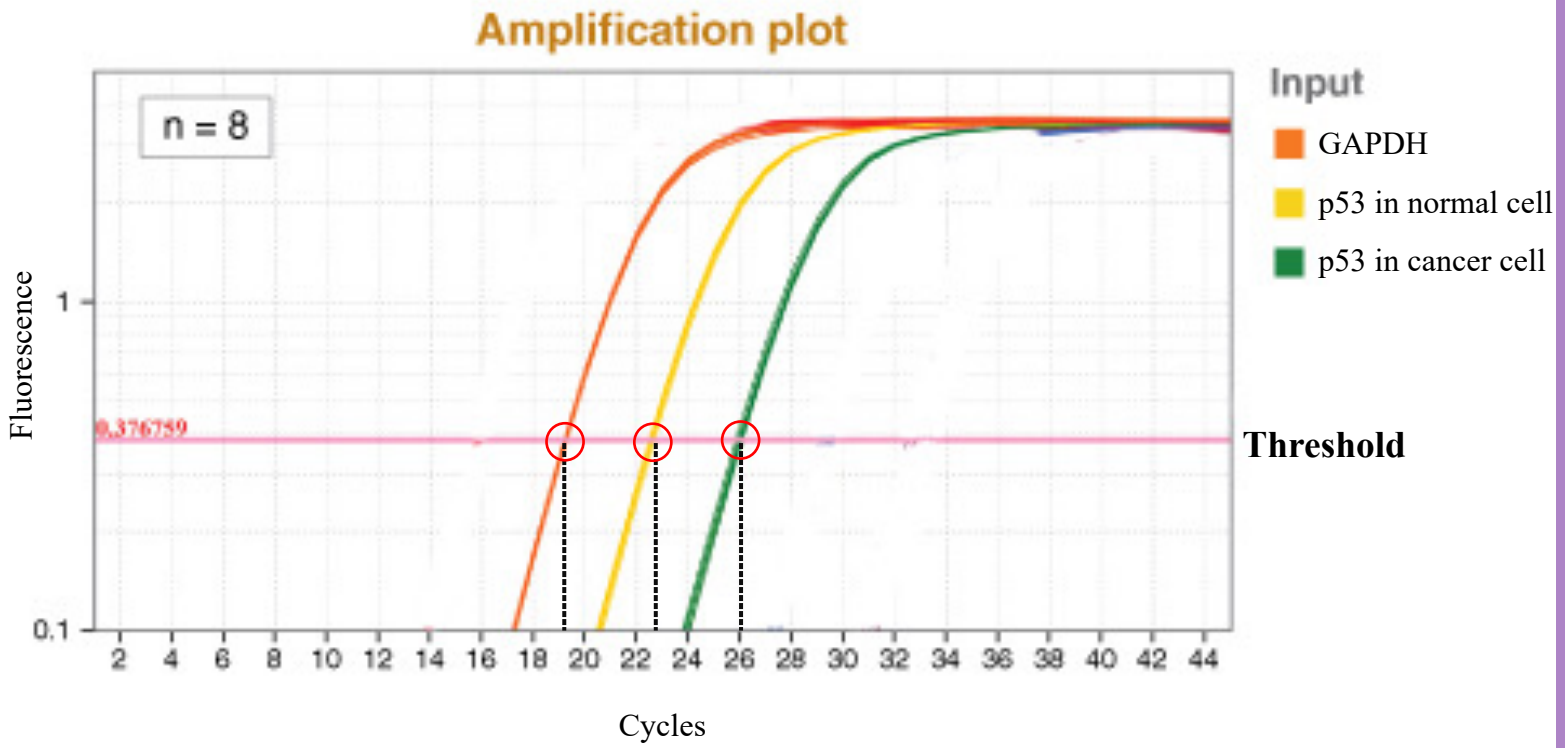
Each row represents <u>one patient</u>



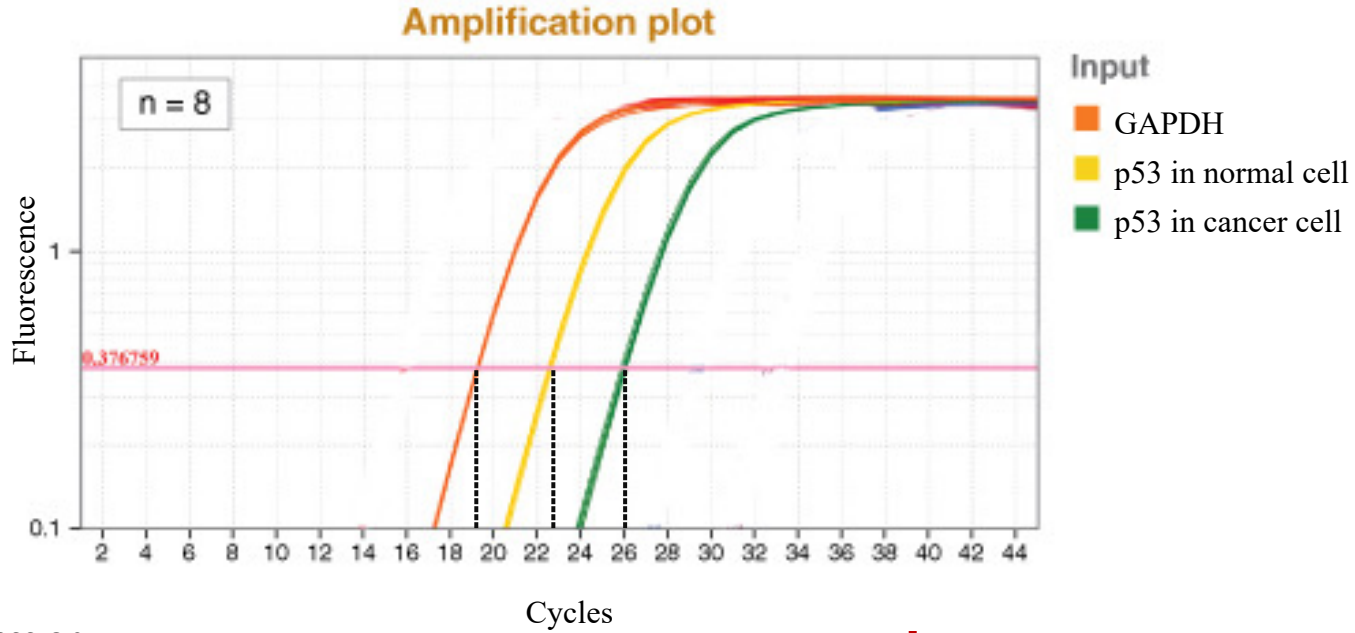
-ve control for each master mix
(no template control)

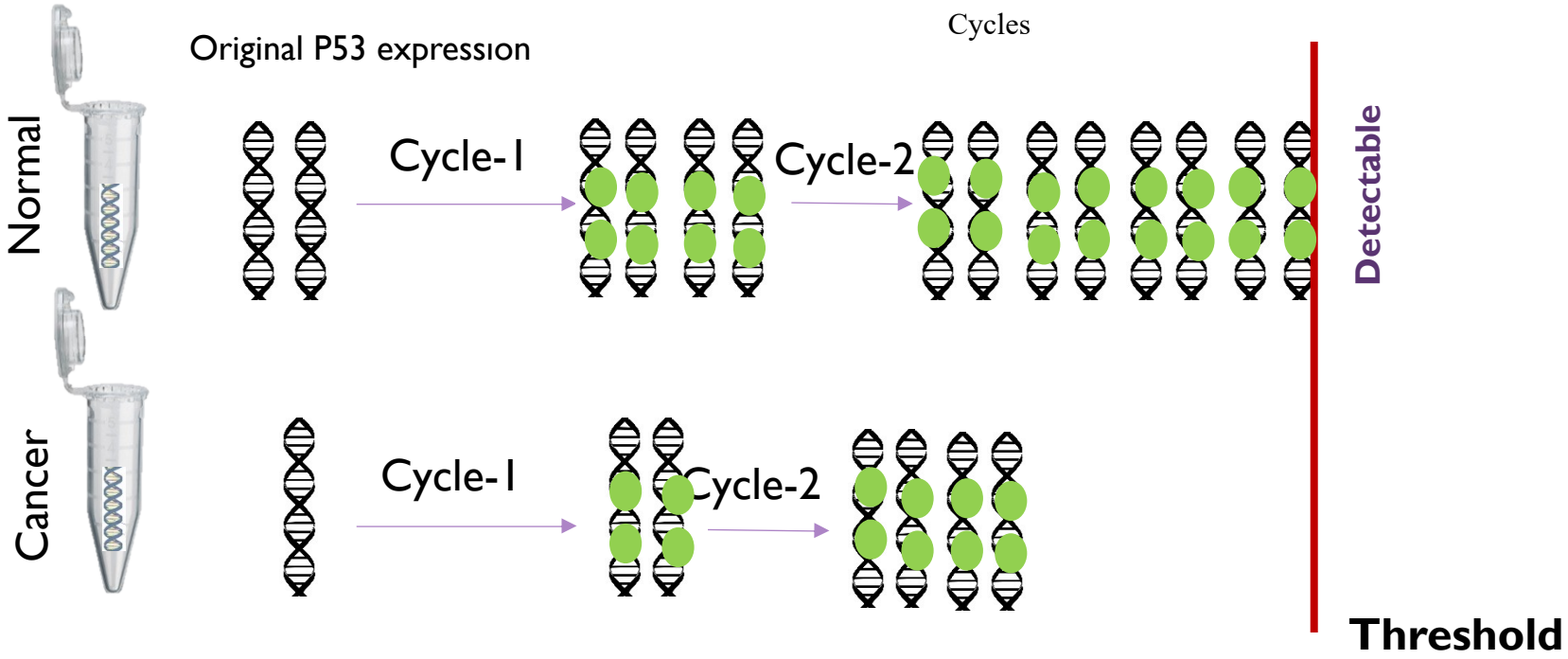
- Ct levels are **inversely proportional** to the amount of target nucleic acid expression in the sample (i.e. **the lower the Ct level the greater the amount of target nucleic acid in the sample).**

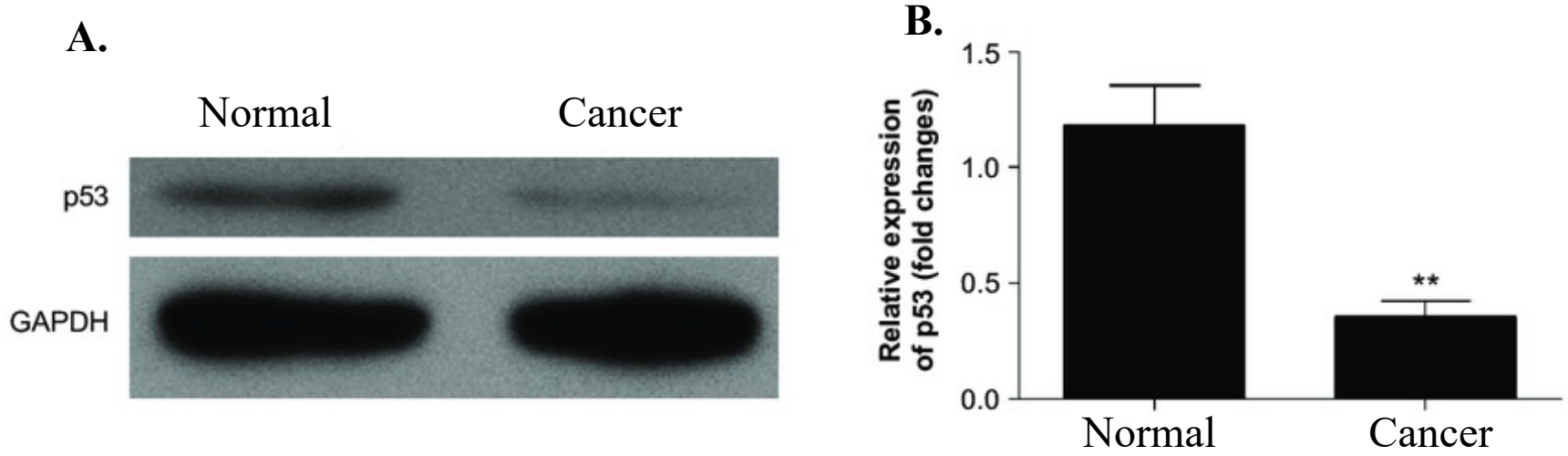
Samples	Ct value	
	GAPDH	p53
Normal 1	19.90	23.00
Normal 2	19.80	22.74
Normal 3	19.85	22.80
Tumor 1	20.00	26.00
Tumor 2	19.75	26.20
Tumor 3	19.72	26.12



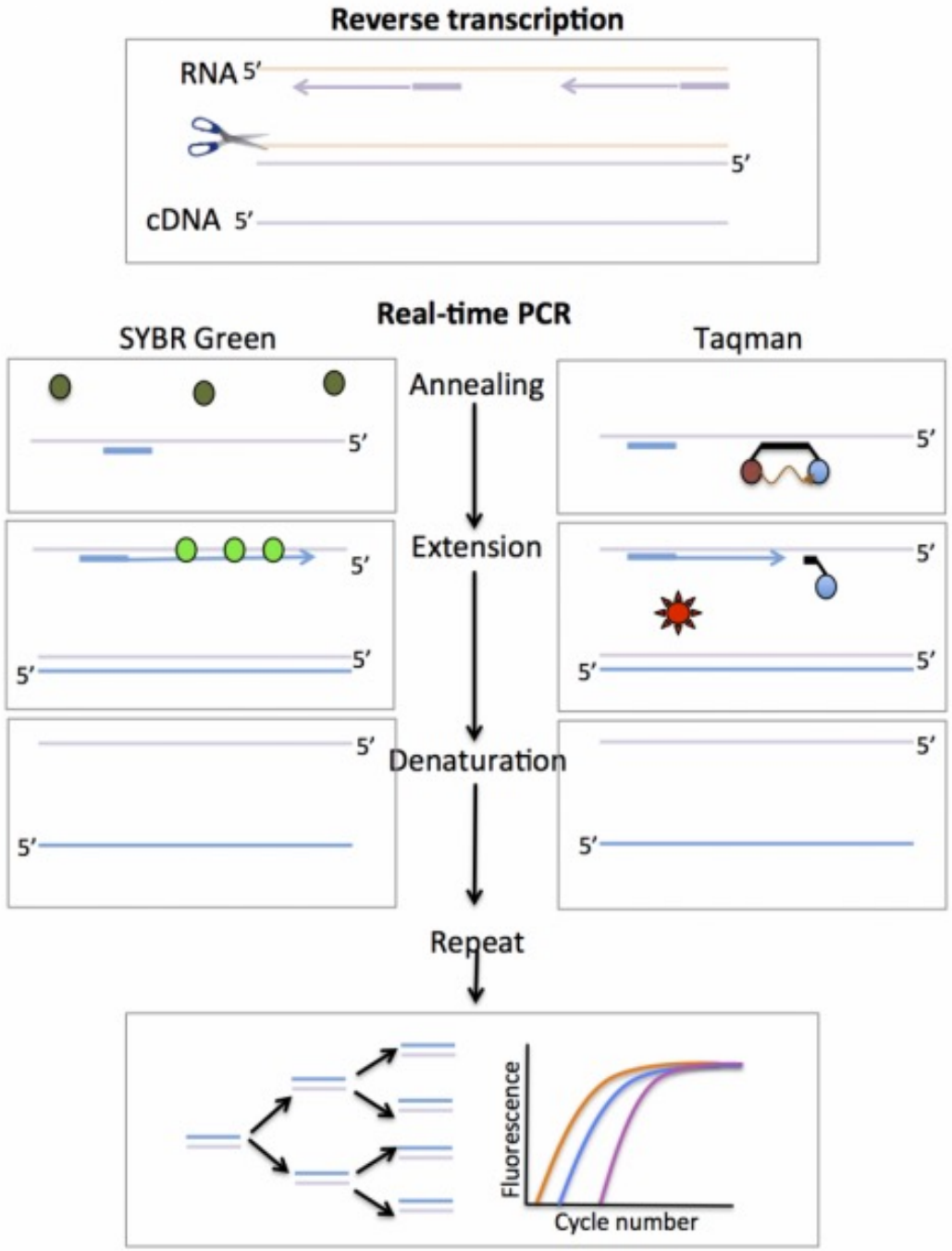
1- Did GAPDH expression change in normal and cancer cell? why?
2- What happened to p53 expression in cancer? Up/down?


Amplification plot
n = 8
Fluorescence
1
0.1
0.376759
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Cycles
Input
GAPDH
p53 in normal cell
p53 in cancer cell


Original P53 expression
Normal
Cycle-1
Cycle-2
Cancer
Cycle-1
Cycle-2
Detectable
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 as ……..gene.



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 ... 💜