# Introduction To Real-Time Quantitative PCR (qPCR)

**SABiosciences, A QIAGEN Company**
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# The Seminar Topics

- Work flow & applications :RNA and DNA
- qPCR for gene expression: What is the change in gene expression during differentiation?
- Factors influencing the performance of a qPCR assay
- RNA purity and integrity
- Reverse Transcription
- qPCR, reporter chemistries and characteristics
assay
- Analyzing qPCR curves
- Data & analysis

# What does Real-Time qPCR Stands for?

- Real-time qPCR is a **sensitive and reliable** method for detection and quantification of nucleic acids (DNA,& RNA (cDNA) levels.
- It is based on **detection and quantification of fluorescence** emitted from a reporter molecule **at real time**.
- This detection occurs during the accumulation of the PCR product **with each cycle of amplification**, thus allows monitoring the **PCR reaction during early & exponential phase** where the first significant increase in the amount of PCR product correlates to the initial amount of target template.

# Applications for qPCR

**RNA**

- Gene Expression Profiling Analysis
- miRNA Expression Profiling Analysis

**DNA**

- SNP Genotyping & allelic discrimination
- Somatic Mutation Analysis
- Copy Number Detection/Variation Analysis
- Chromatin IP Quantification
- DNA Methylation Detection
- Pathogen Detection
- Viral Quantification

# Work Flow: A Brief Look

Samples

RNA (total, mRNA, small RNA)

DNA

Reverse transcription

cDNA

Sample quality control

SYBR® or Probe

Assay Design

Assay Optimization

Real Time PCR Set Up

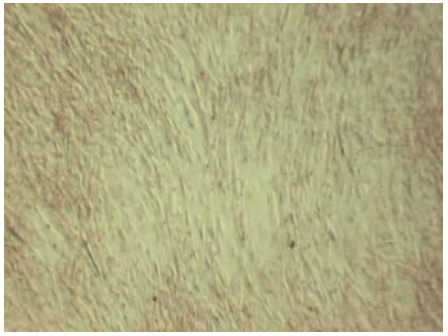
Instrument Set up & thermal cycling

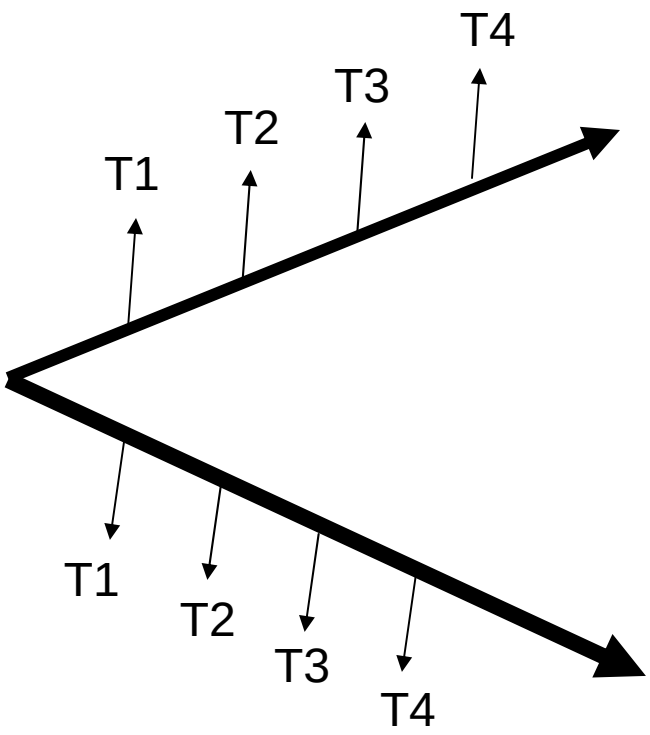
Data Output & Analysis

*

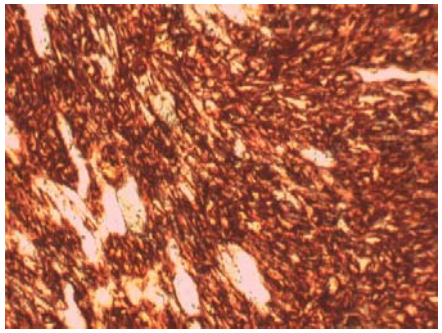
# Application example: gene expression changes during differentiation

**hMSC**






**Osteogenesis – Day 16**



**Neurogenesis – 72 hr**

Differentiation protocol
Collect Total RNA at different time points
Measure 1 HKG and 1 GOI (TNFα)
Repeat experiment 3x (biological replicates)

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DNA

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cDNA

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Data Output & Analysis

# Factors Critical For A Successful Assay

- **DNA or RNA sample preparation --- Template quality**

  - Choose appropriate sample preparation kits/reagents (inhibitors can compromise RT or PCR Reaction

- **Reverse transcription for converting RNA to cDNA**

  - Choose RT kits (type of RT, which type of primers, controls?)

- **Assay design: chemistry, specificity, PCR efficiency, & throughput & cost**

  - Choose validated assay, or need to validate our own?

- **Running PCR**

  - Choose commercial mastermix or make own (primer, probe, master mix)

- **Data analysis tool**

  - User friendly & streamlined data analysis module

# RNA Isolation

- **RNA Isolation:**
  - **Qiazol?**
  - **Column based method (RNeasy?)**
  - **Both: Efficient lysis and inhibition of RNases; molecular grade RNA**
  - **miRNA? Use a kit specific for miRNA and mRNA**

RNeasy Lipid tissue mini Kit

Qiazol:
phenol/guanidine-based lysis

Instant inactivation of RNases
Instant end of biological activities

Column cleanup:
Molecular biology grade RNA

# RNA Sample Quality

Spectroscopic: measure 260/280 and 230/280

OD260 is used to calculate amount of nucleic acid
260/280 ratio (typical minimum value 1.8)
260/230 ratio (typical minimum value 1.7)

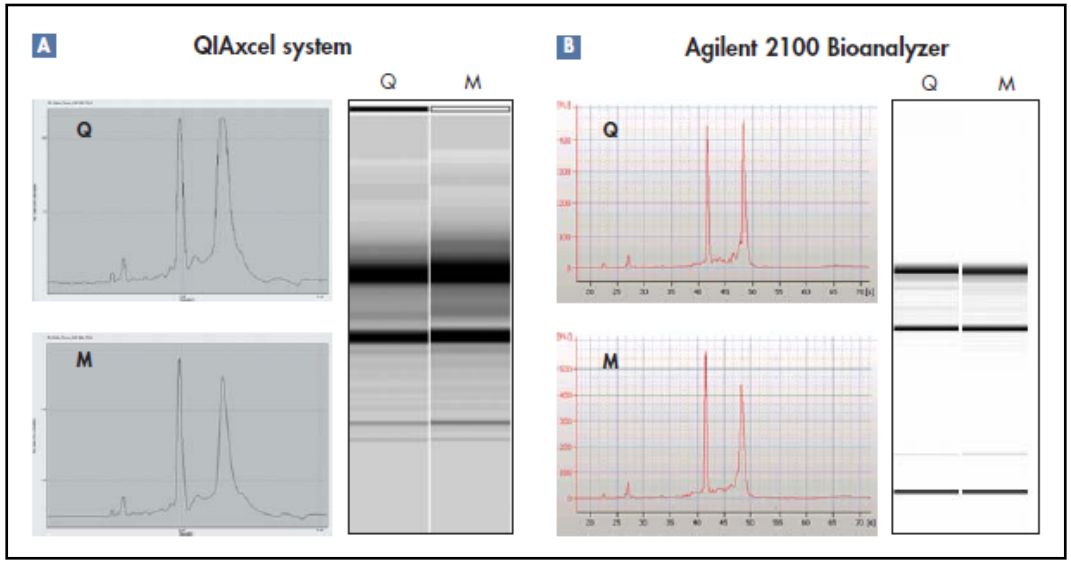
Low ratio may indicate protein, QIAzol, Carbohydrates, Guanidine HCL,
Absorbance measurements do not show integrity of RNA

Denaturing RNA Agarose Gel

Used to detect integrity of RNA (usually through ribosomal bands)

QIAxcel

Automate
RNA integrity
analysis



# qPCR Components & Steps: Overview

## A. Templates

10 – 1000 copies of nucleic acids
100 pg to 1 μg RNA

↓↑

Reverse Transcription

↓↑

Two-step qPCR: (1) RT (2) qPCR

One-step qPCR: one tube reaction

## B. Primers/Probes

## C. Master Mix

DNA Polymerase
Mg++
dNTP
Buffer
*Passive reference dye

Denaturation → Annealing → Extension

Denaturation → Annealing/Extension

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Denaturation → Annealing → Extension

Denaturation → Annealing/Extension

# Reverse Transcription

Used to make cDNA copy of RNA

Reagents:

- Reverse transcriptase – many different kinds
- dNTPs
- Buffers for RT
- Primers
  - Random pentamers or hexamers?
  - Oligo-dT?
  - Both?

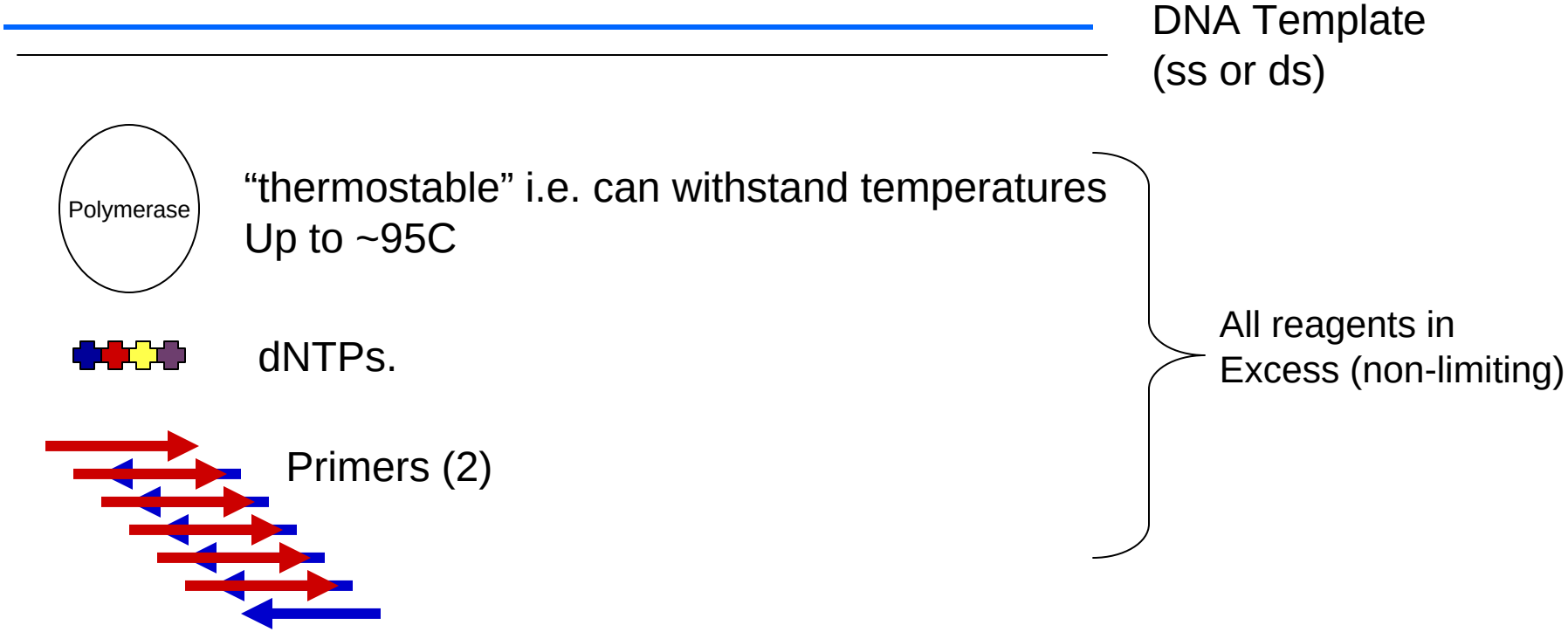
- Control RNA to monitor reverse transcription kit?

Note:

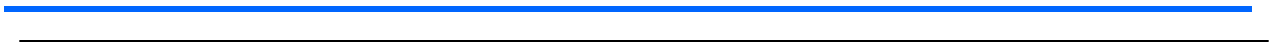
- Make sure that RT reaction is linear
- Do not try to reverse transcript too much RNA
- Sensitivity of qPCR step is dependent on good RT reaction
- Monitor RT reaction to ensure equal RT efficiency across all samples

# What is in a PCR Reaction?

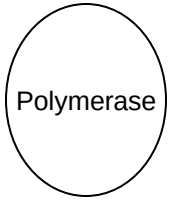
**PCR**= **P**olymerase **C**hain **R**eaction
Exponential Amplification of DNA in single tube

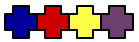


# PCR Reaction in Action

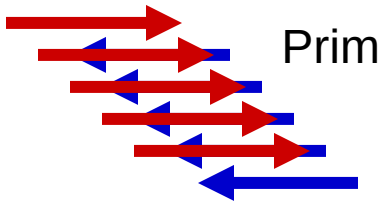


DNA Template
(ss or ds)



dNTPs.



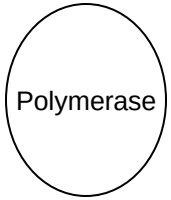
Primers (2)

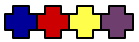
1. Heat denature template (~95C)
2. Anneal Primer (~60C)
3. Extend primer (~60C)
4. Repeat (~95C)

# PCR Reaction in Action

Heat denature

DNA Template
(ss or ds)



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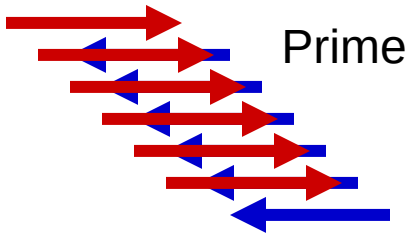

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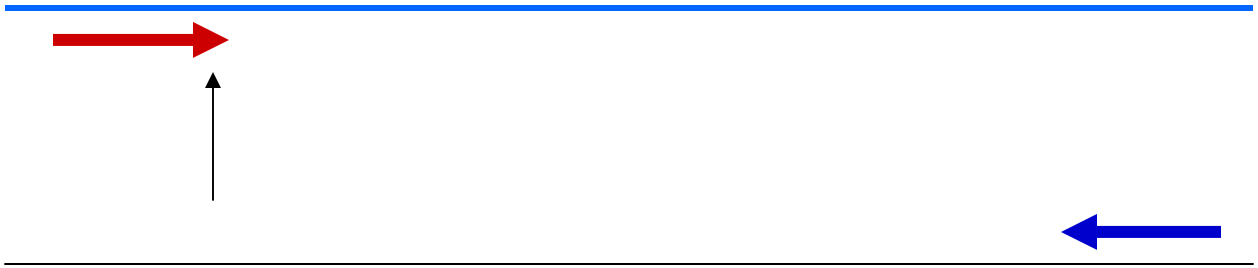

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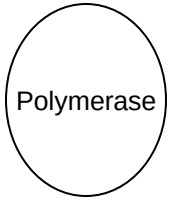


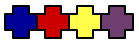

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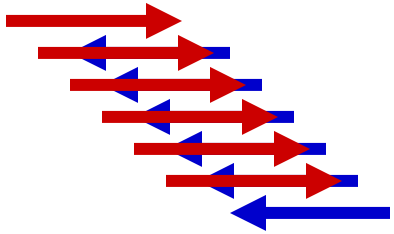

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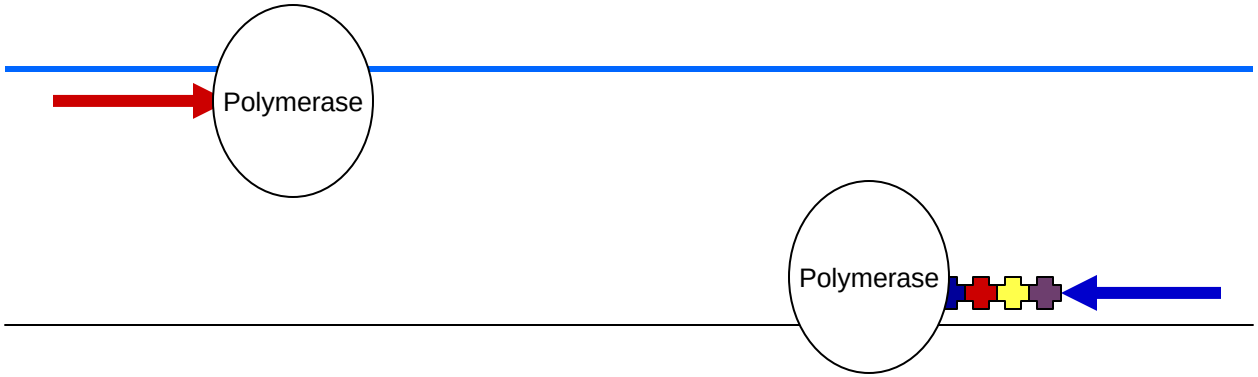


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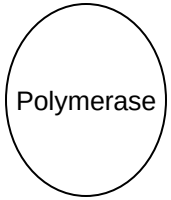


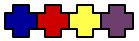

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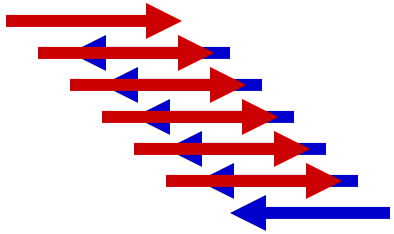

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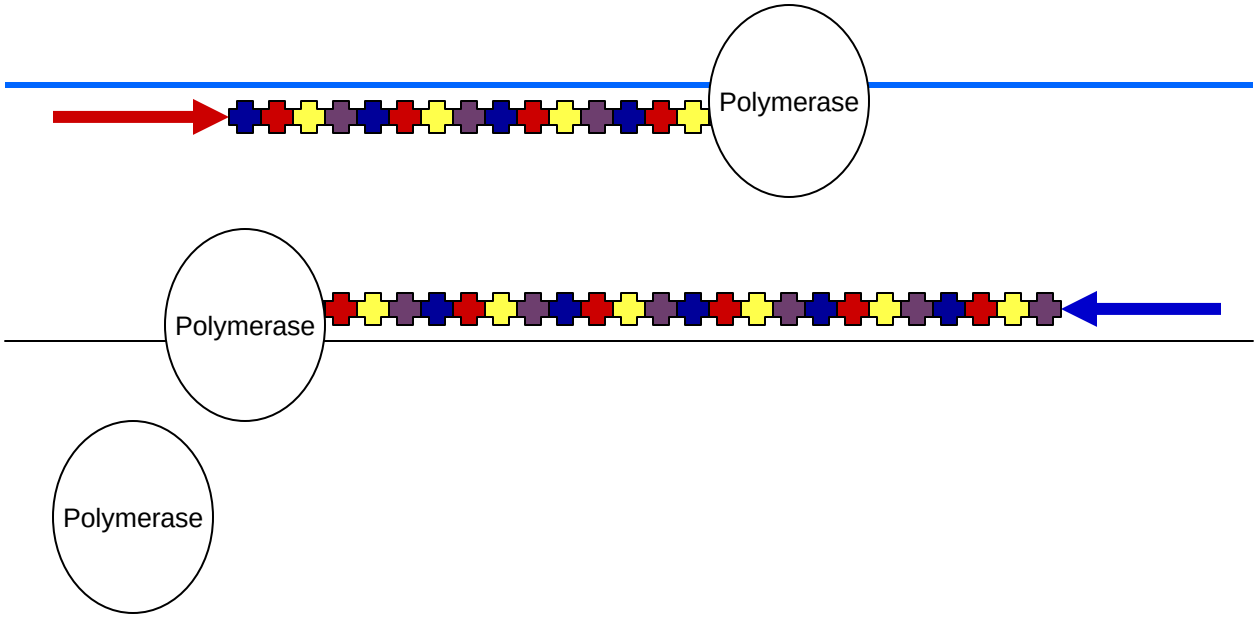


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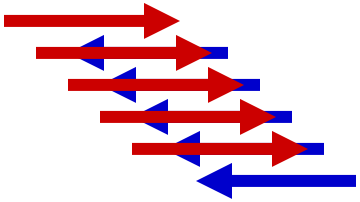

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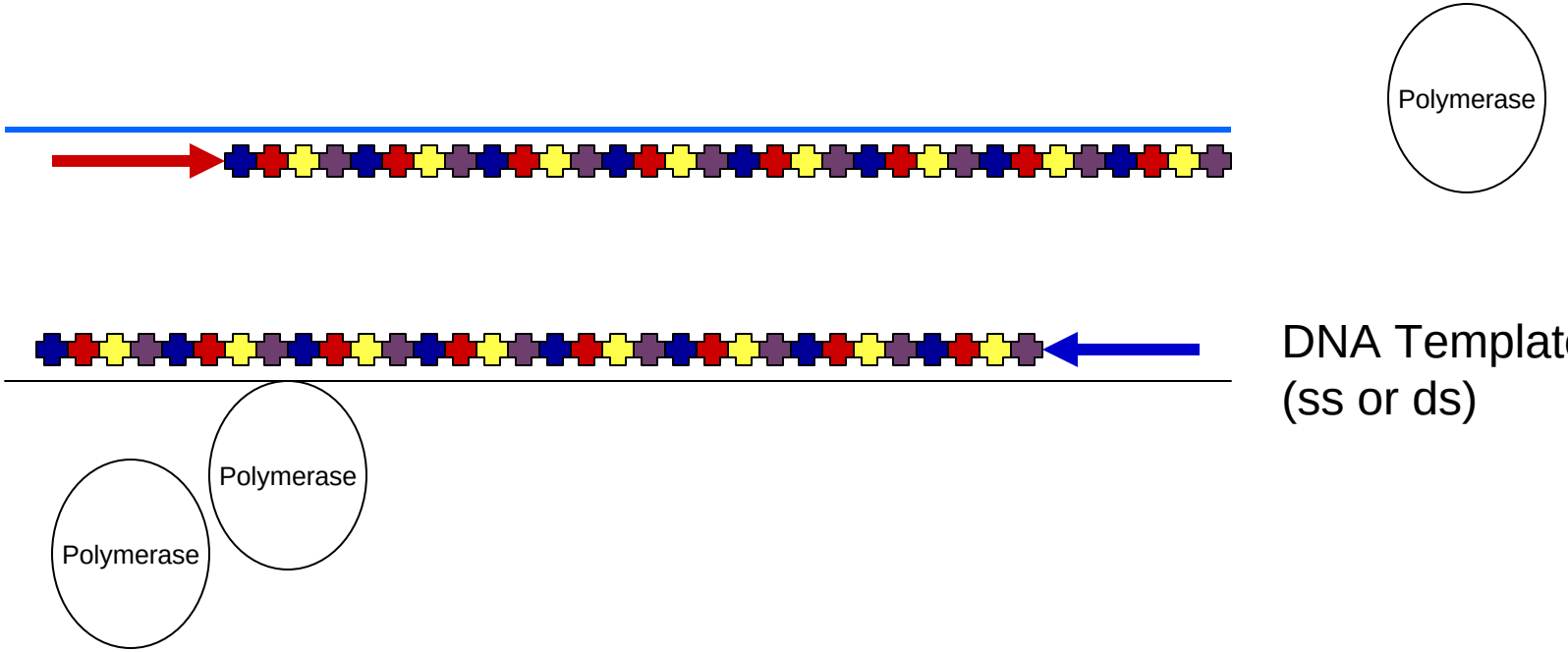


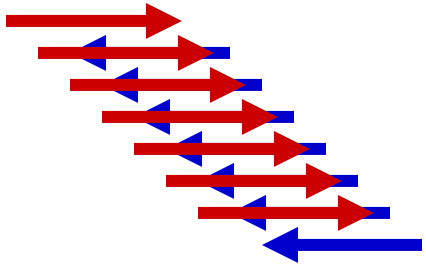

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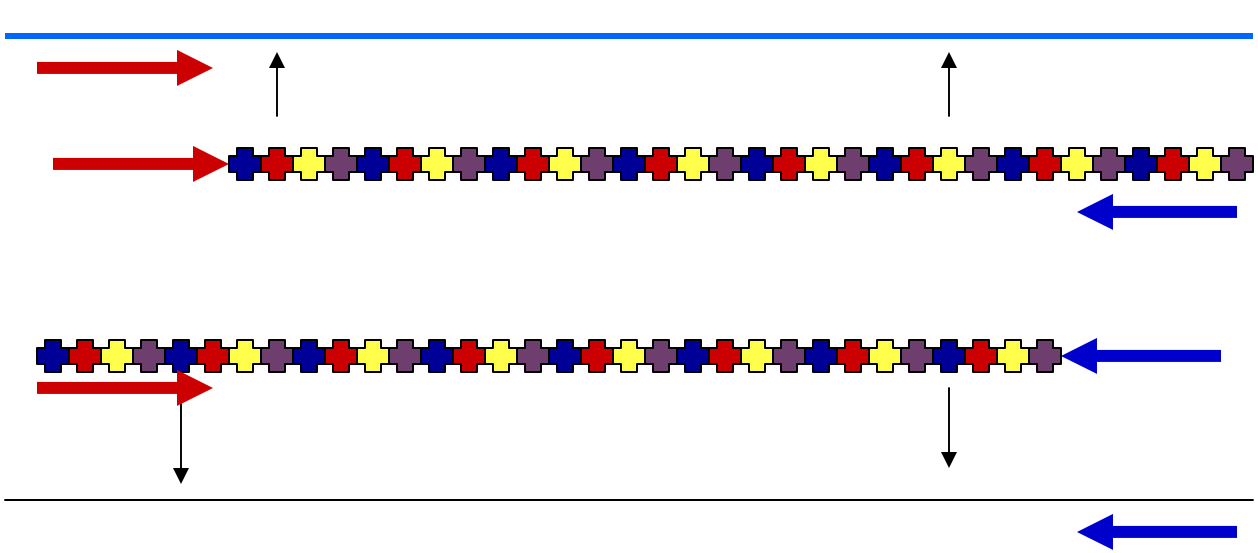


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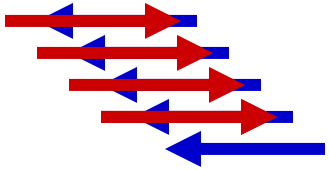

1. Heat denature template (~95C)
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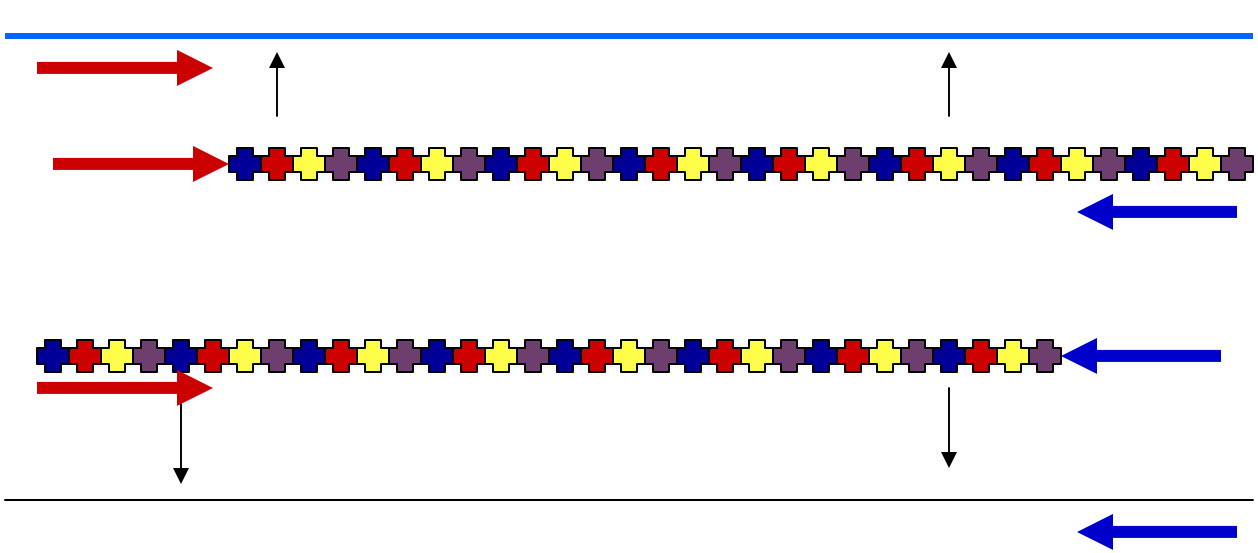
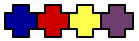
3. Extend primer (~50 to ~70C)
4. Repeat (~95C)

# qPCR Reaction: Measure DNA amount at end of each cycle to get ratio of DNA or absolute amount (if using a standard)



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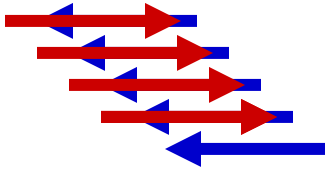

1. Heat denature template (~95C)
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3. Extend primer (~50 to ~70C)
4. Measure Amount of PCR Product
5. Repeat (~95C)

# Real-Time qPCR Fluorescence Chemistry

- DNA binding agents

SYBR® I Dye,

- Hydrolysis Probes

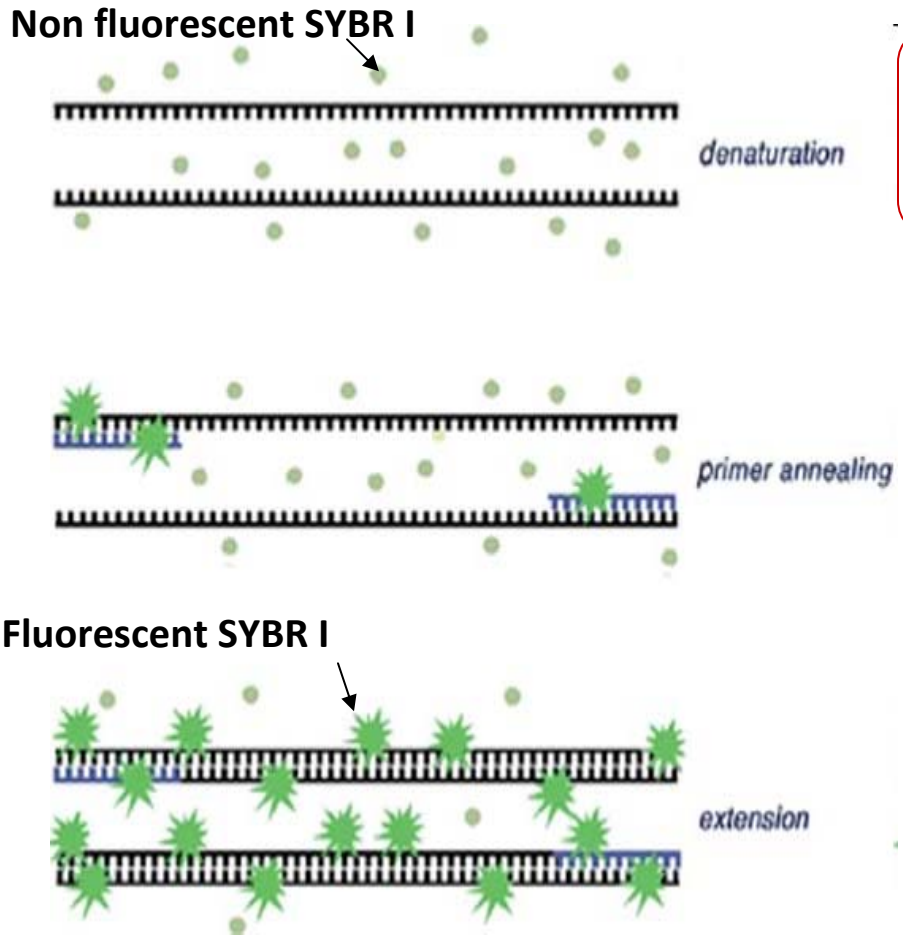
Dual-labeled Hydrolysis (Taqman®) probe

Two most commonly used chemistries in qPCR community

Others, such as hybridization probes

-Molecular beacon and scorpion probes

# SYBR® Green I Assay: Fluorescent DNA Binding Dye



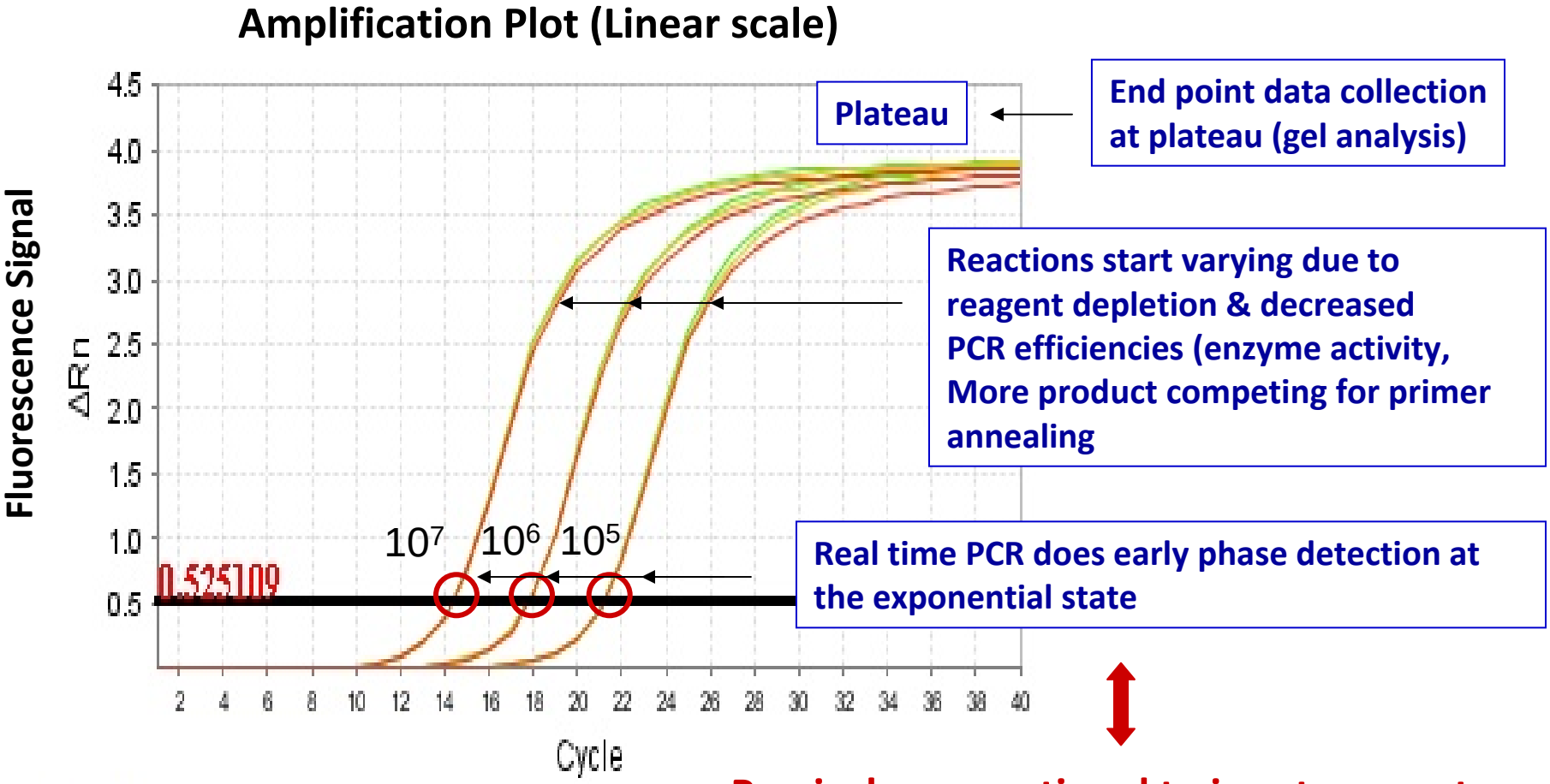

**SYBR I binds to double-strand DNA but not single strand DNA. Little fluorescence emitted from SYBR I in solution.**

**SYBR I upon binding to double-strand DNA emits fluorescence very brightly**
**Simple & cost saving**

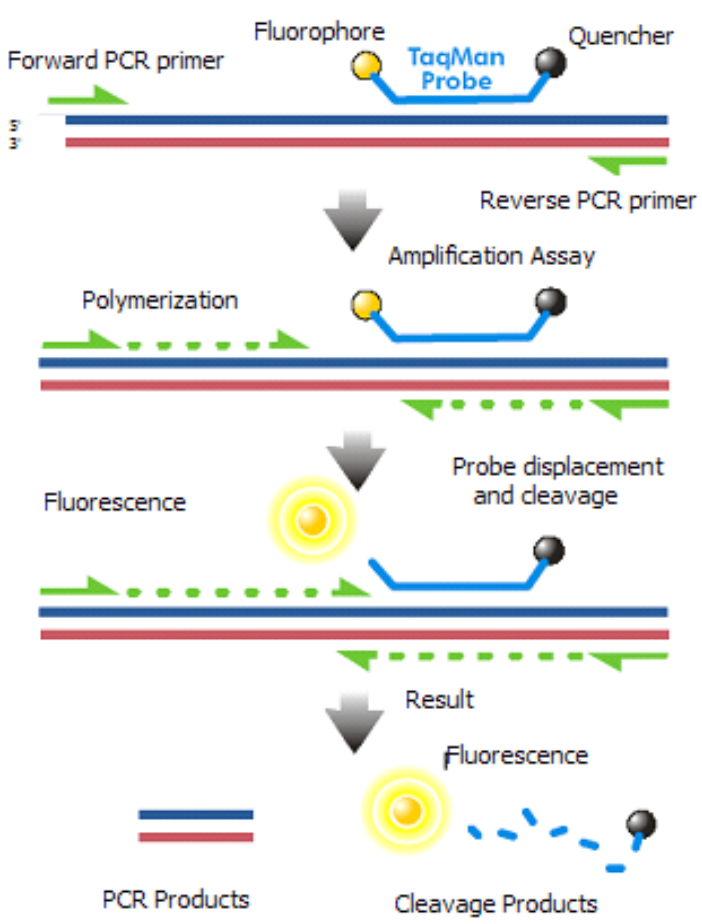
The SYBR I signal intensities correlate with DNA amplified (amplicon amount) thus the initial **sample** input amounts

**High Specificity Is Required when using SYBR Green since SYBR I binds all double-strand DNA (non-specific or primer dimmer).**

# Understanding Kinetics in PCR



# Hydrolysis Based Probe - - - Taqman® Probe Assay



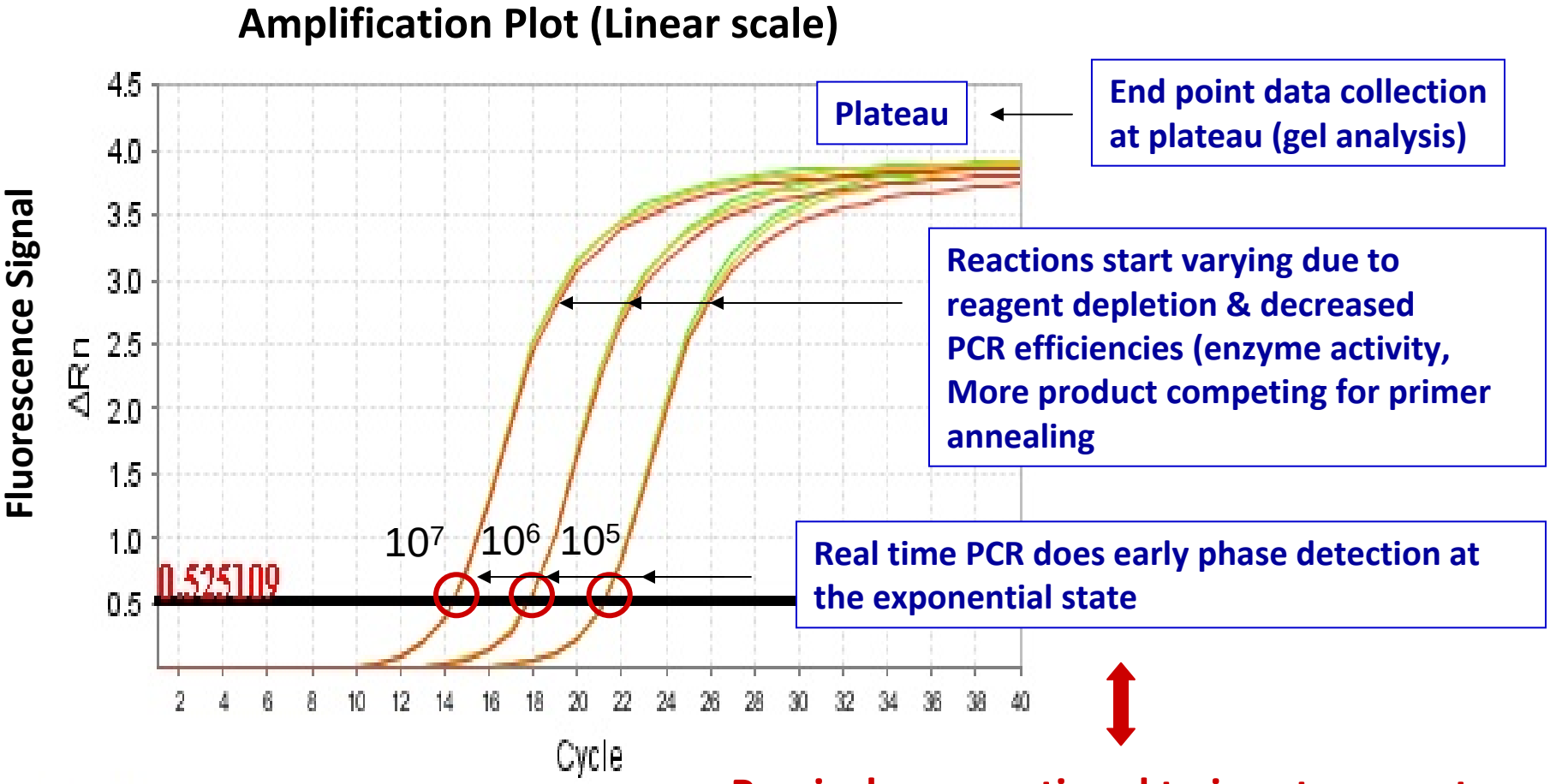

**The fluorescence of the reporter dye is suppressed by the quencher**

**Primer binding followed by extension**

**Probe cleavage by Taq to free the reporter dye thus the fluorescence intensity correlates with the initial sample input amounts.**
**Taq has 5'→ 3' exonuclease activity**

**Each amplicon needs a sequence-specific probe (cost & time)**

# Understanding Kinetics in PCR



# Characteristics of a good qPCR Assay

Amplification efficiency: 100% during exponential phase

Sensitivity: Able to detect down to reasonable quantities of template in 1 reaction (10-50 copies)

Specificity: 1 assay, 1 target: (no off-target amplification or primer-dimers)

# Amplification Analysis: standard curve and single curve analysis

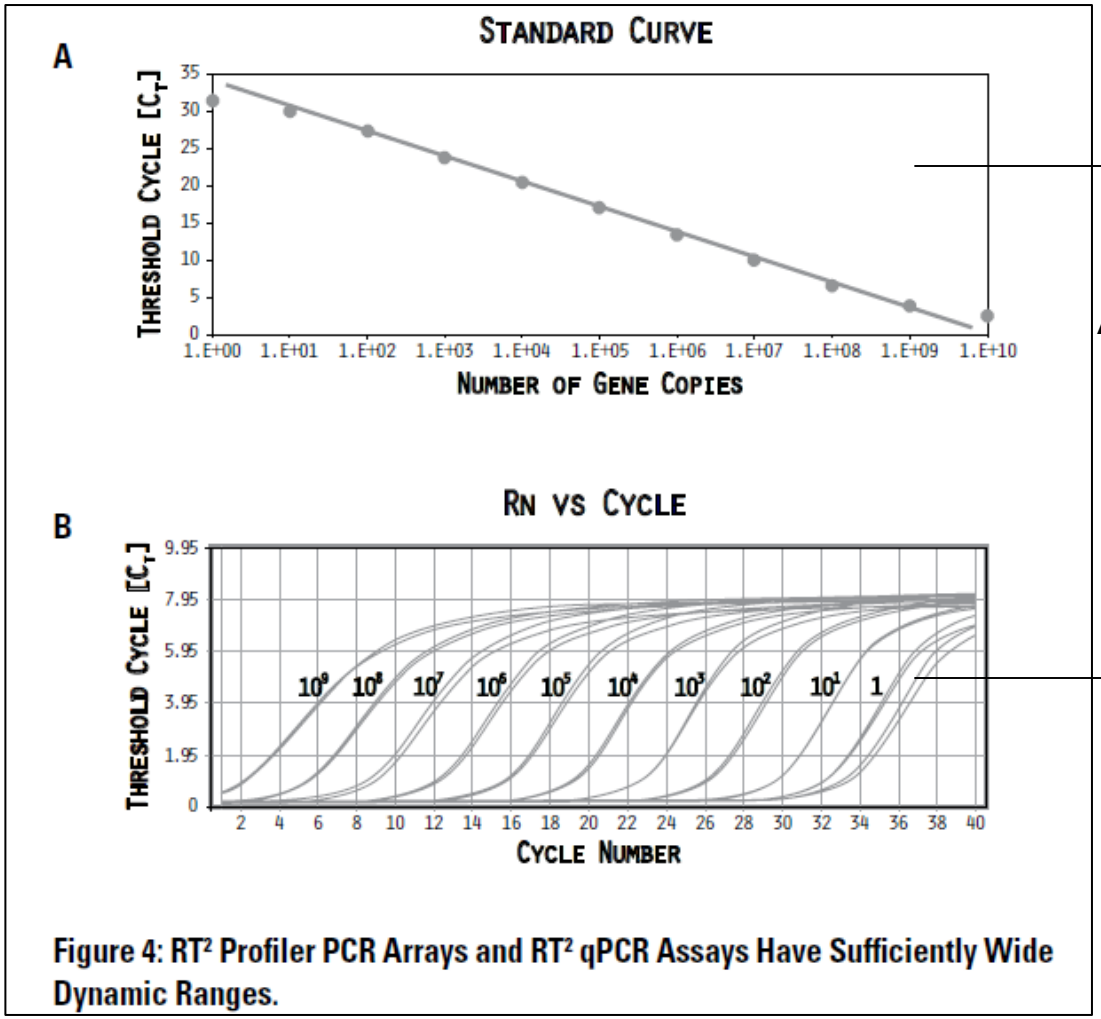



**Figure 4: RT² Profiler PCR Arrays and RT² qPCR Assays Have Sufficiently Wide Dynamic Ranges.**

Plot:
x axis dilution
Y axis Ct value

Amp efficiency = $10^{(-1/\text{slope})}$ -1 *100

Single curve analysis

PCR Miner
http://miner.ewindup.info/version2

"DART"
www.gene-quantification.de/DART_PCR_version_1.0.xls

# Sensitivity: How many copies can my assay detect?

Sensitivity is very important for low expressed genes or where there is limited sample

Method 1: Use primers to make PCR product, T/A clone, grow-up, isolate, quantitate and use for qPCR reactions

Method 2: Use gDNA as template and use mass of gDNA to calculate copy number and assume 1 target per genome (or actually calculate targets using bioinformatics)

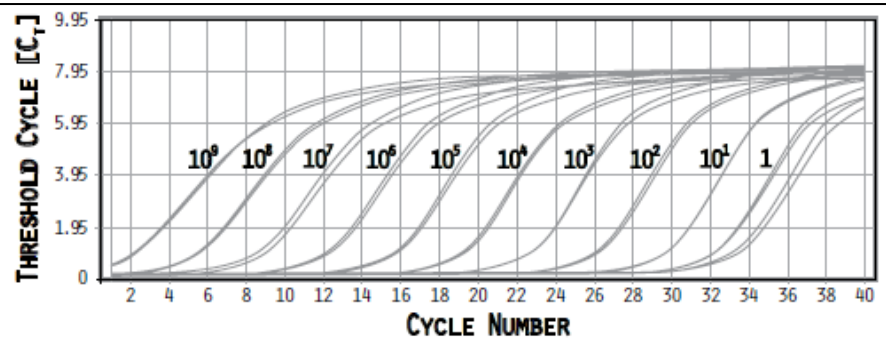
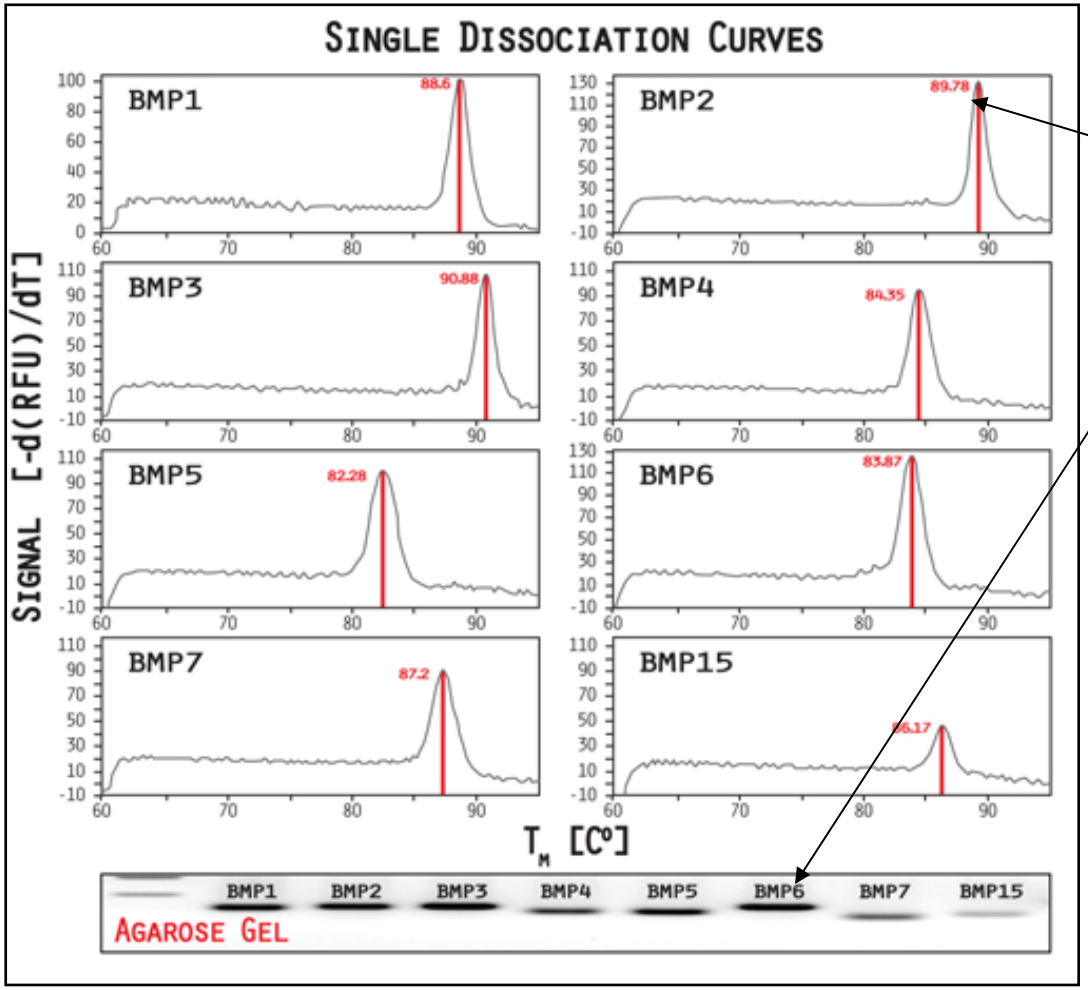



**Figure 4: RT² Profiler PCR Arrays and RT² qPCR Assays Have Sufficiently Wide Dynamic Ranges.**

# Specificity: SYBR Green




Single peak dissociation curves

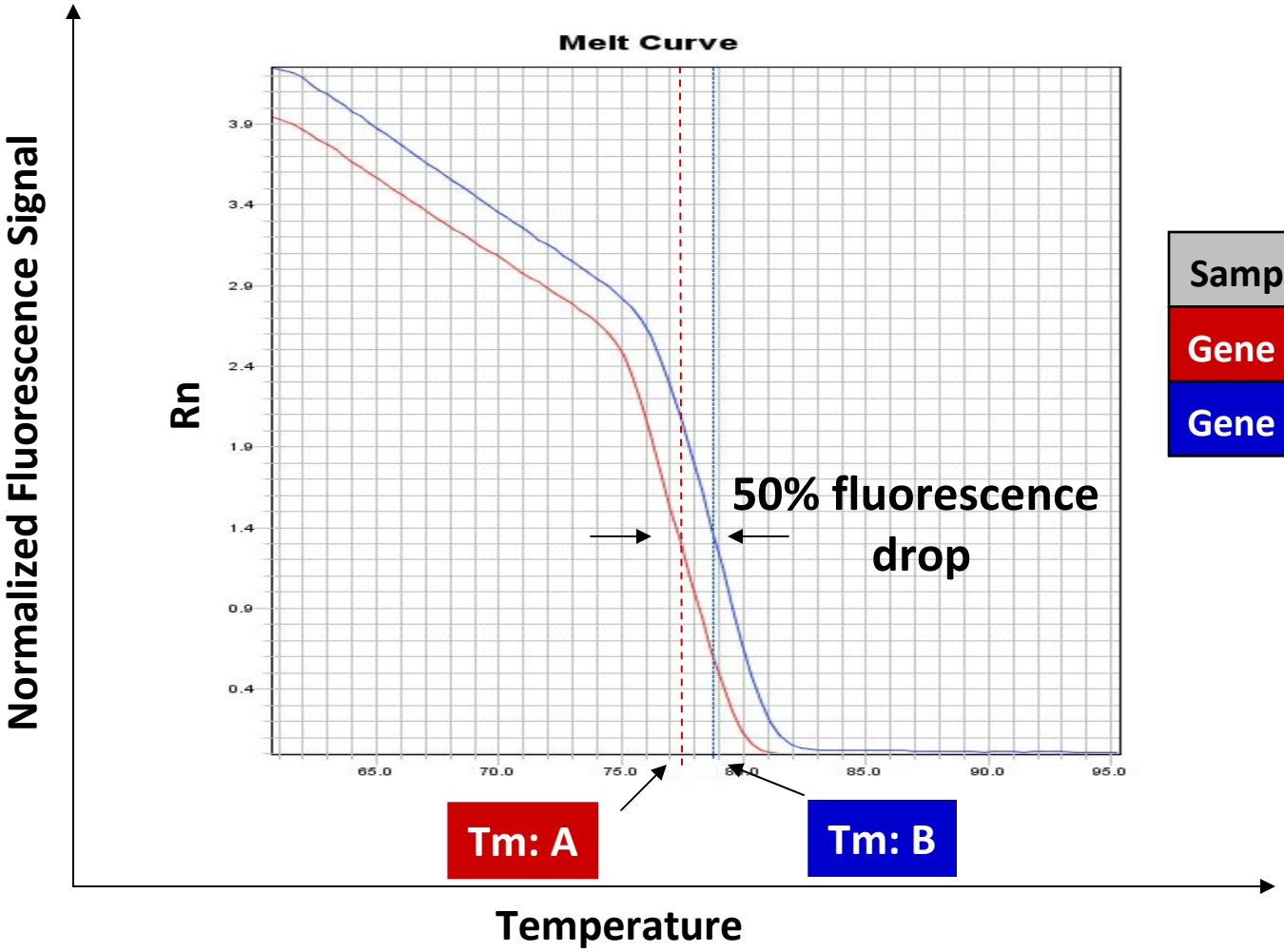
Single gel bands of predicted size

# Melt Curve Analysis: The General Program Steps

- Rapid heating of amplified samples to 94°C to denature the DNA
- Cooling the sample to 60°C to let DNA double strands anneal
- Slowly heating (by increasing the temperature, usually 0.2°C/sec) the sample while plotting the fluorescent signal versus temperature.
- As the temperature increases, and DNA melts, the fluorescent signal should decrease.
- There will be a significant drop of the signal when 50% DNA melts.

# Melting Curve Analysis --- Normalized Reporter Plot

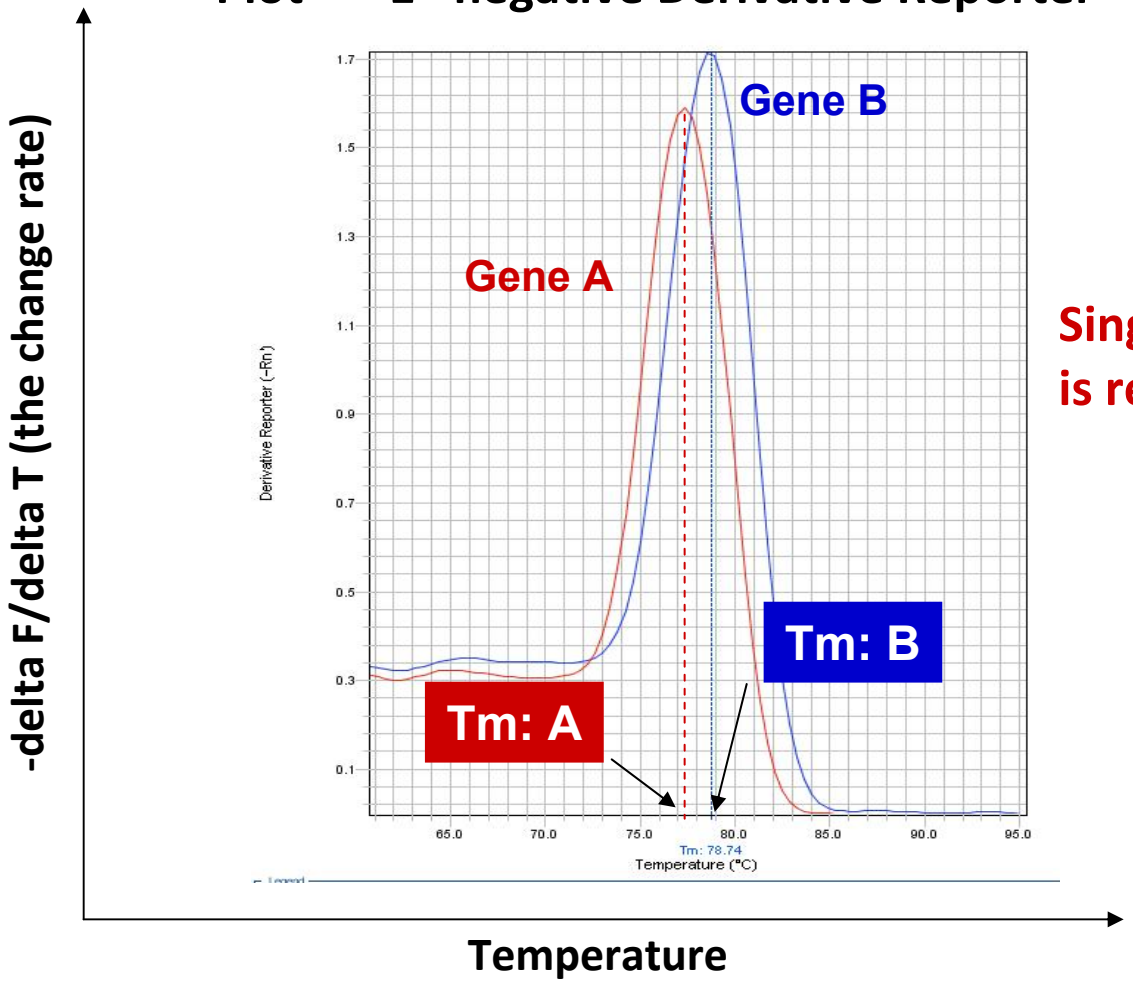
## Plot - Normalized Reporter (Fluorescence/Passive dye signal)




| Samples | Tm |
|---|---|
| Gene A | 77.36 |
| Gene B | 78.94 |

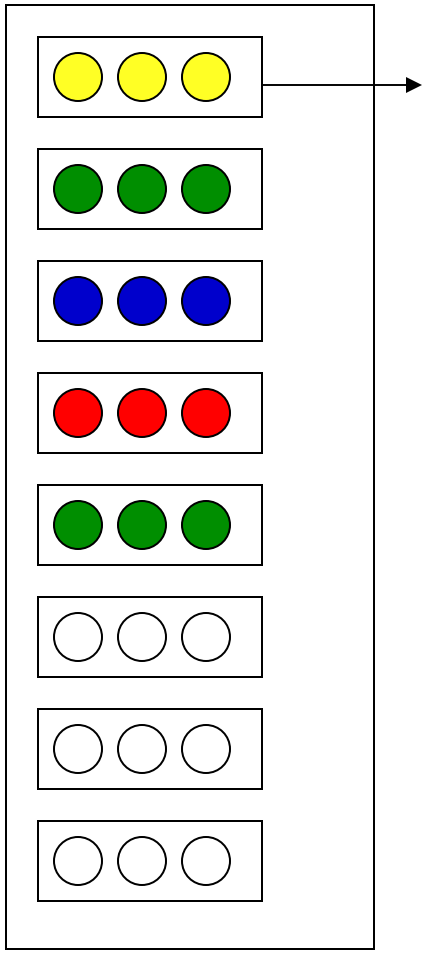
# Melt Curve Analysis --- 1st Negative Derivative Plot

**Plot - - -1st negative Derivative Reporter**



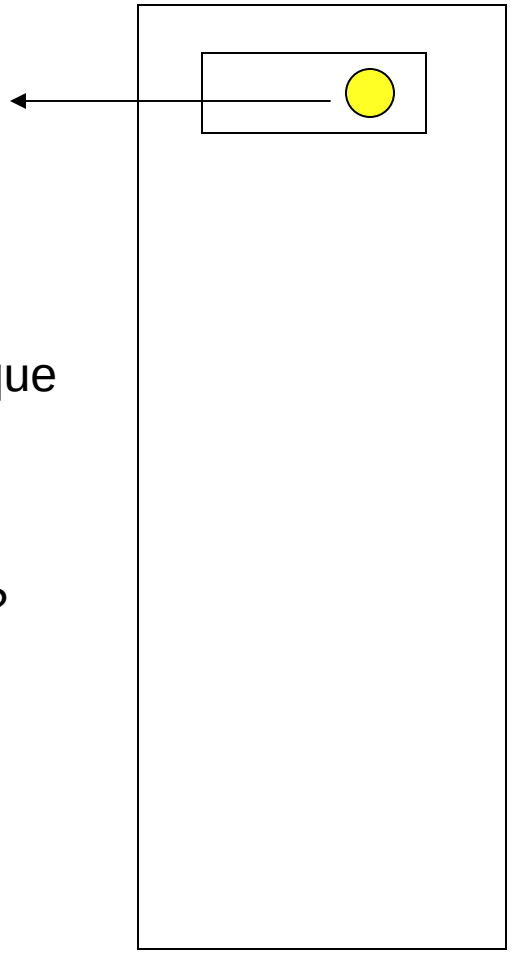

**Single melt curve of each amplicon is required for specificity validation!**

# Experimental Setup



Gene 1
sample 1
(n=3)

Gene 1
sample 1
(n=1)



Do I need technical replicates?
Technical = variation of technique
-machine dependent
-pipetting accuracy

Do I need biological replicates?
-biological variation

# Biological replicates are better than technical replicates

- Biological Replicates:  3 different experiments
  - Shows variability due to experiment

- Technical replicates: 3 different measurements for same step
  - Shows variability due to pipetting, machine, enzymes, etc.

Sacrifice Technical replicates for biological replicates, always do at least 3 to

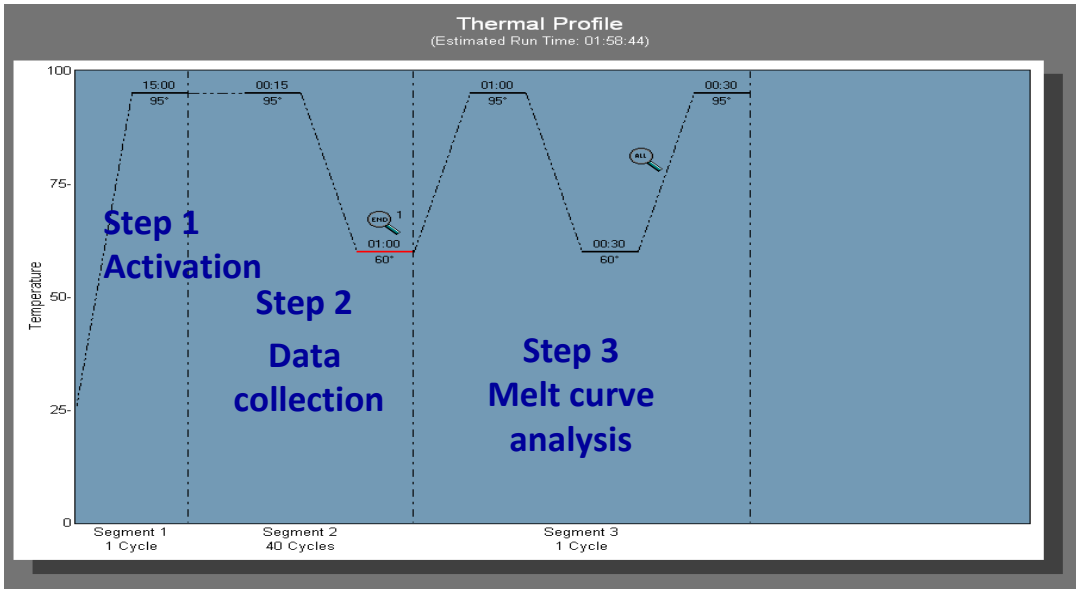
Get fold change and p value (or other statistics such as 95% confidence interval)

# Thermal Cycling Programs

**Table 4. Cycling conditions* for Applied Biosystems, Bio-Rad,† Stratagene, and Eppendorf‡ cyclers**

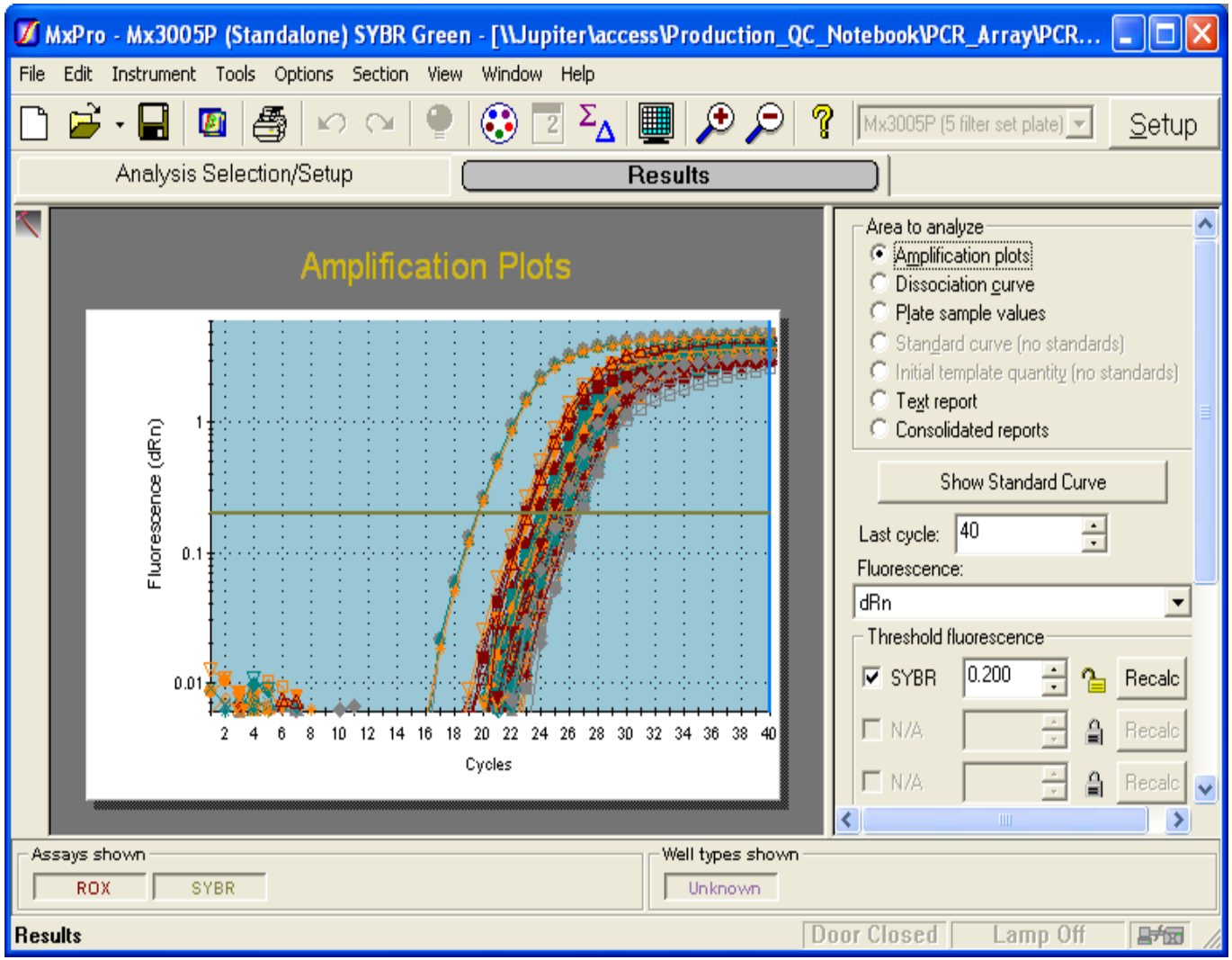
| Cycles | Duration | Temperature | Comments |
|---|---|---|---|
| 1 | 10 min | 95°C | HotStart DNA *Taq* Polymerase is activated by this heating step. |
| 40 | 15 s | 95°C | |
| | 1 min | 60°C | Perform fluorescence data collection. |

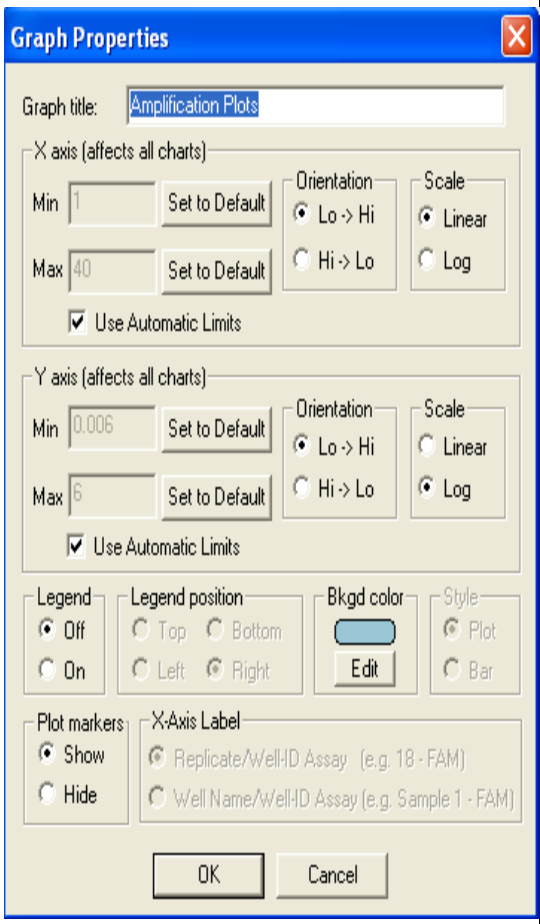
1 **Instrument default melt curve program** **Melt curve analysis (SYBR Only)**




**Stratagene Mxp3005p**

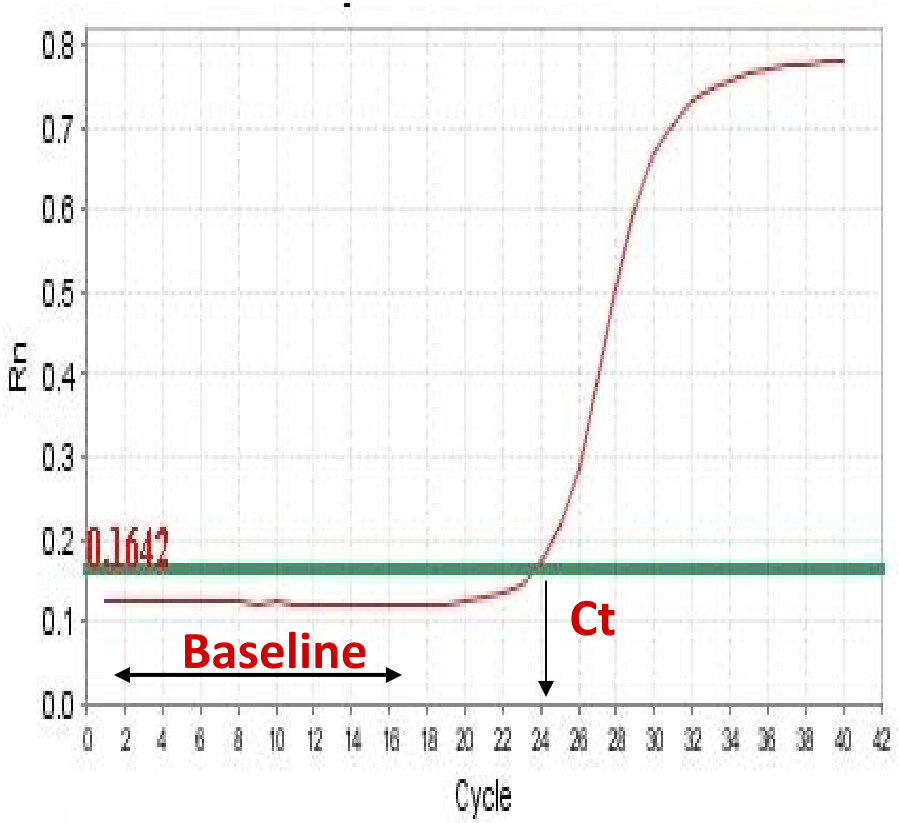
# Run qPCR - - - Results



# How To Define/Set Up The Baseline

**Linear Amplification Plot**




**-Automated Baseline Option**

if an instrument has a adaptive baseline function

**-Define manually:**

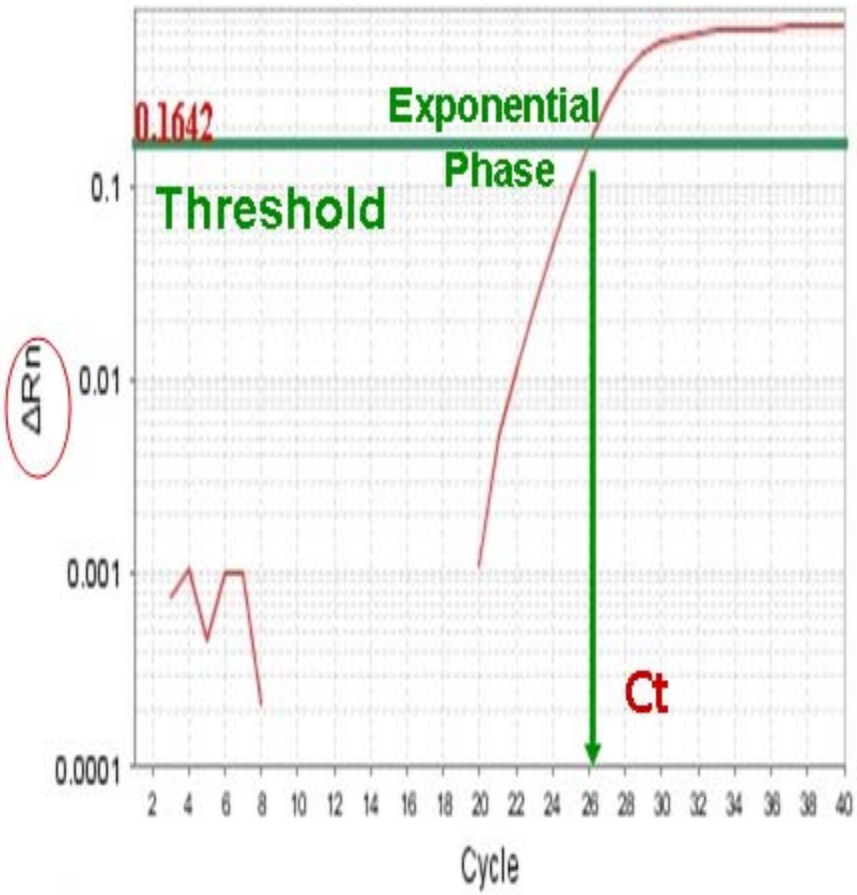
(1) Use linear view of the plot

(2) Set up the baseline reading from cycle #2 to the cycle that 2 cycles before the earliest visible amplification

(3) Usually a baseline falls in **3-15** cycles

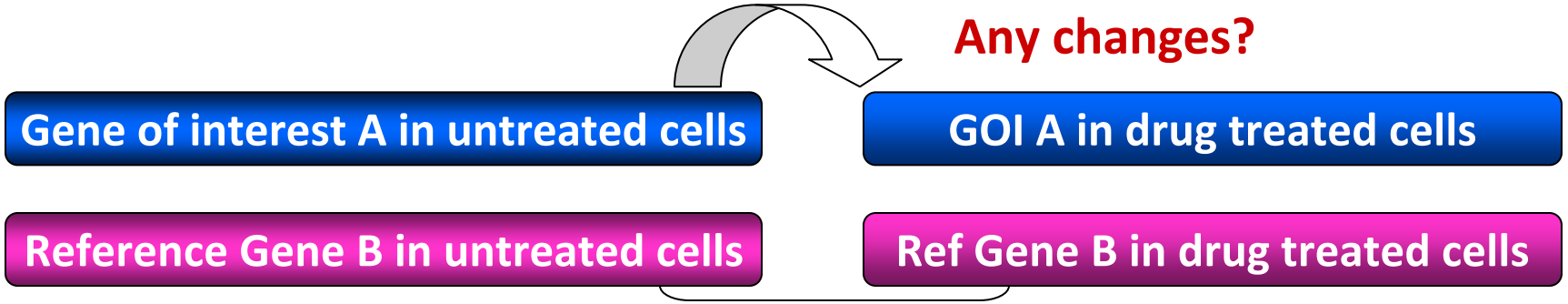
# How To Define Threshold

## Log View Amplification Plot




- Use **log view** of amplification plot
- Threshold should be higher than baseline (higher than the noise level)
- Threshold should at **LOWER** 1/3 or 1/2 of the linear phase of amplification
- **Linear phase = exponential phase**
- Different runs across samples for the same experiments should have **the same threshold** for comparison

# Reference Genes (Housekeeping Genes) For Normalization




**The expression level of a reference gene remain consistent under experimental conditions or different tissues**

- **A Reference Gene** is aimed to normalize possible variations during:
  - Sample prep & handling (e.g use the same number of cells from a start)
  - RNA isolation (RNA quality and quantity)
  - Reverse transcription efficiency across samples/experiments
  - PCR reaction set up
  - PCR reaction amplification efficiencies

# Commonly Used Housekeeping Genes

**Table 7. Housekeeping genes commonly used as endogenous references**

| | Gene symbol | | Relative expression level* | |
|---|---|---|---|---|
| **Gene** | **Human** | **Mouse** | **Human** | **Mouse** |
| 18S ribosomal RNA | RRN18S | Rn18s | ++++ | ++++ |
| Actin, beta | ACTB → | Actb | +++ | +++ |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH → | Gapdh | +++ | +++ |
| Phosphoglycerate kinase 1 | PGK1 | Pgk1 | +++ | ++ |
| Peptidylprolyl isomerase A | PPIA | Ppia | +++ | +++ |
| Ribosomal protein L13a | RPL13A → | Rpl13a | +++ | +++ |
| Ribosomal protein, large, P0 | RPLP0 | | +++ | |
| Acidic ribosomal phosphoprotein P0 | | Arbp | | +++ |
| Beta-2-microglobulin | B2M → | B2m | ++ – +++ | ++ – +++ |
| Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide | YWHAZ | Ywhaz | ++ – +++ | + |
| Succinate dehydrogenase complex, subunit A, flavoprotein (Fp) | SDHA | Sdha | ++ | + |
| Transferrin receptor | TFRC | Tfrc | ++ | + |
| Aminolevulinate, delta-, synthase 1 | ALAS1 | Alas1 | + | + |
| Glucuronidase, beta | GUSB | Gusb | + | + |
| Hydroxymethylbilane synthase | HMBS | Hmbs | + | ++ – +++ |
| Hypoxanthine phosphoribosyltransferase 1 | HPRT1 → | Hprt1 | + | + |
| TATA box binding protein | TBP | Tbp | + | + |
| Tubulin, beta | TUBB | | + | |
| Tubulin, beta 4 | | Tubb4 | | + |

* "+" indicates relative abundance of the transcripts.

→ **HKGs in RT² Profiler PCR Arrays**

# Data Analysis website

1.) Average Ct values for all gene replicates

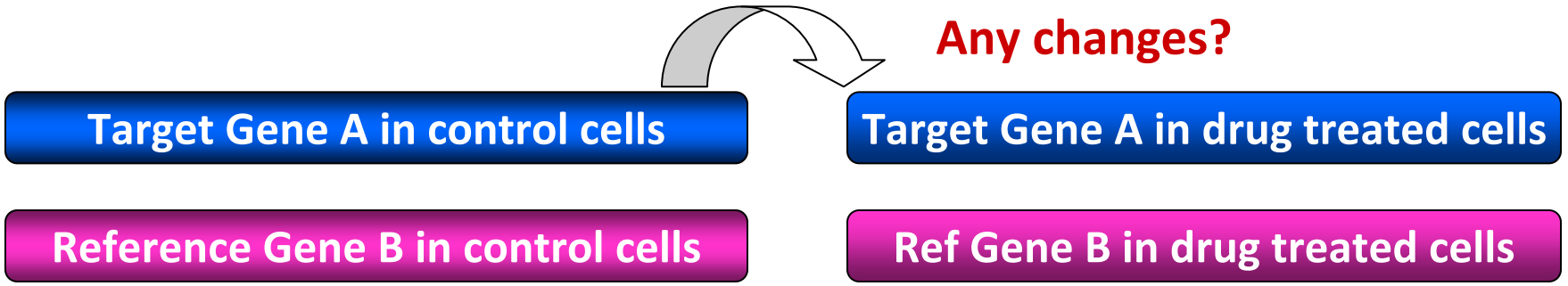
2.) Calculate Delta Ct value between GOI and HKG for each experiment

3.) Average Delta Ct values between experiments (replicates)

4.) Calculate Delta-Delta Ct values ( Delta Ct experiment- Delta Ct control)

5.) Calculate Fold Change $2^{(-\text{Delta Delta Ct})}$

# Normalized Gene Expression Level



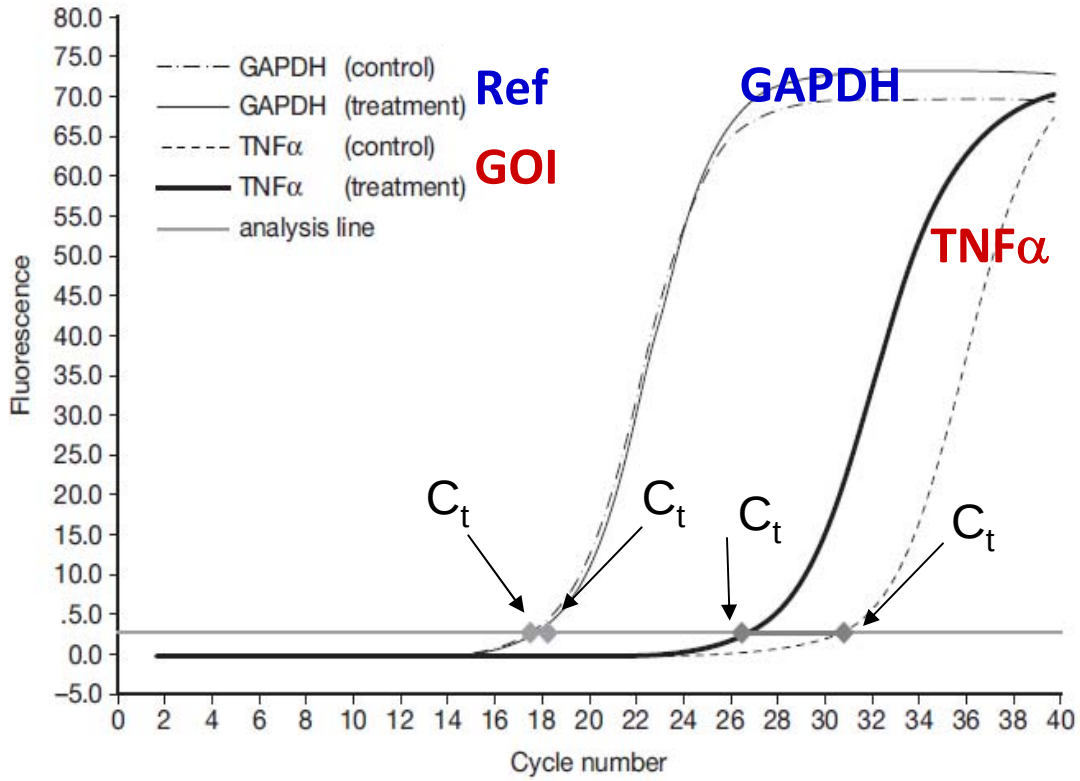

→ ΔCt = Ct (Target A -treated) – Ct (Ref B-treated)

→ ΔCt = Ct (Target A-control) – Ct (Ref B-control)

→ ΔΔ Ct = Δ Ct (treated) – Ct (control)

**Normalized target gene expression level = $2^{(-\Delta\Delta Ct)}$**

# Delta Delta Ct Method: A Look of Amplification Plots




$\Delta\Delta Ct = \Delta Ct\ (TNF\alpha_{treat} - GAPDH_{treat}) - \Delta ct\ (TNF\alpha_{control} - GAPDH_{control})$

**The fold change = $2^{(-\Delta\Delta Ct)}$**

# Data Analysis website

1.) Average Ct values for all gene replicates

| | Control 1 | Control 2 | Control 3 | Exp 1 | Exp 2 | Exp 3 |
|---|---|---|---|---|---|---|
| GAPDH | 17.2 | 18 | 19 | 17 | 18 | 17.5 |
| TNFa | 31 | 32.3 | 33.4 | 26 | 27.2 | 26.8 |

2.) Calculate Delta Ct value: GOI-HKG

| | | | | | | |
|---|---|---|---|---|---|---|
| TNFa-GAPDH | 13.8 | 14.3 | 14.4 | 9 | 9.2 | 9.3 |

3.) Average Delta Ct values between experiments (replicates)

| | | | | |
|---|---|---|---|---|
| Average | 14.17 | | | 9.17 |

4.) Calculate Delta-Delta Ct values (Delta Ct experiment- Delta Ct control)

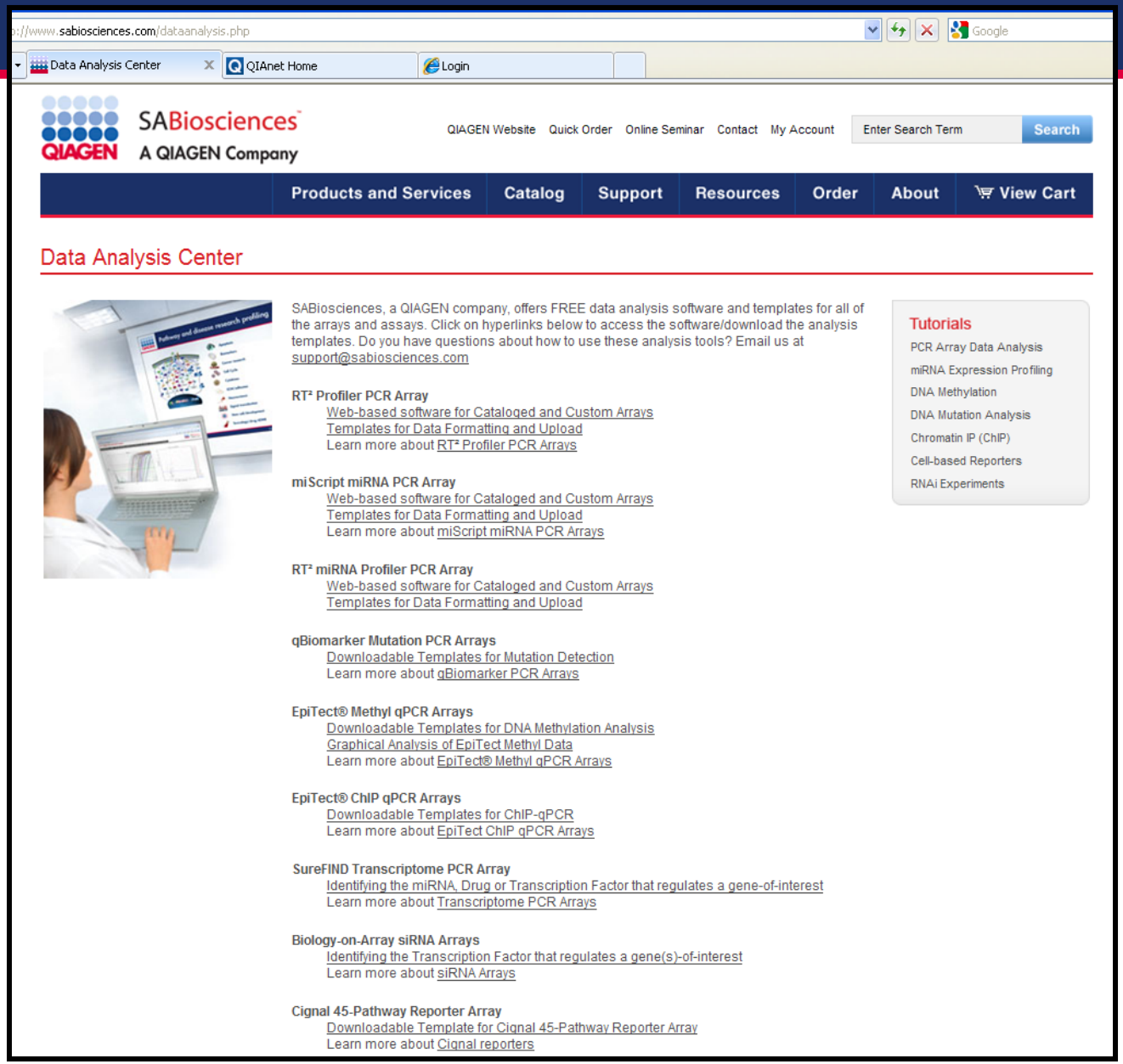
| | |
|---|---|
| DD Ct | -5.00 |

5.) Calculate Fold Change $2^{(-\text{Delta Delta Ct})}$

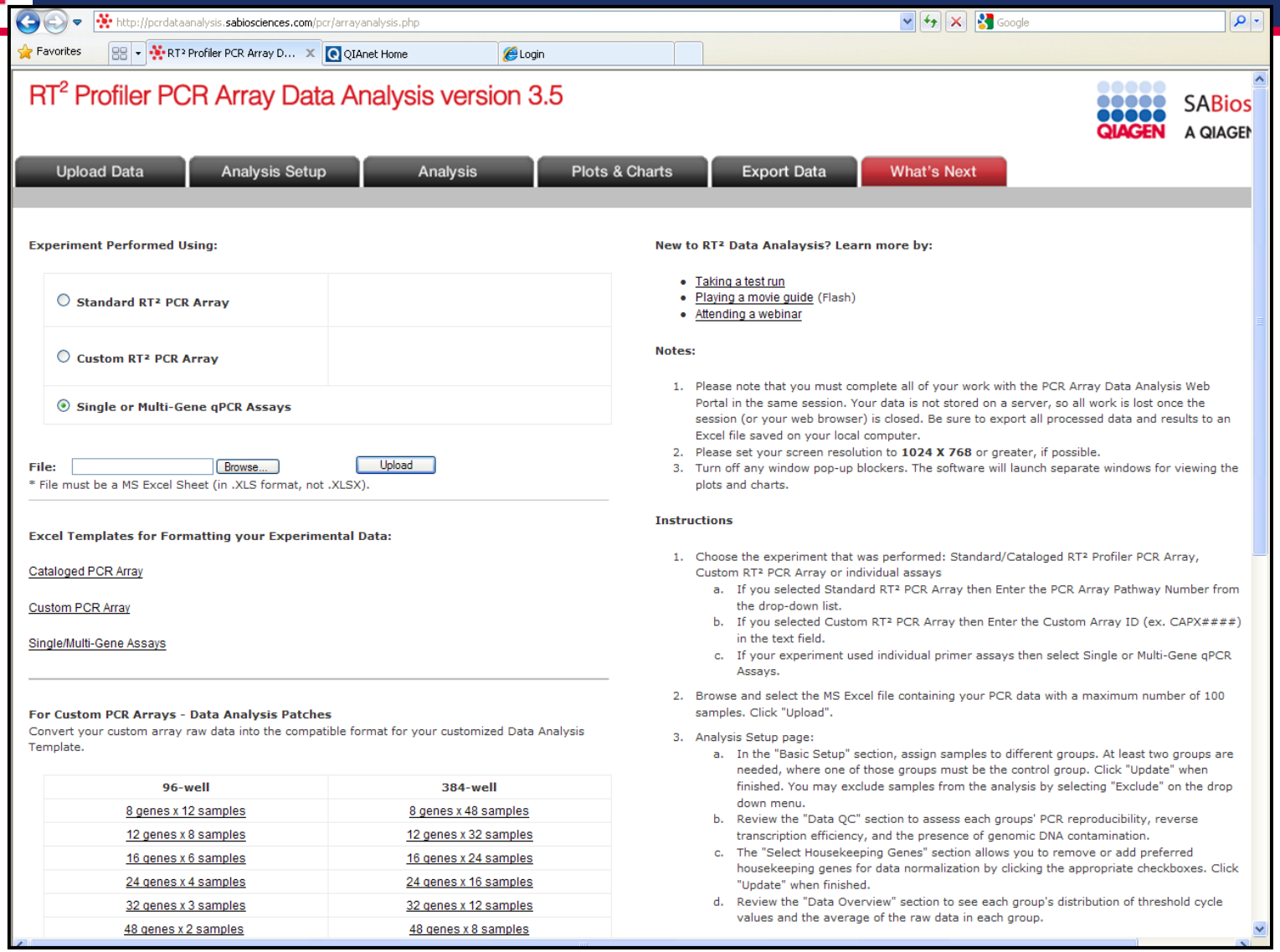
| | |
|---|---|
| Fold Change | 32.00 |

TNF$\alpha$ is up-regulated 32 fold in the treated cells versus the control

# http://www.sabiosciences.com/dataanalysis.php



# http://www.sabiosciences.com/dataanalysis.php



# Questions?

**Ask now or contact Technical Support M – F, 9 AM – 6 PM EST**

**Telephone: (888) 503-3187**

**Email: support@SABiosciences.com**

**Thank you!**