QUANTITATIVE REVERSE TRANSCRIPTION PCR (RT-QPCR)
" REAL-TIME PCR"

BCH462- Practical

Real- time PCR:

- 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.
- Traditional PCR **VS** RT-qPCR.
- Device.

Real-Time PCR

Measured as the reaction progresses

"In real time"

Amplification

Concept of Real- time PCR :

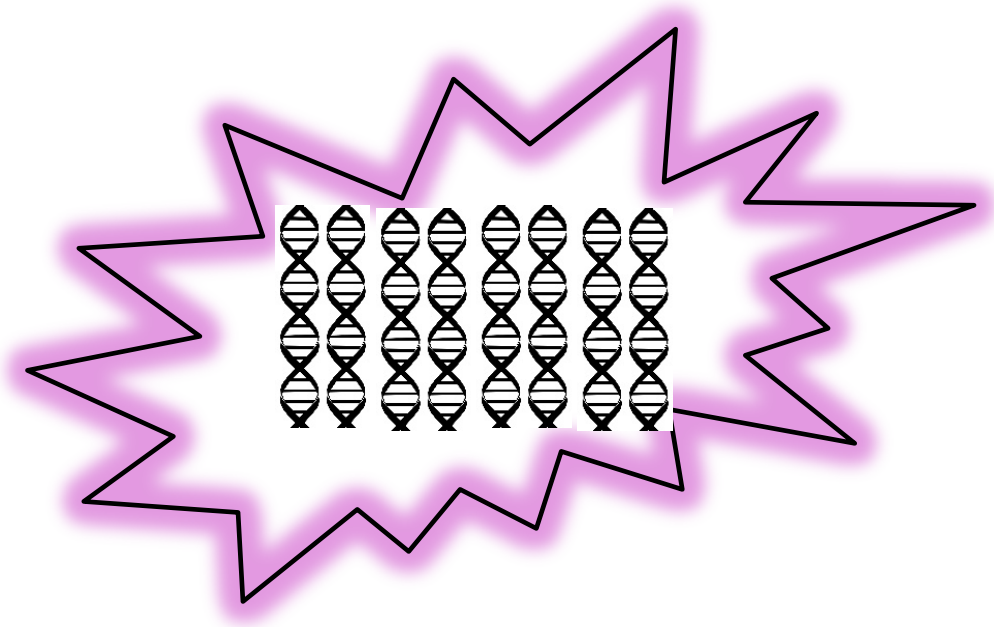
- Fluorescent signal is proportional to the amount of DNA ➔ Measuring.
- PCR product is measured at each cycle , via fluorescent dyes that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules (amplicons) generated.

- So….

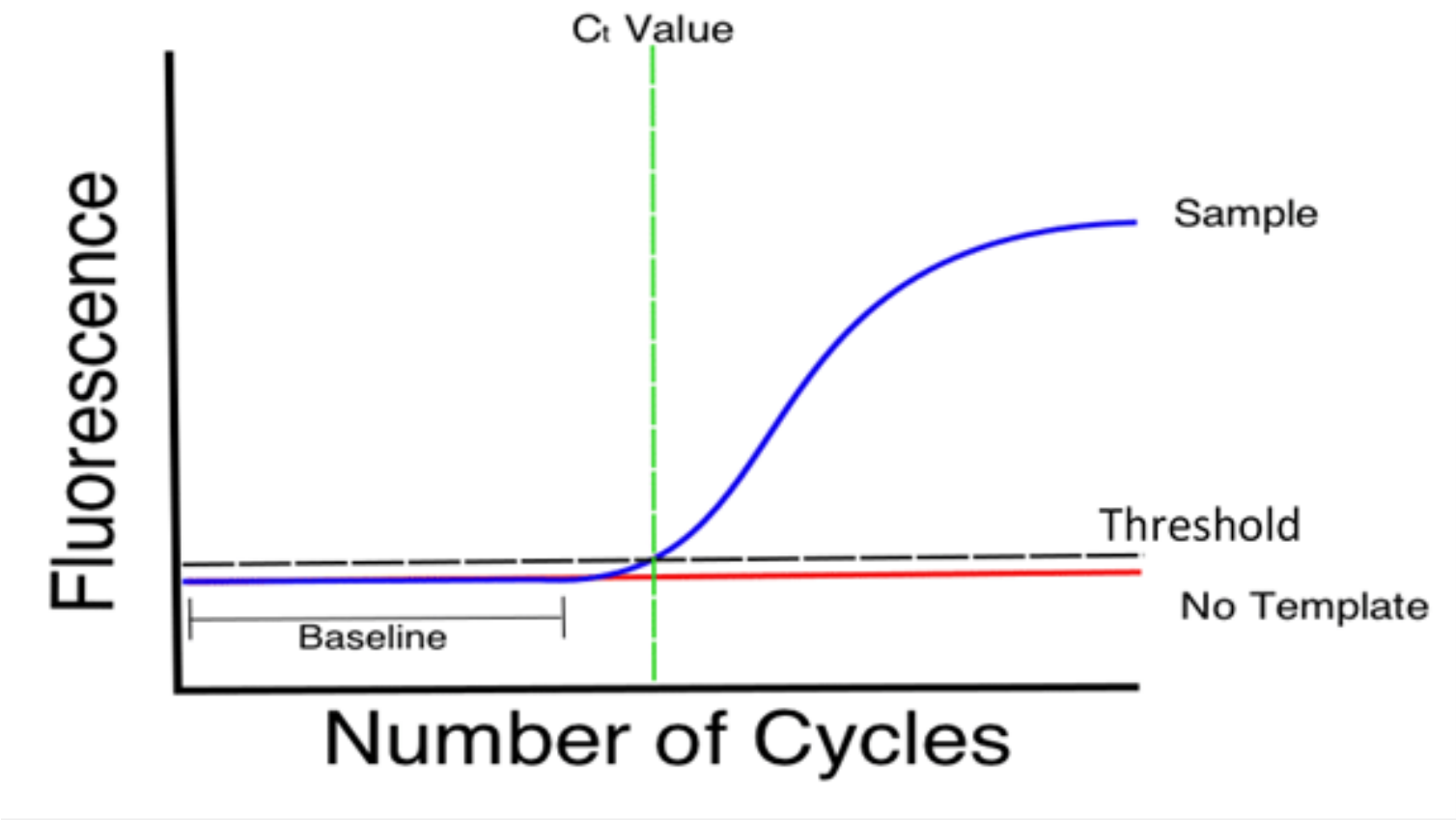
Fluorescence signal indicate the amount of DNA that being amplified in the PCR Reaction.

(FLOURECENCE SINGLA + AMPLIFICATION)

AMPLIFICATION + FLUORESCENCE

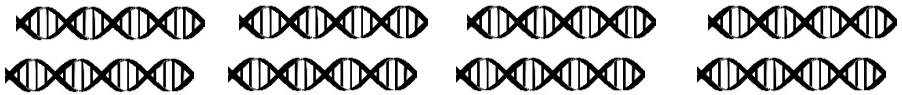


RT-qPCR amplification curve:

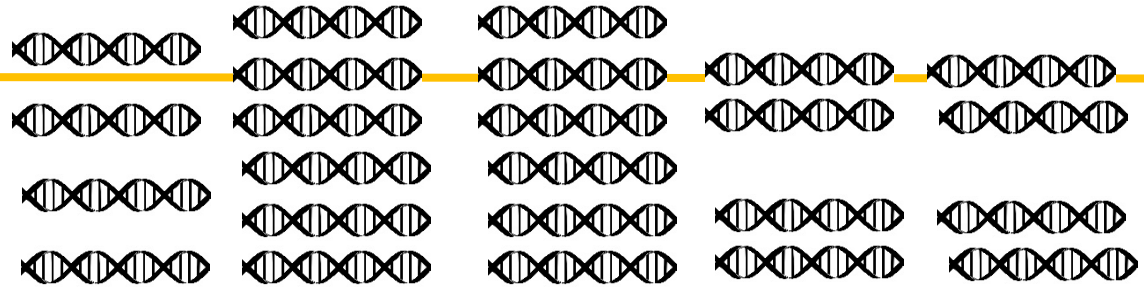


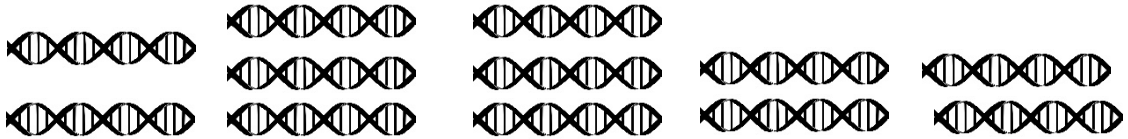
DETECTION

Cycle Threshold



Sample A

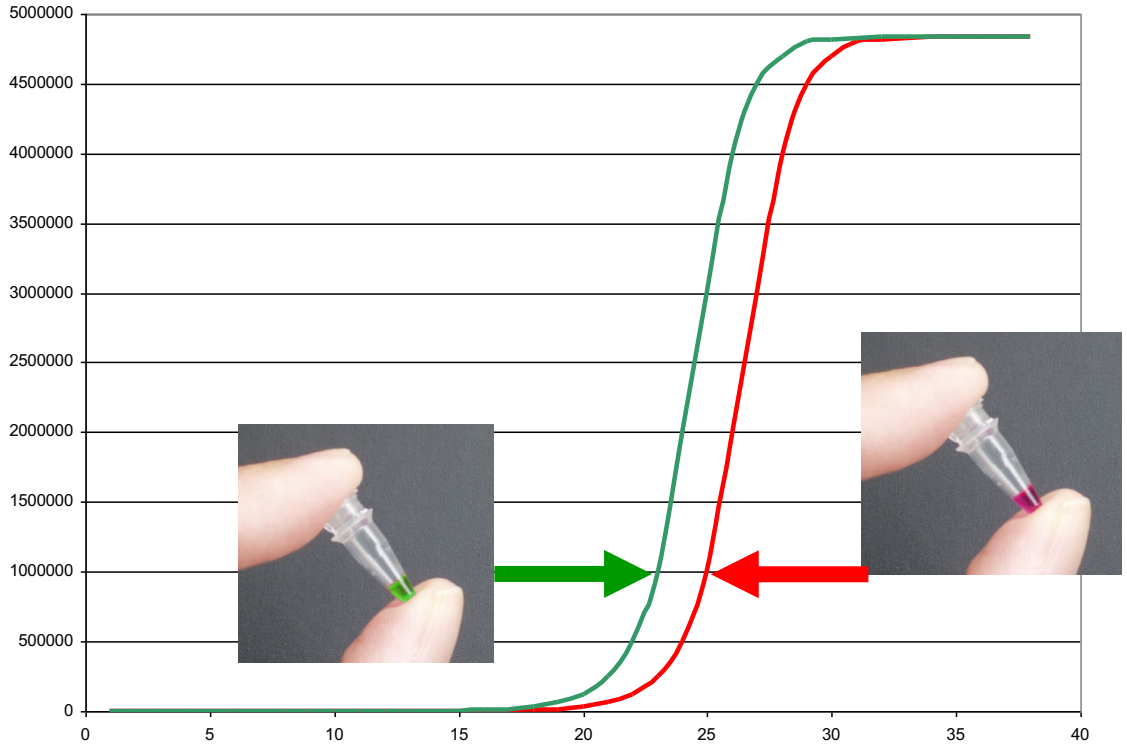




Sample B

So, if YOU started with FOUR times as much DNA template as I did…

…Then you'd reach 1,000,000 copies exactly TWO cycles earlier than I would!



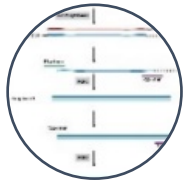
RT-qPCR applications:

- Gene expression.
- qualitative detection could be used.

Evaluating gene expression by RT-qPCR steps:

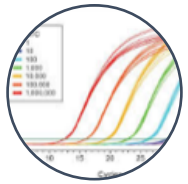
RNA Extraction from Tissue/ Cell line or blood

(Tissue must be :Fresh, Stored in RNlater, or Liqued nitrogen)

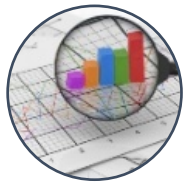


Reverse transcription to convert RNA to cDNA

(Very important Step, RNA is very unstable)



Determination of cDNA using real time PCR (flurosenec)



Data analysis

RT-qPCR types:

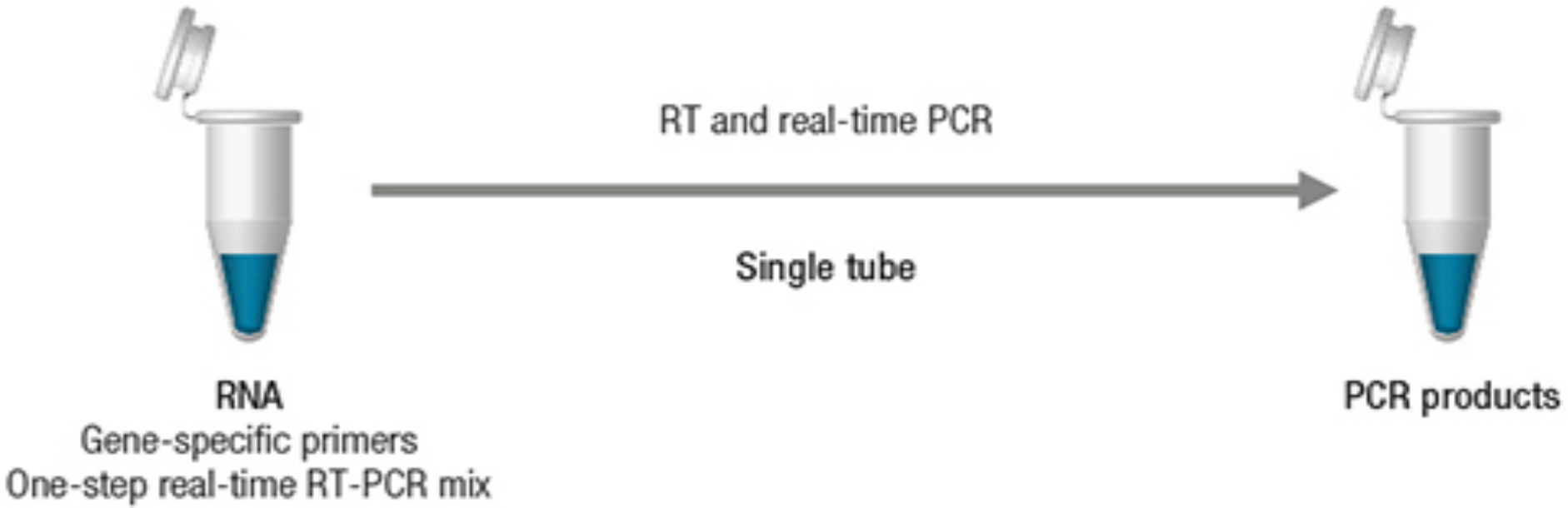
- Two methods:

➔One-step RT-qPCR.

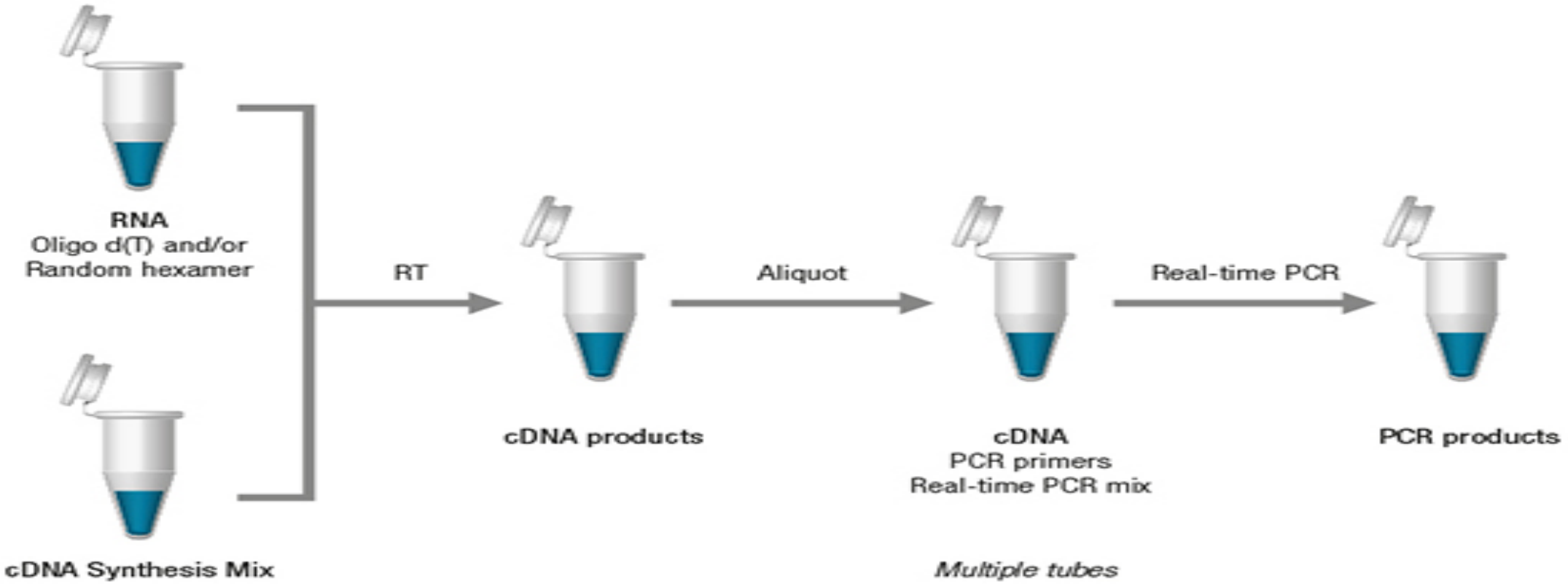
➔Two-step RT-qPCR.

- Primers.
- Advantages and disadvantages.

One-step RT-qPCR



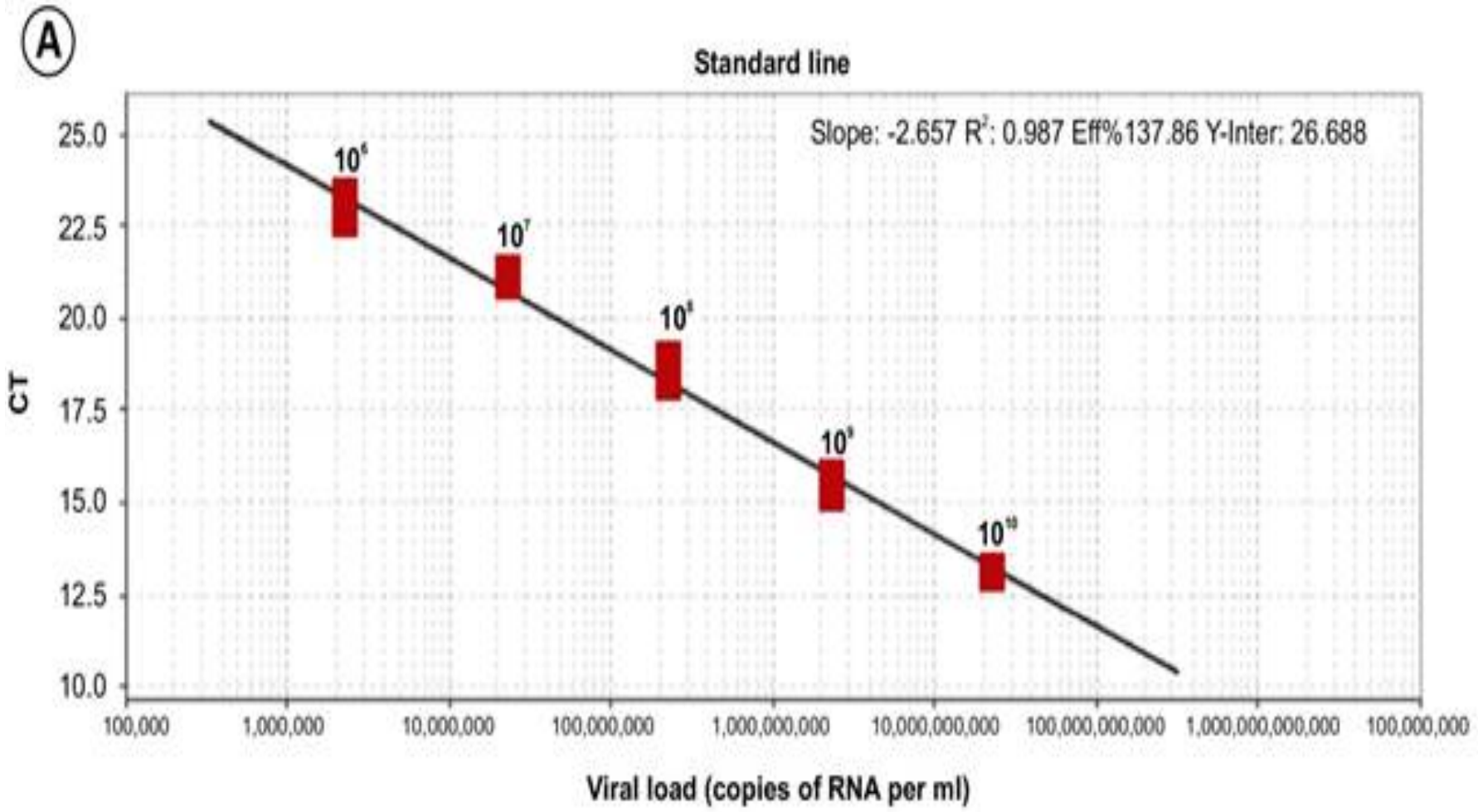
Two-step RT-qPCR



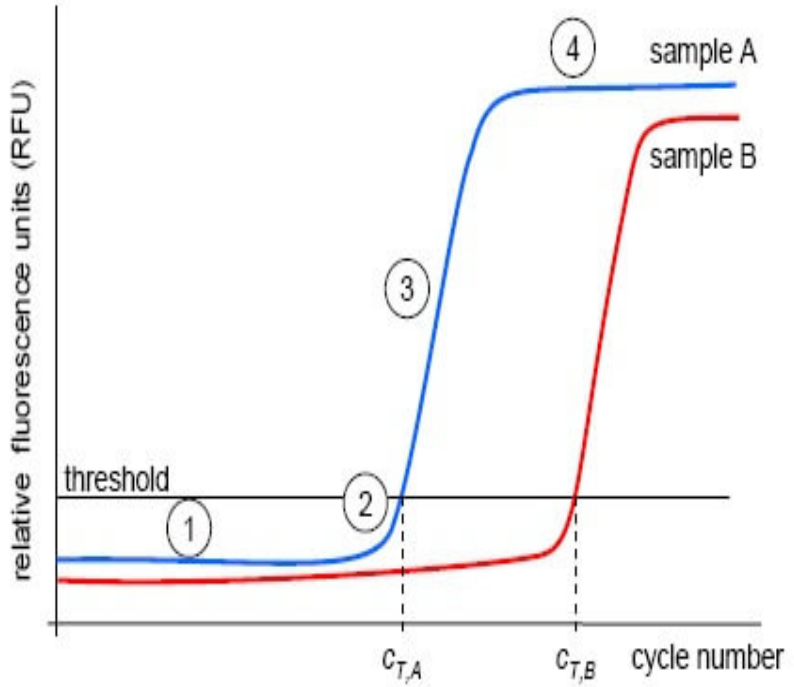
RT-qPCR quantification methods:

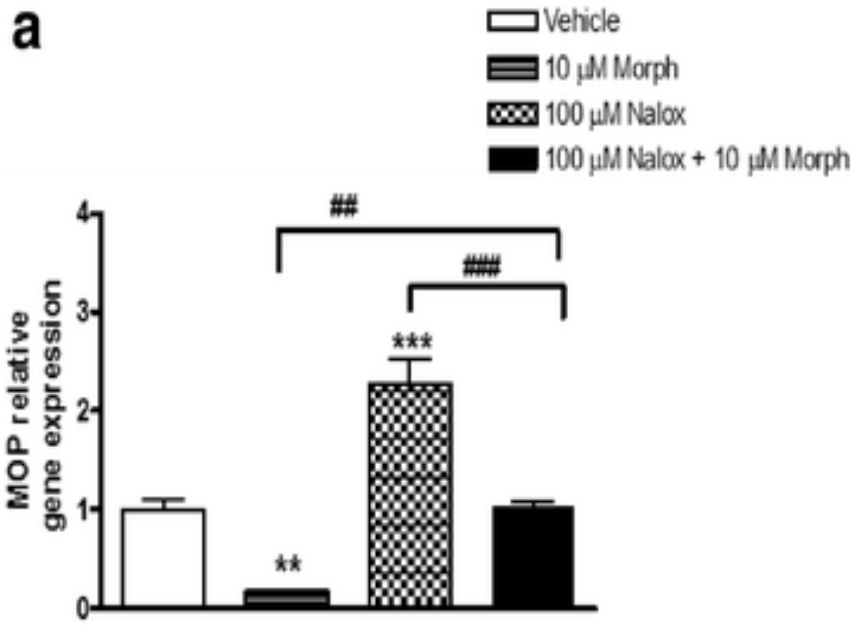
- Absolute quantification.
- Relative/ comparative quantification.

Absolute quantification



Relative quantification



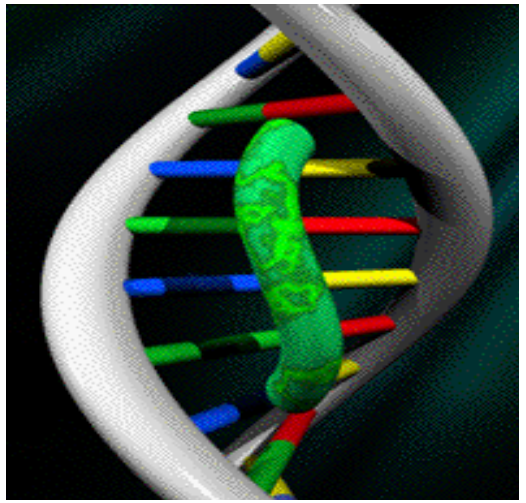


Normalizer gene… Why?

	Ct Target	Ct Control	ΔCt
Untreated 1	25.3	18.6	6.7
Untreated 2	26.2	19.3	6.9
Untreated 3	25.8	19.4	6.4
Treated 1	27.9	19.8	8.1
Treated 2	28.2	20.1	8.1
Treated 3	27.7	19.3	8.4

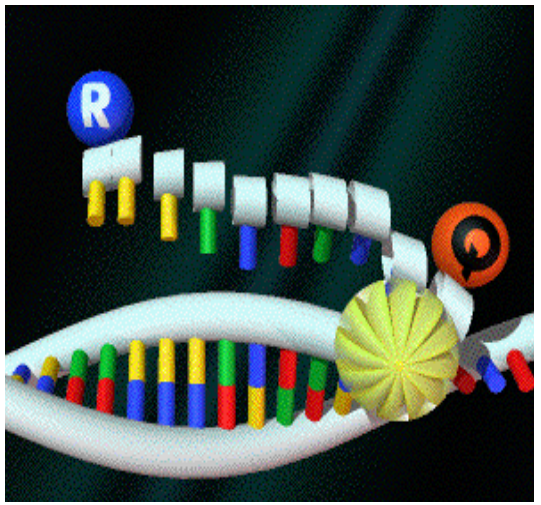
RT-qPCR chemistries (detection systems):

SYBR® Green I dye



Binds double-stranded DNA

Fluorogenic 5' Nuclease Assay



Uses a TaqMan® probe

Principle of SYBR®-green based assay:

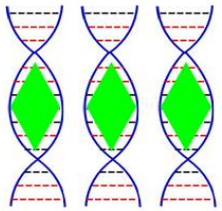
SYBR green master mix having SYBR green dye, Taq, buffer and dNTPs

SYBR green dye

DNA template

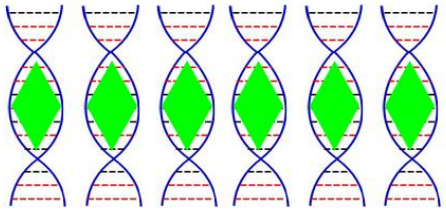
Reaction master mix having DNA template and primers

Ready for reaction



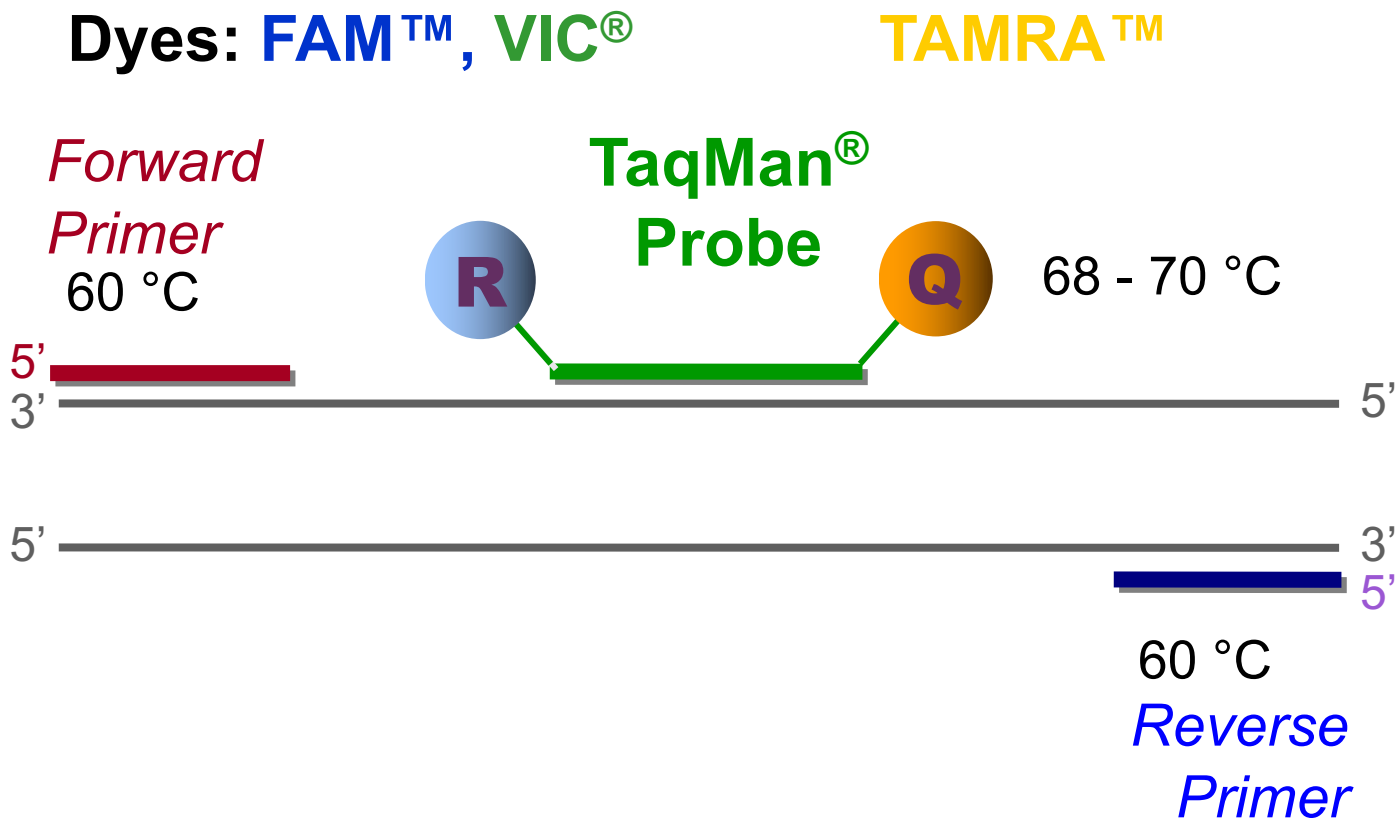
SYBR green binds to the double stranded DNA template.

PCR amplification



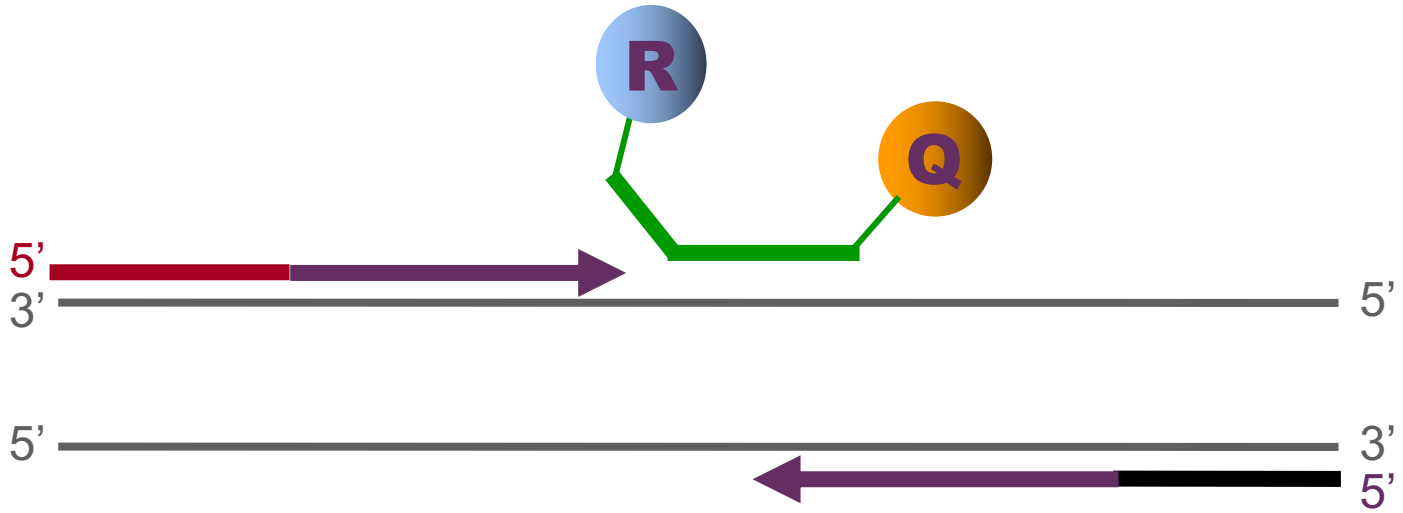
With every PCR cycle the double stranded DNA increases to which SYBR green binds leading to increase in fluorescence.

Principle of fluorogenic 5' nuclease assay (TaqMan® probe assay):



- PCR specificity (primer).
- Hybridization specificity (probe).

Principle of fluorogenic 5' nuclease assay (TaqMan® probe assay):



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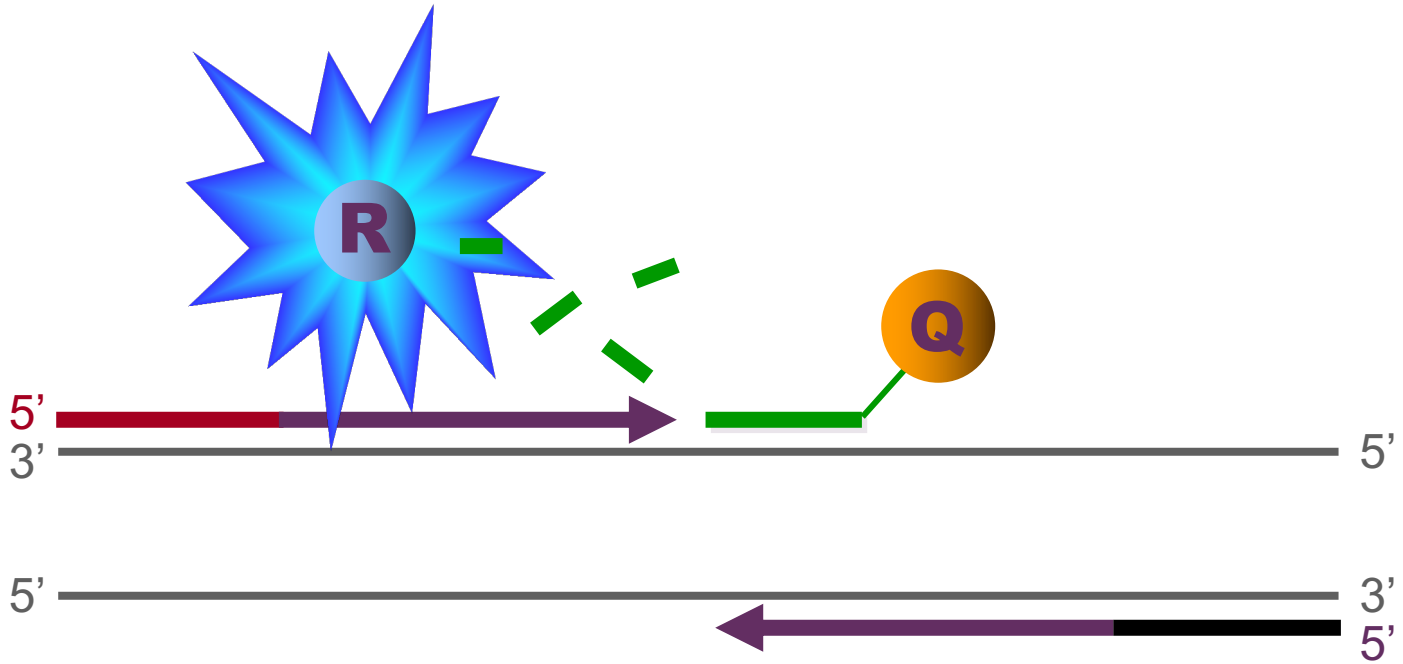

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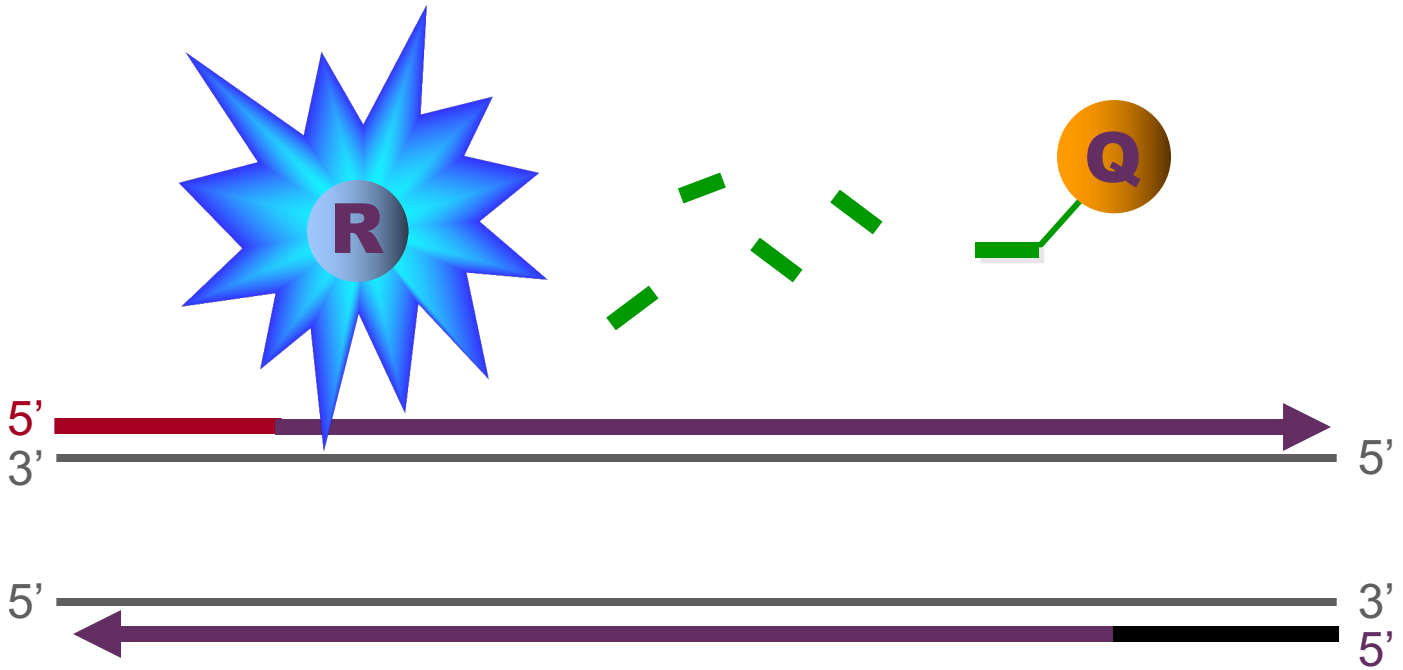


Comparison between SYBR green and Taq man assay:

- Specificity.
- Applications.
- Optimization.

Practical Part 🧬

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

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

Patient 1

Normal

Target gene (P53)

Reference gene (GAPDH)

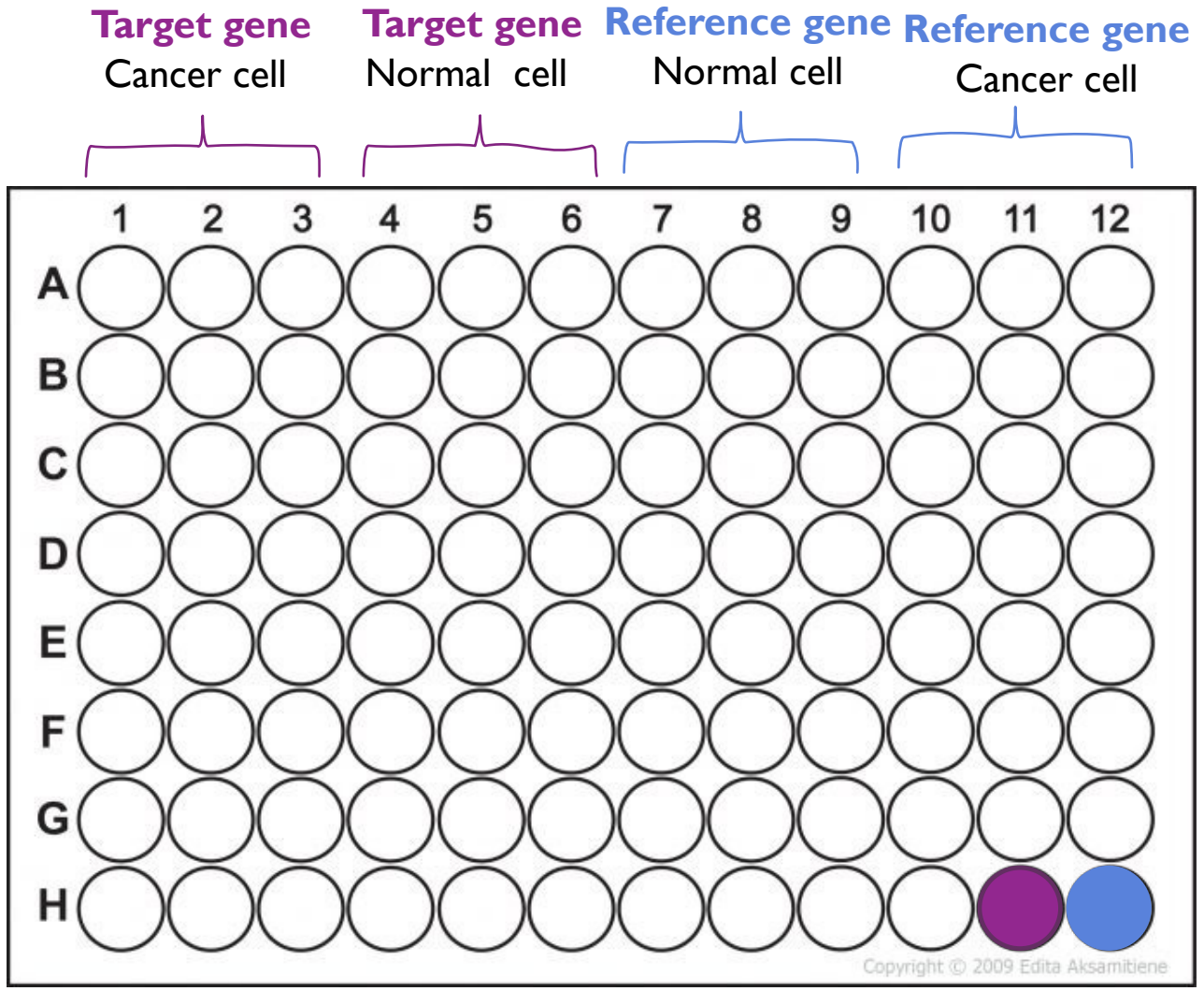
Cancer

Target gene (P53)

Reference gene (GAPDH)

Each As triplicate

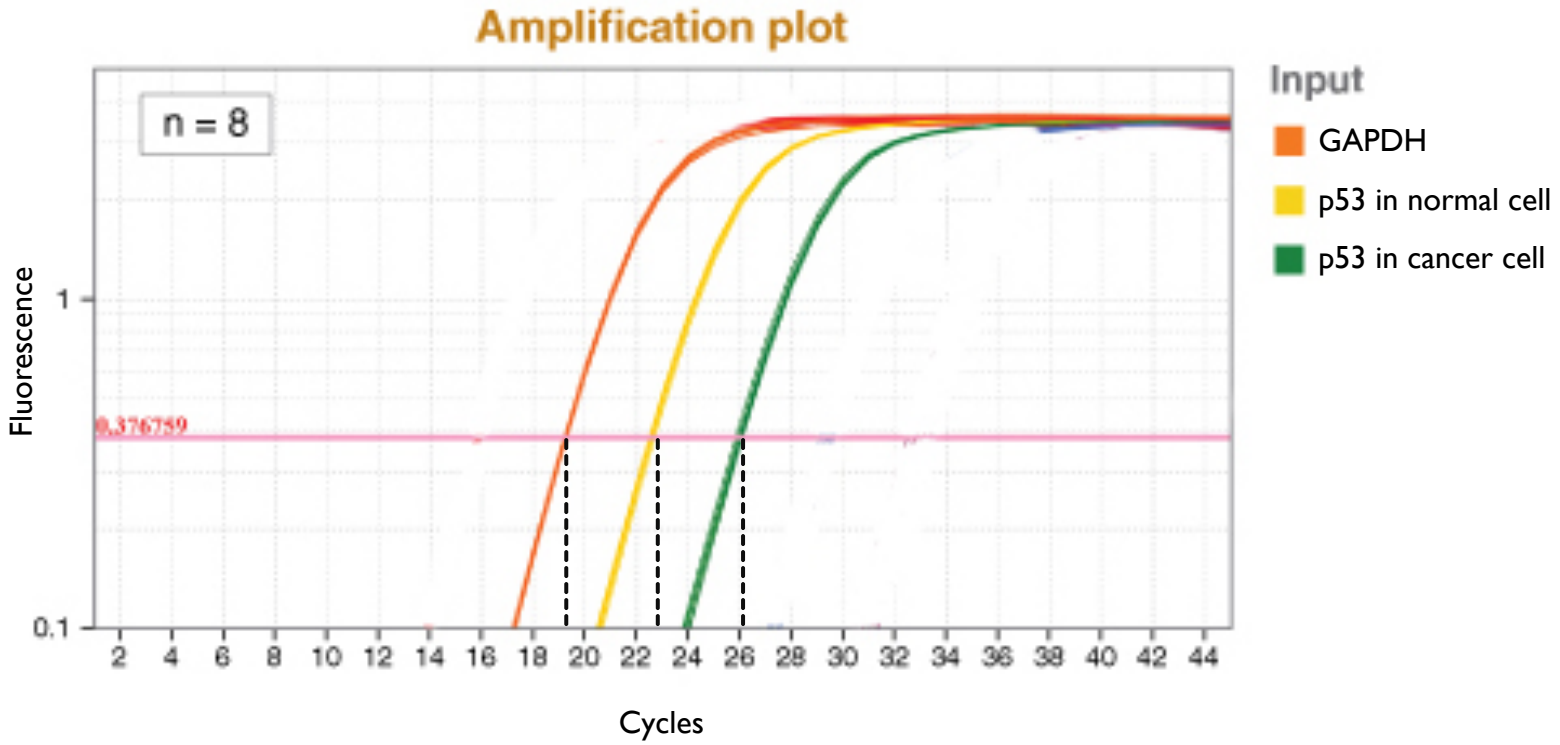
Each line represent <u>one patient</u>



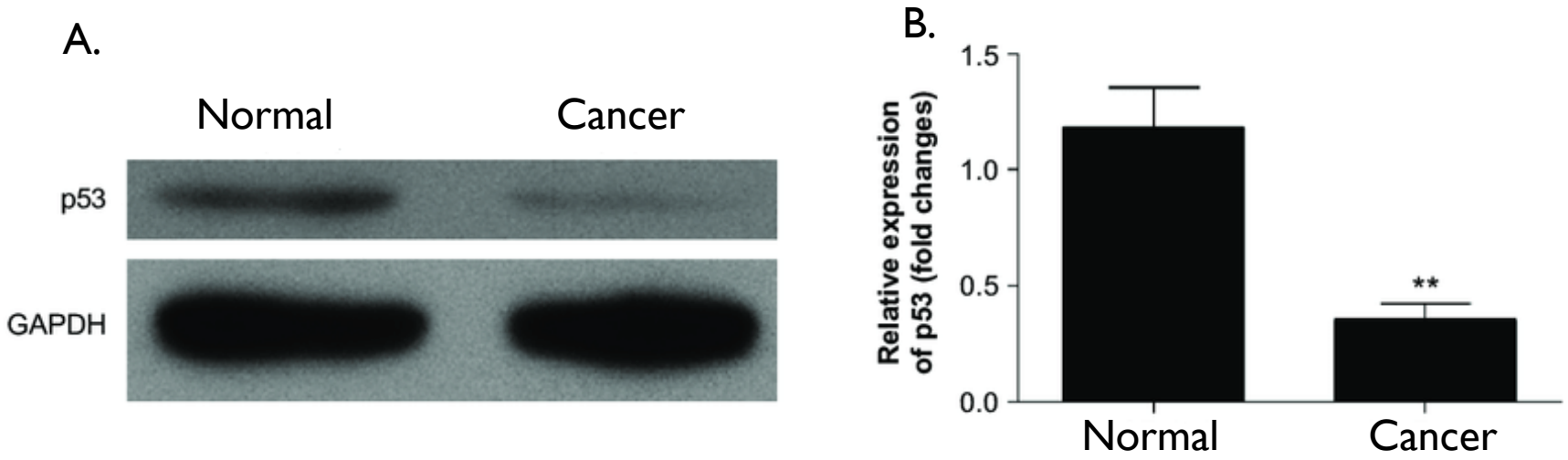
-Ve control for each master mix

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

Samples	Ct value	
	GAPDH	p53
Tumor 1	21.00	23.00
Tumor 2	20.50	22.00
Tumor 3	20.60	22.50
Normal 1	20.00	26.00
Normal 2	20.50	26.20
Normal 3	20.30	26.40



- Below is a RT-PCR and Western blot results, you can conclude that…..



HomeWork:

- By referring to real-time PCR handbook from applied biosystem (https://www.thermofisher.com/content/dam/LifeTech/global/Forms/PDF/real-time-pcr-handbook.pdf) , answer the following:

➔What is melting curve ?

➔How it is used to check the reaction specificity of SYBR green I ?