

*Lecture by:
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qPCR



Quantitative Polymerase Chain Reaction



INTRODUCTION

This section will discuss the foundations of quantitative PCR technology

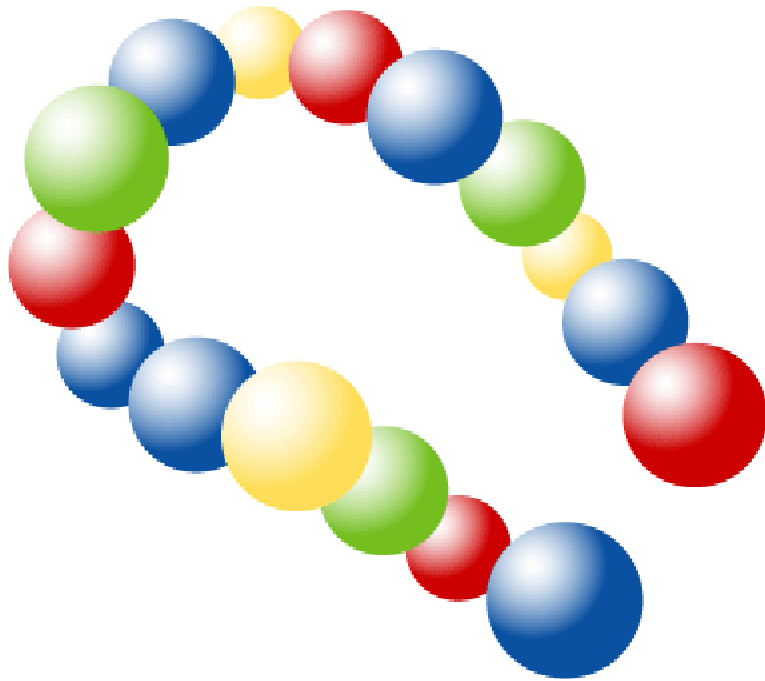
Quantitative PCR

- Commonly known as **Real Time PCR** or **Kinetic PCR**
- Based on polymerase chain reaction (**PCR**) principle
- **Amplify** and simultaneously **quantify** a targeted DNA molecule
- It enables both **detection** and **quantification** of a specific sequence in a DNA sample.
- The amplified DNA is **quantified** as it accumulates in the reaction in **real time** after each amplification cycle.
- Two common methods of quantification:
 - ▣ **fluorescent dyes** that **intercalate** with double-strand DNA
 - ▣ **modified DNA oligonucleotide probes** that fluoresce when hybridized with a complementary DNA.



http://www.gaininternational.com/PCR%20LABEL.100_0879CROP.jpg

Expression Quantification



<http://www.altabioscience.bham.ac.uk/services/oligo/beacons.shtml>

- Expression level of a gene can be estimated by some other method such as **northern blotting** or **microarray**
- Gene amplification by PCR → Detect gene expression at minute levels from single/small number of cells
- For **mRNA-based PCR** the RNA sample first needs to be **reverse transcribed** to cDNA via the enzyme, **reverse transcriptase**.
- The amplified product is measured at the end of each PCR cycle → using **Fluorophores**
- The data can be analyzed by computer software to calculate **relative gene expression** between several samples, or **mRNA copy number** based on a standard curve.

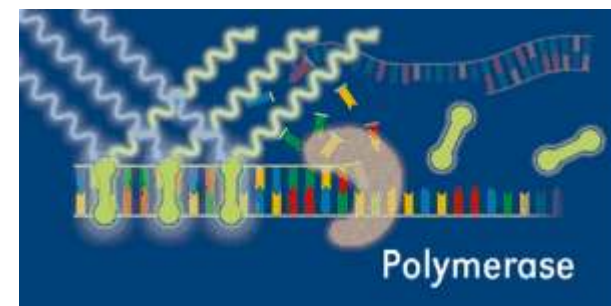
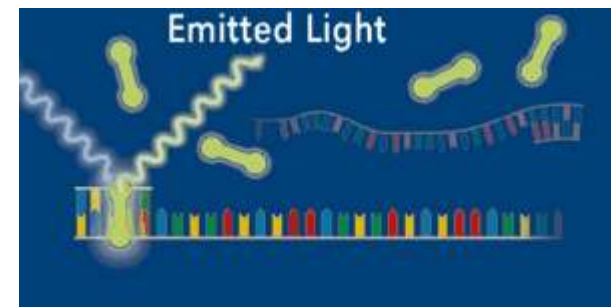
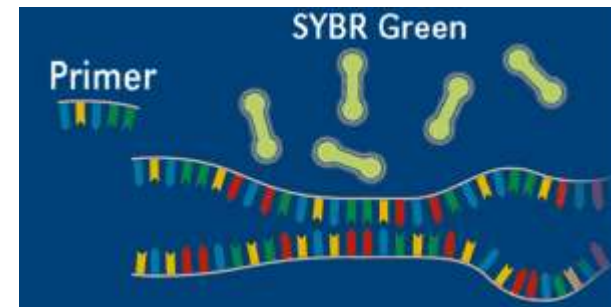
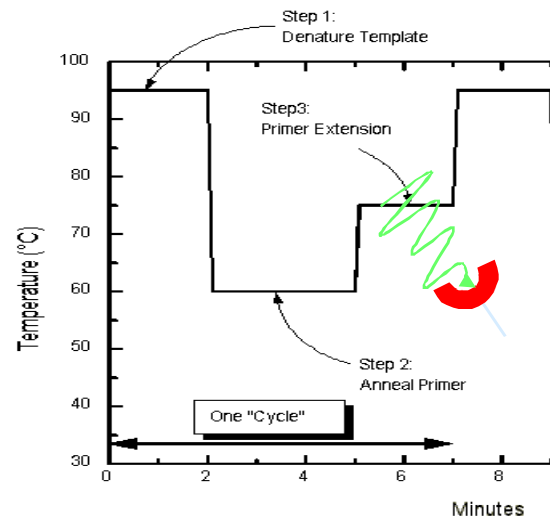
Detection method

How is DNA detected during PCR process?

Double-Stranded DNA Dyes

- Binds to all double-stranded (ds)DNA → **fluorescence** of the dye.
- An increase in DNA product → increase in fluorescence intensity
- Also binds to **nonspecific PCR products** (e.g. primer dimers).
- The reaction = PCR + fluorescent dsDNA dye.
- With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

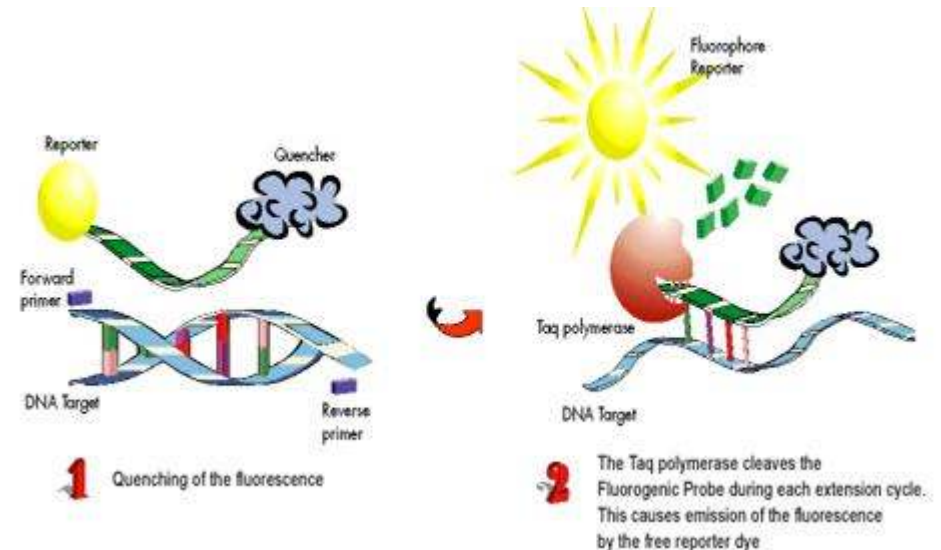
The fluorescence measurement is performed **during extension step**.



<http://www.gene-quantification.de/chemistry.html>

Fluorescent Reporter Probe

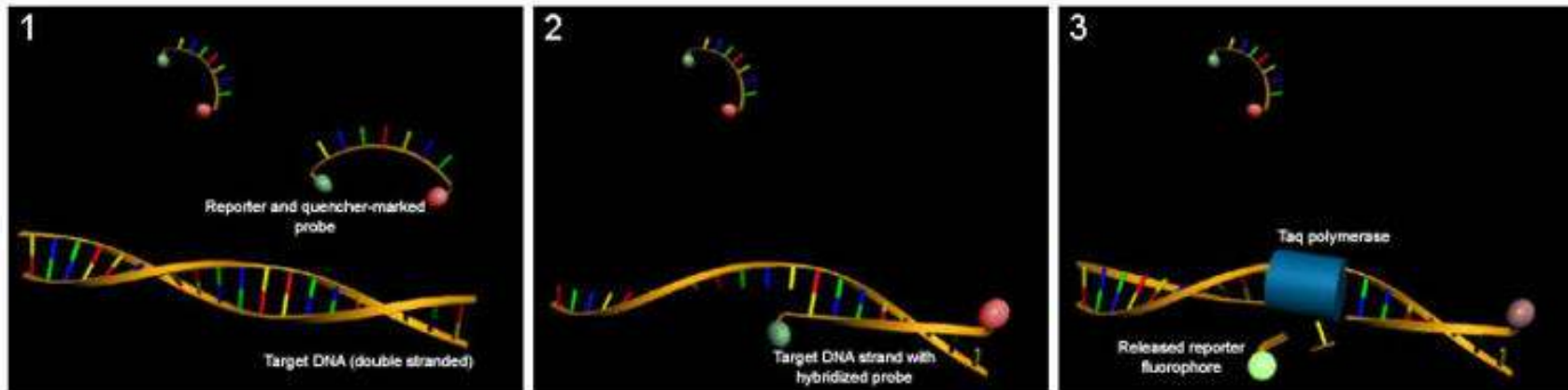
- The most accurate and most reliable (but also the most expensive)
- It uses a **sequence-specific** RNA or DNA-based probe to quantify only the DNA containing the probe sequence
- Significantly increases **specificity**, and allows quantification even in the presence of some **non-specific** DNA amplification.
- Allows for **multiplexing** by using specific probes with different-coloured labels



<http://www.shinegene.org.cn/image/image004.jpg>

Fluorescent Reporter Probe

Taqman Probe

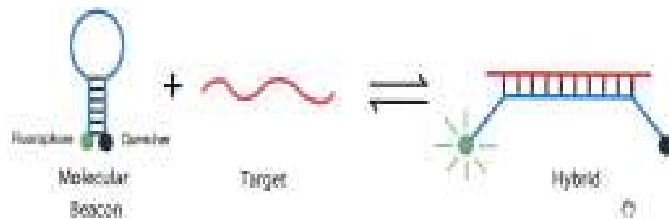


http://en.wikipedia.org/wiki/Image:TaqMan_Probes.jpg

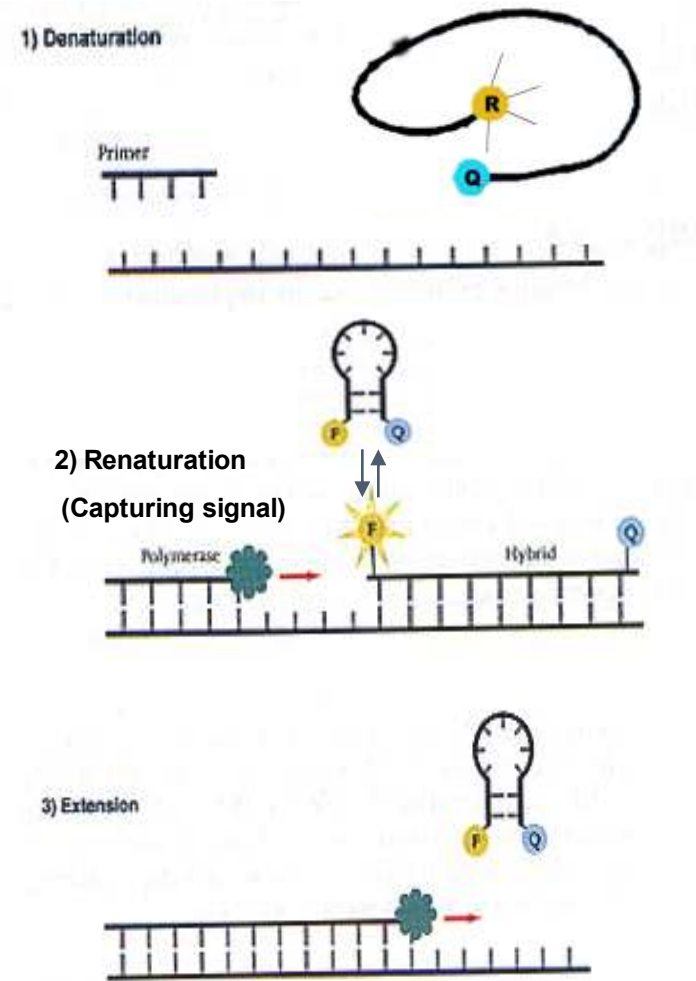
- TaqMan probes are **oligonucleotides** that have a fluorescent **reporter** dye attached to the 5' end and a **quencher** moiety coupled to the 3' end.
- These probes are designed to **hybridize** to an **internal region** of a PCR product.
- In the unhybridized state, the proximity of the Fluor and the quench molecules **prevents the detection** of fluorescent signal from the probe.
- During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'-nuclease activity of the polymerase **cleaves the probe**.
- This decouples the fluorescent and quenching dyes and **FRET no longer occurs**. Thus, fluorescence increases in each cycle, **proportional** to the amount of probe cleavage

Fluorescent Reporter Probe

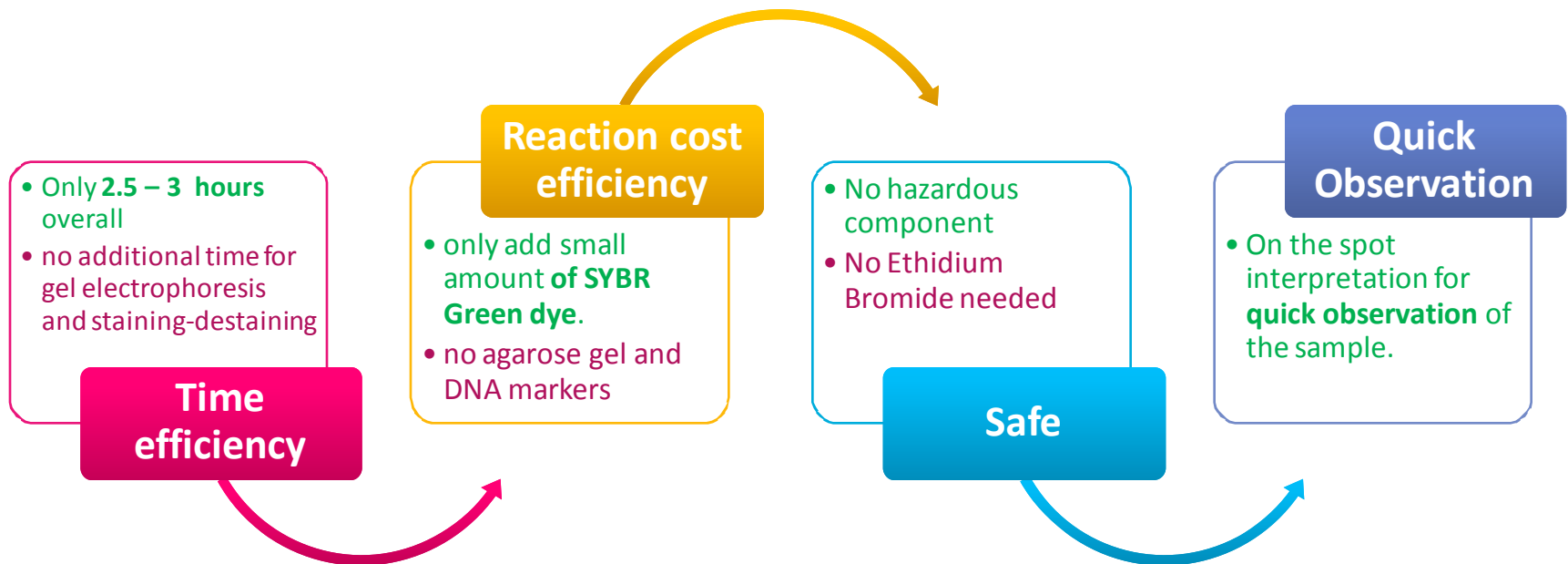
Molecular Beacons



- Designed to **remain intact** during the amplification reaction, and must **rebind to target** in every cycle for signal measurement.
- Form a **stem-loop structure** when free in solution. → prevents the probe from fluorescing.
- When a Molecular Beacon **hybridizes** to a target, the fluorescent dye and quencher are separated → the fluorescent dye emits light upon irradiation.
- Can be used for **multiplex assays** by using spectrally separated fluor/quench moieties on each probe.



The Benefit



Quantitation of Results

Two strategies are commonly employed to quantify the results obtained by real-time RT-PCR;

- ❖ the standard curve method
- ❖ the comparative threshold method

Standard Curve

First constructed from an **RNA of known concentration**

Used as a **reference standard** for extrapolating quantitative information for mRNA targets of unknown concentrations.

RNA stability can be a **source of variability**

It would involve the construction of cDNA plasmids → in vitro transcribed into the RNA standards → accurately quantitated
a time-consuming process.

The use of absolutely quantitated RNA standards will help **generate absolute copy number data.**

Standard Curve

Other nucleic acid samples can be used to construct the standard curve (e.g. **purified plasmid dsDNA**, **in vitro generated ssDNA** or any **cDNA** sample expressing the target gene)

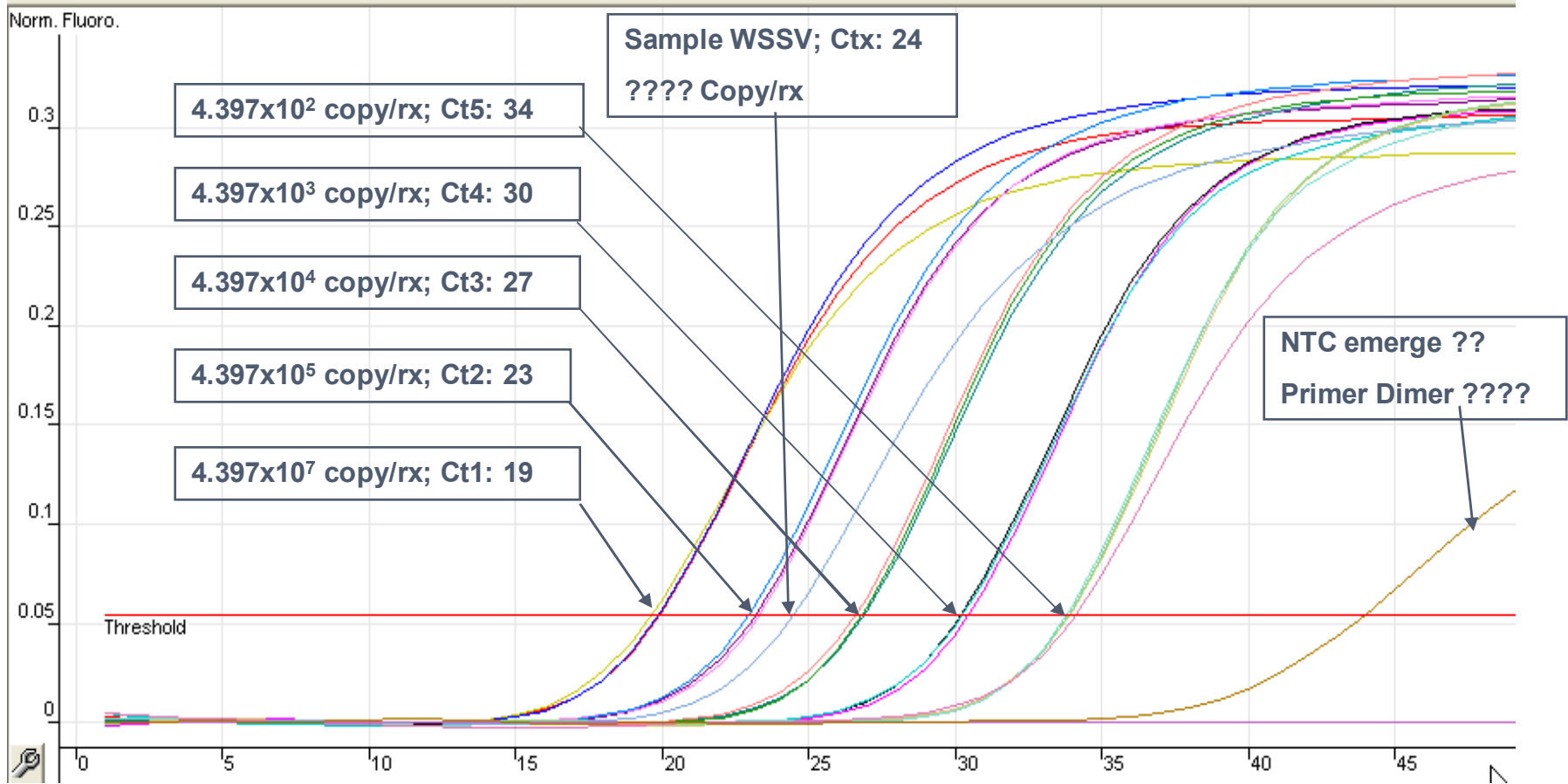
Spectrophotometric measurements at 260 nm can be used to assess the concentration of these DNAs, which can then be converted to a copy number value based on the molecular weight of the sample used.

cDNA plasmids are the preferred standards for standard curve quantitation.

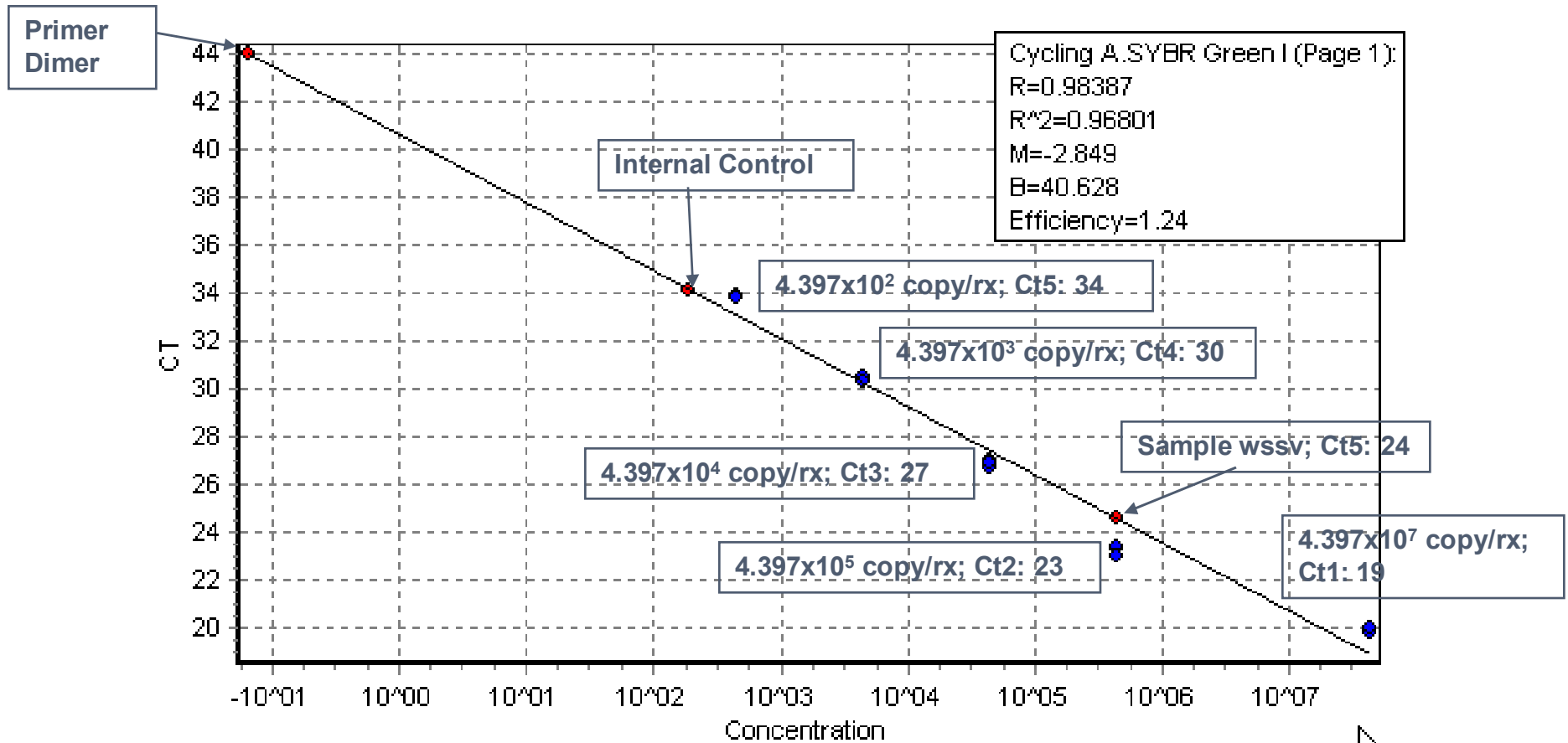
This method will only yield information on **relative changes in mRNA expression**.

This, and variation introduced due to variable RNA inputs, can be **corrected by normalization** to a housekeeping gene.

Amplification Curve

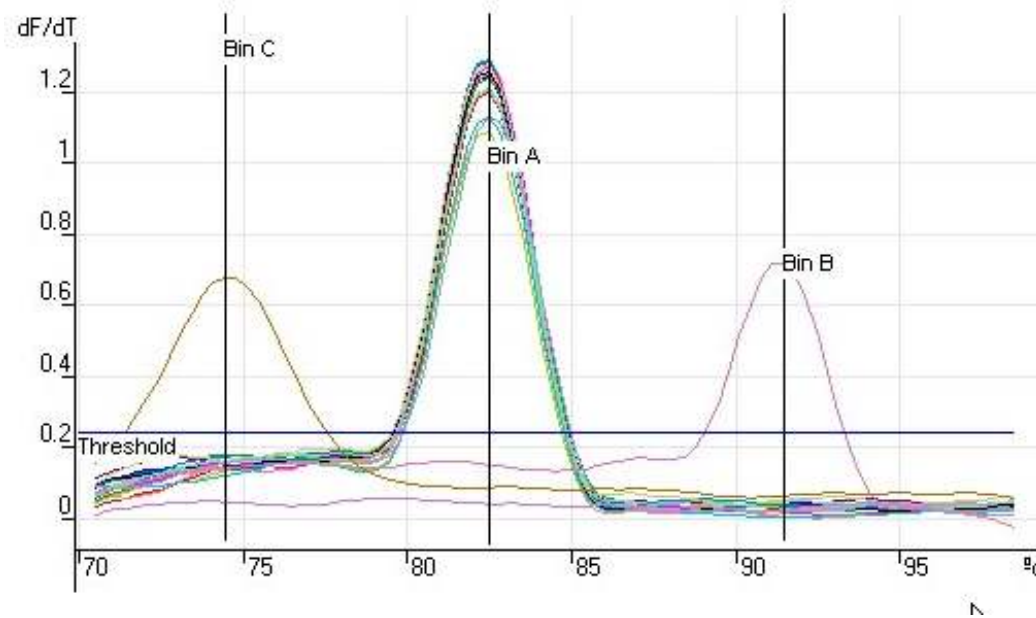


Standard Curve



Melt Curve

Melt data for Melt A.SYBR Green I



No.	Colour	Name	Peak 1
1	Red	STD_10 ⁷	82.5 (Bin A)
2	Yellow	STD_10 ⁷	82.5 (Bin A)
3	Blue	STD_10 ⁷	82.5 (Bin A)
4	Purple	STD_10 ⁵	82.5 (Bin A)
5	Pink	STD_10 ⁵	82.5 (Bin A)
6	Light Blue	STD_10 ⁵	82.5 (Bin A)
7	Teal	STD_10 ⁴	82.5 (Bin A)
8	Light Red	STD_10 ⁴	82.5 (Bin A)
9	Green	STD_10 ⁴	82.5 (Bin A)
10	Bright Pink	STD_10 ³	82.5 (Bin A)
11	Black	STD_10 ³	82.5 (Bin A)
12	Cyan	STD_10 ³	82.5 (Bin A)
13	Gold	STD_10 ²	82.5 (Bin A)
14	Light Green	STD_10 ²	82.5 (Bin A)
15	Light Cyan	STD_10 ²	82.5 (Bin A)
16	Blue	Sample A	82.5 (Bin A)
18	Purple	NTC	
19	Pink	Internal Control A	91.5 (Bin B)
21	Brown	NTC	74.5 (Bin C)

Amplicon

Primer dimer



Thank you