

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 of a qPCR 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.

RNA

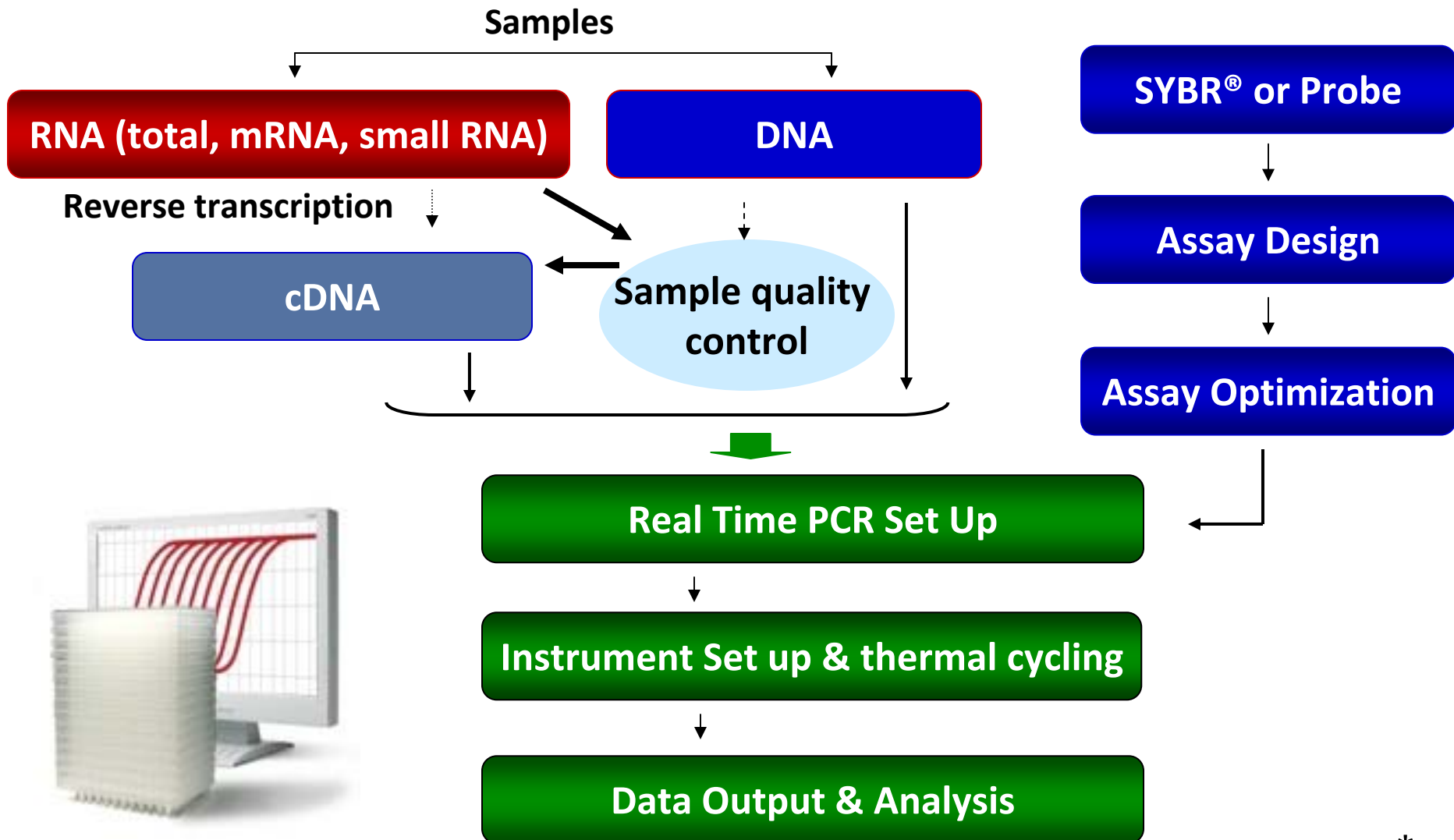
- Gene Expression Profiling Analysis
- miRNA Expression Profiling Analysis

DNA

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



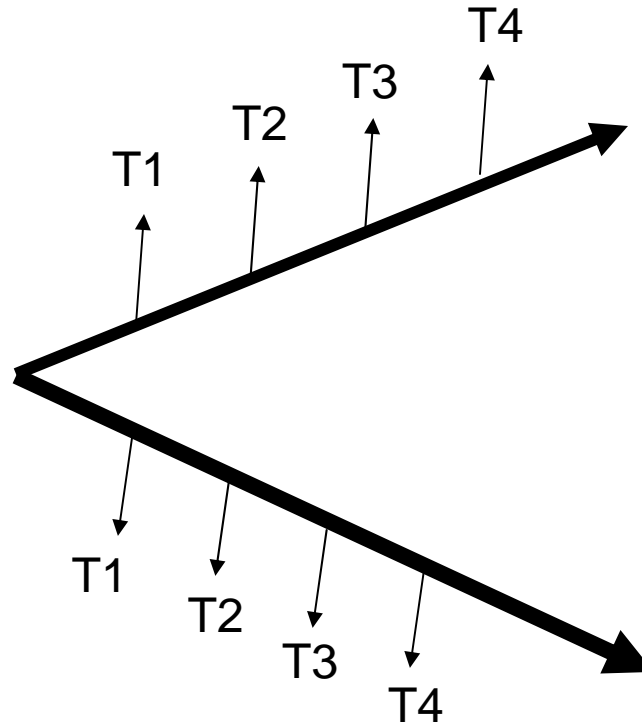
Work Flow: A Brief Look



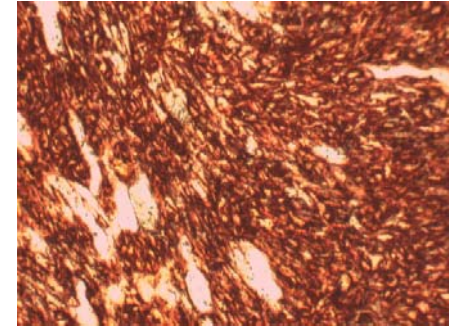
*

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 ($\text{TNF}\alpha$)
Repeat experiment 3x (biological replicates)

RNA

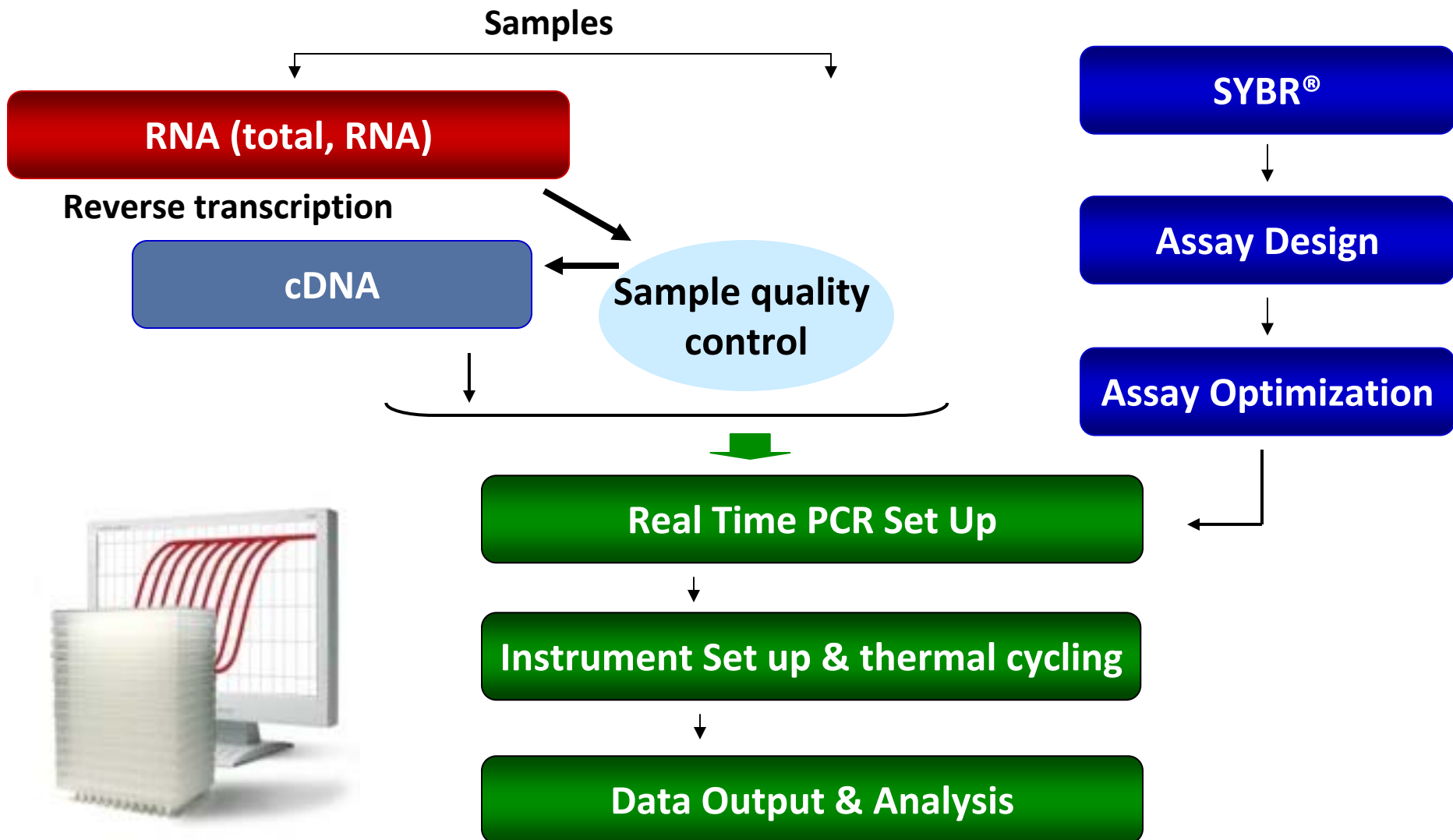
- **Gene Expression Profiling Analysis**
- miRNA Expression Profiling Analysis

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



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



Qiazol:
phenol/guanidine-based lysis

Instant inactivation of RNases
Instant end of biological activities

Column cleanup:
Molecular biology grade RNA

RNeasy Lipid tissue mini Kit

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)

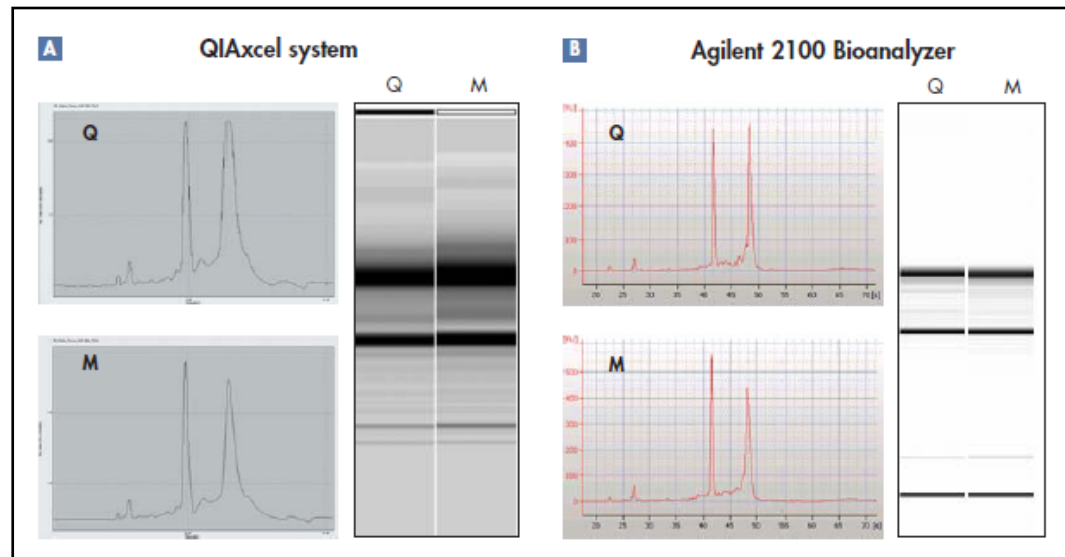
Low ratio may indicate protein, QIAzol, Carbohydrates, Guanidine HCL,
Absorbance measurements do not show integrity of RNA

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

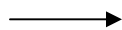
Mg⁺⁺

dNTP

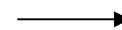
Buffer

*Passive reference dye

Denaturation

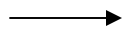


Annealing



Extension

Denaturation



Annealing/Extension

qPCR Components & Steps: Overview

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Reverse Transcription



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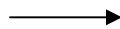
Mg⁺⁺

dNTP

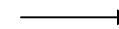
Buffer

*Passive reference dye

Denaturation

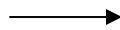


Annealing



Extension

Denaturation



Annealing/Extension

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

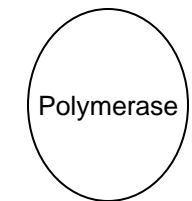
Sensitivity of qPCR step is dependent on good RT reaction

Monitor RT reaction to ensure equal RT efficiency across all samples

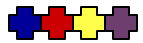
What is in a PCR Reaction?

PCR = **P**olymerase **C**hain **R**eaction

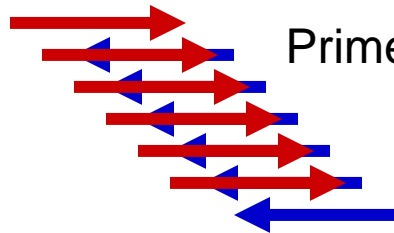
Exponential Amplification of DNA in single tube



“thermostable” i.e. can withstand temperatures
Up to ~95C



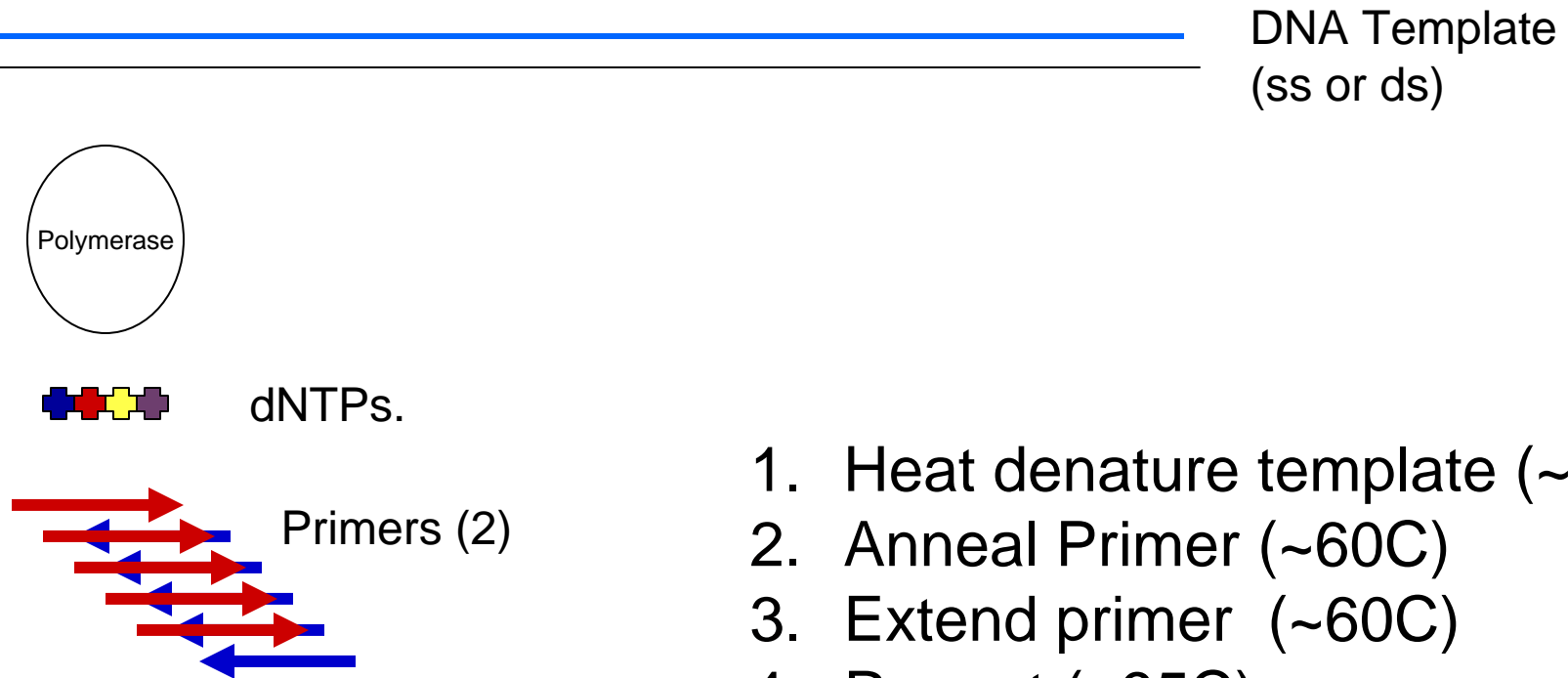
dNTPs.



Primers (2)

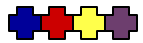
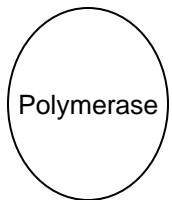
DNA Template
(ss or ds)

All reagents in
Excess (non-limiting)

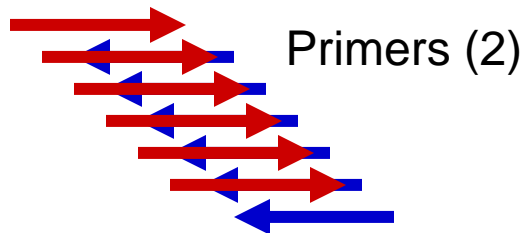


↑
Heat denature

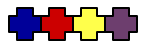
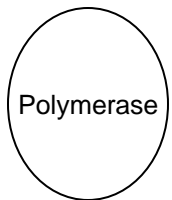
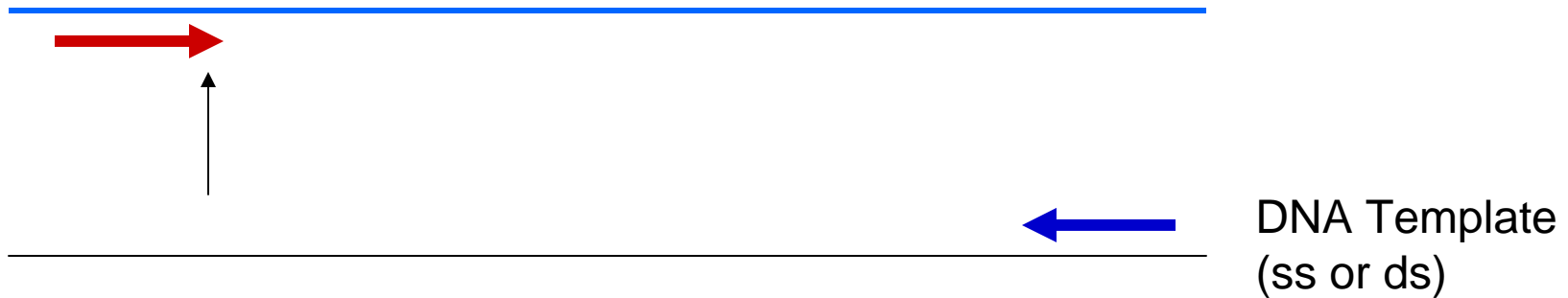
DNA Template
(ss or ds)



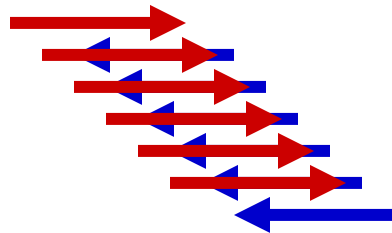
dNTPs.



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

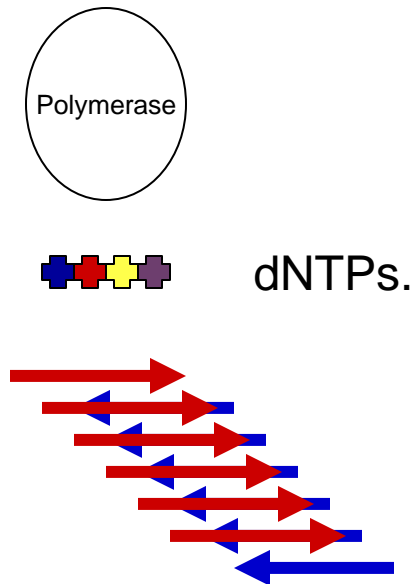
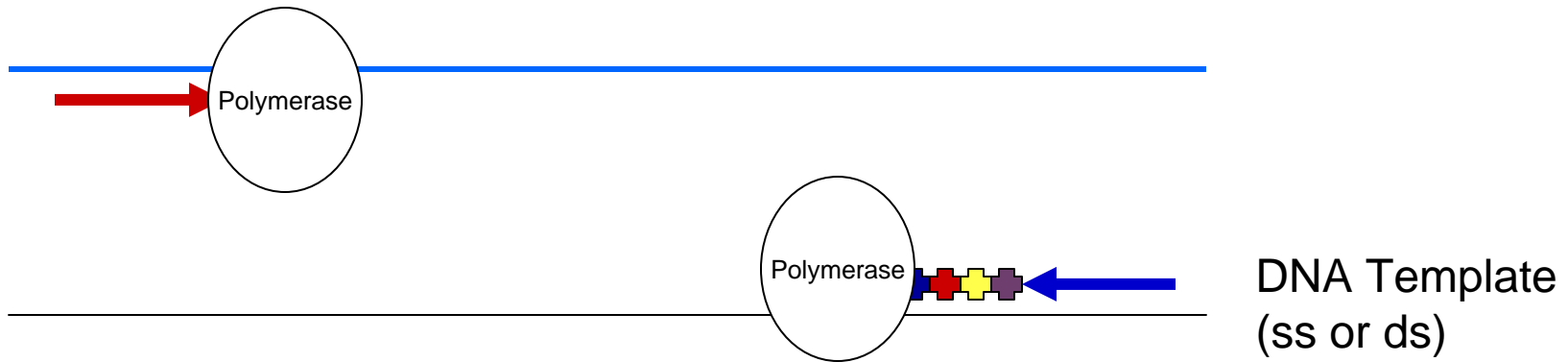


dNTPs.



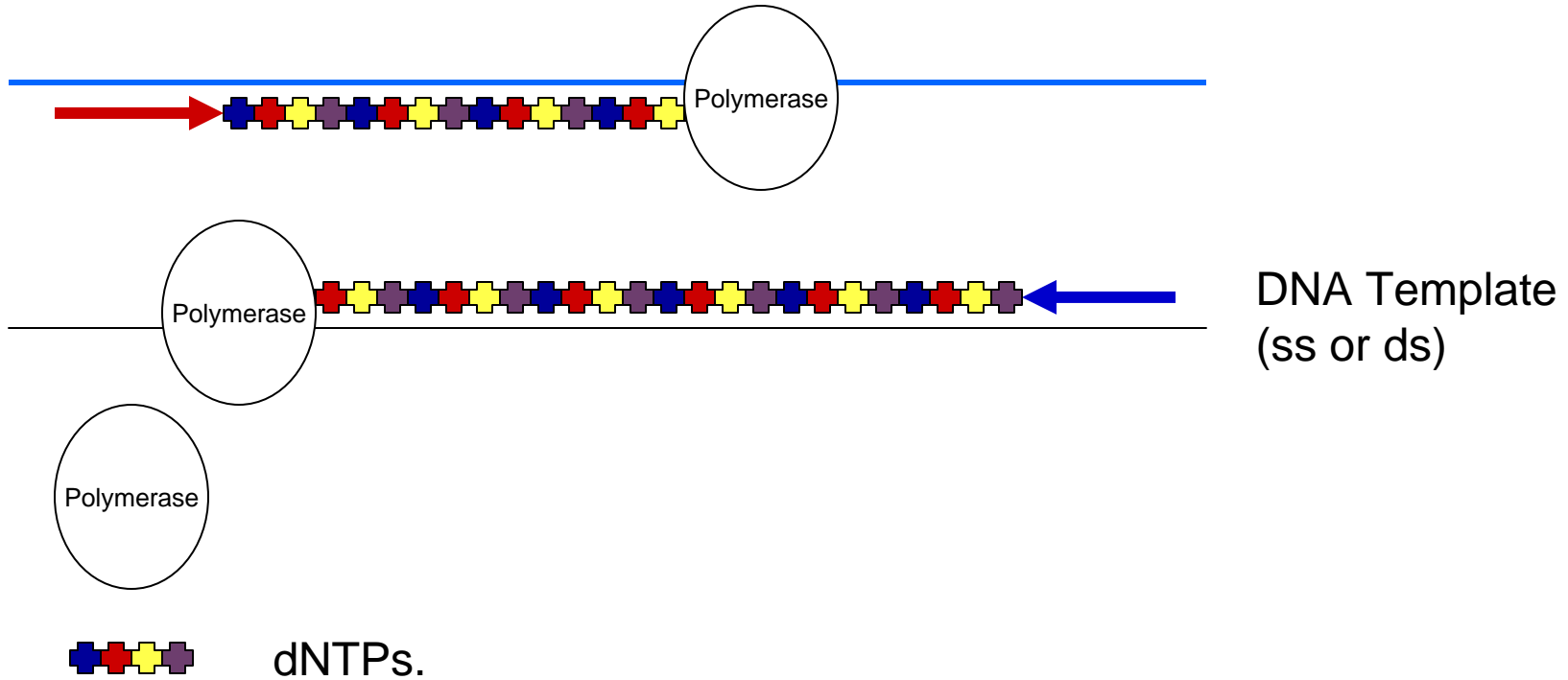
1. Heat denature template (~95C)
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PCR Reaction in Action

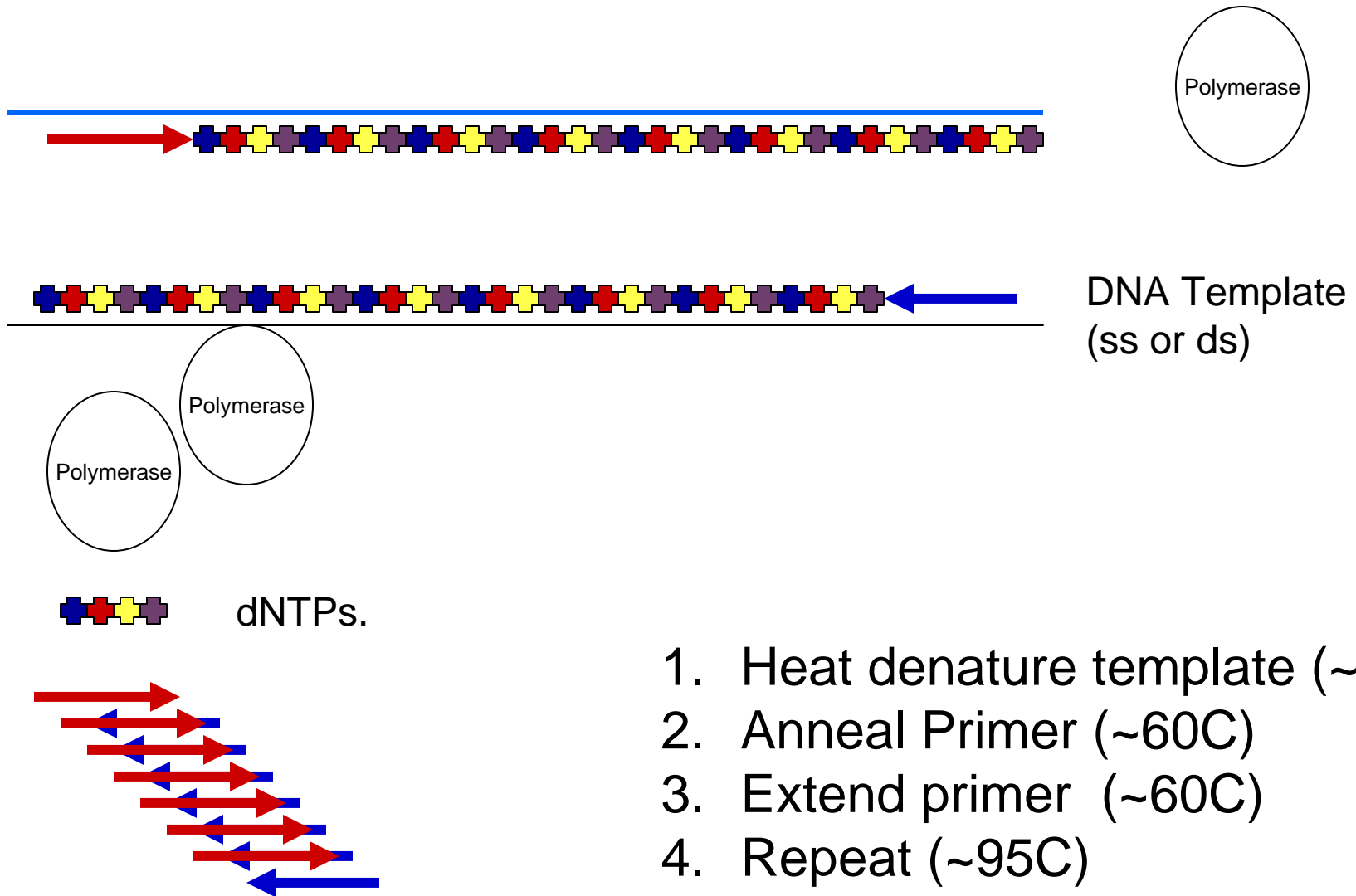


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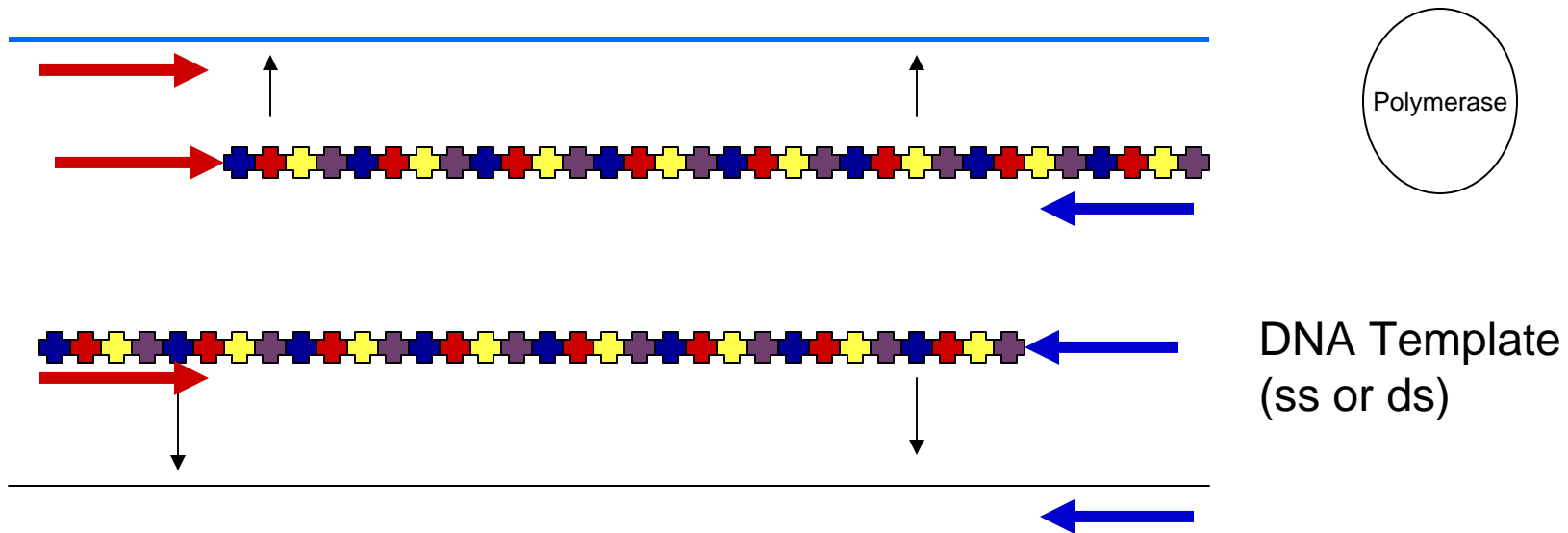
PCR Reaction in Action



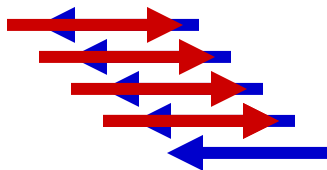
PCR Reaction in Action



PCR Reaction in Action

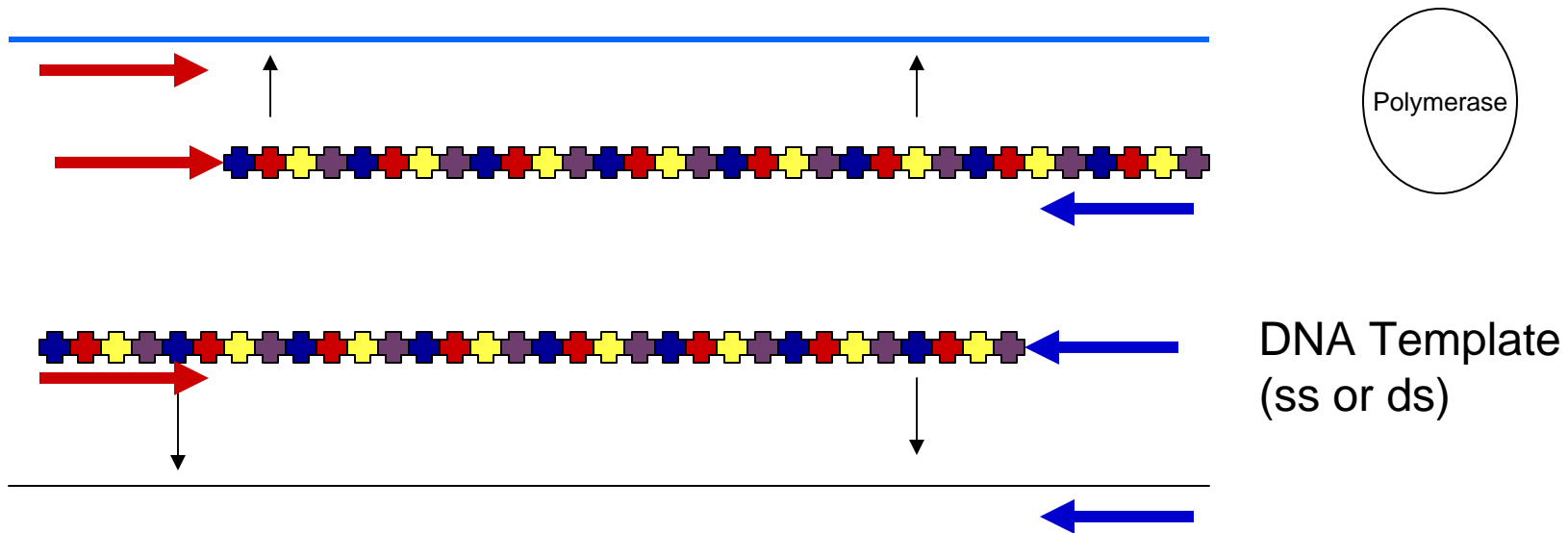


 dNTPs.

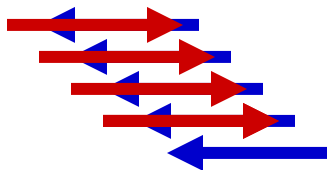


1. Heat denature template (~95C)
2. Anneal Primer (~60C)
3. Extend primer (~50 to ~70C)
4. Repeat (~95C)

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



 dNTPs.



1. Heat denature template (~95C)
2. Anneal Primer (~60C)
3. Extend primer (~50 to ~70C)
4. Measure Amount of PCR Product
5. Repeat (~95C)

■ DNA binding agents

SYBR[®] I Dye

Two most commonly used chemistries in qPCR community

■ Hydrolysis Probes

Dual-labeled Hydrolysis (Taqman[®]**) probe**

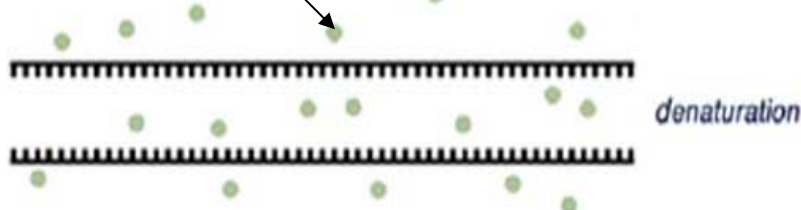
Others, such as hybridization probes

-Molecular beacon and scorpion probes

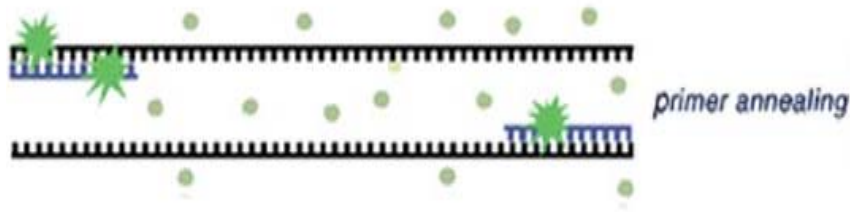


SYBR® Green I Assay: Fluorescent DNA Binding Dye

Non fluorescent SYBR I

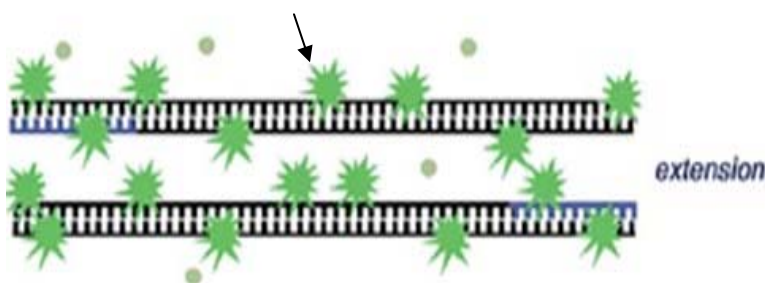


SYBR I binds to double-strand DNA but not single strand DNA. Little fluorescence emitted from SYBR I in solution.



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

Fluorescent SYBR I

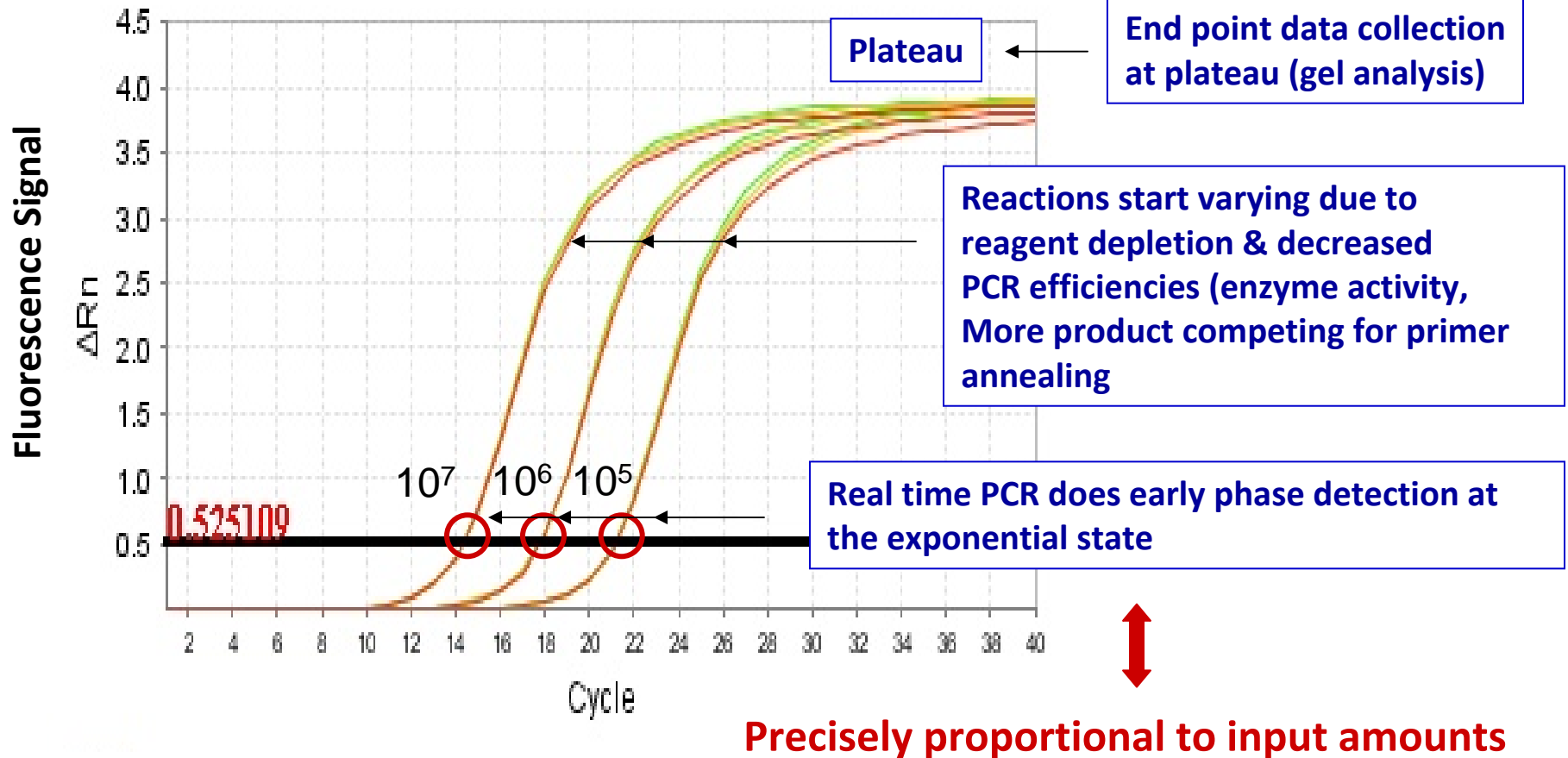


The SYBR I signal intensities correlate with DNA amplified (amplicon amount) thus the initial **sample** input amounts

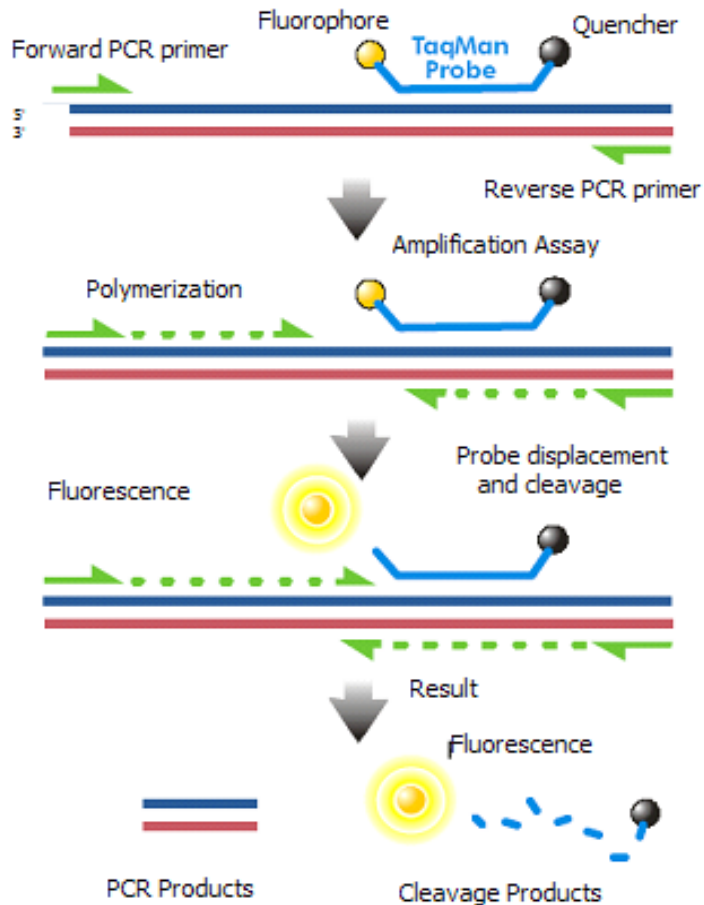
High Specificity Is Required when using SYBR Green
since SYBR I binds all double-strand DNA (non-specific or primer dimmer).

Understanding Kinetics in PCR

Amplification Plot (Linear scale)



Hydrolysis Based Probe - - Taqman® Probe Assay



The fluorescence of the reporter dye is suppressed by the quencher

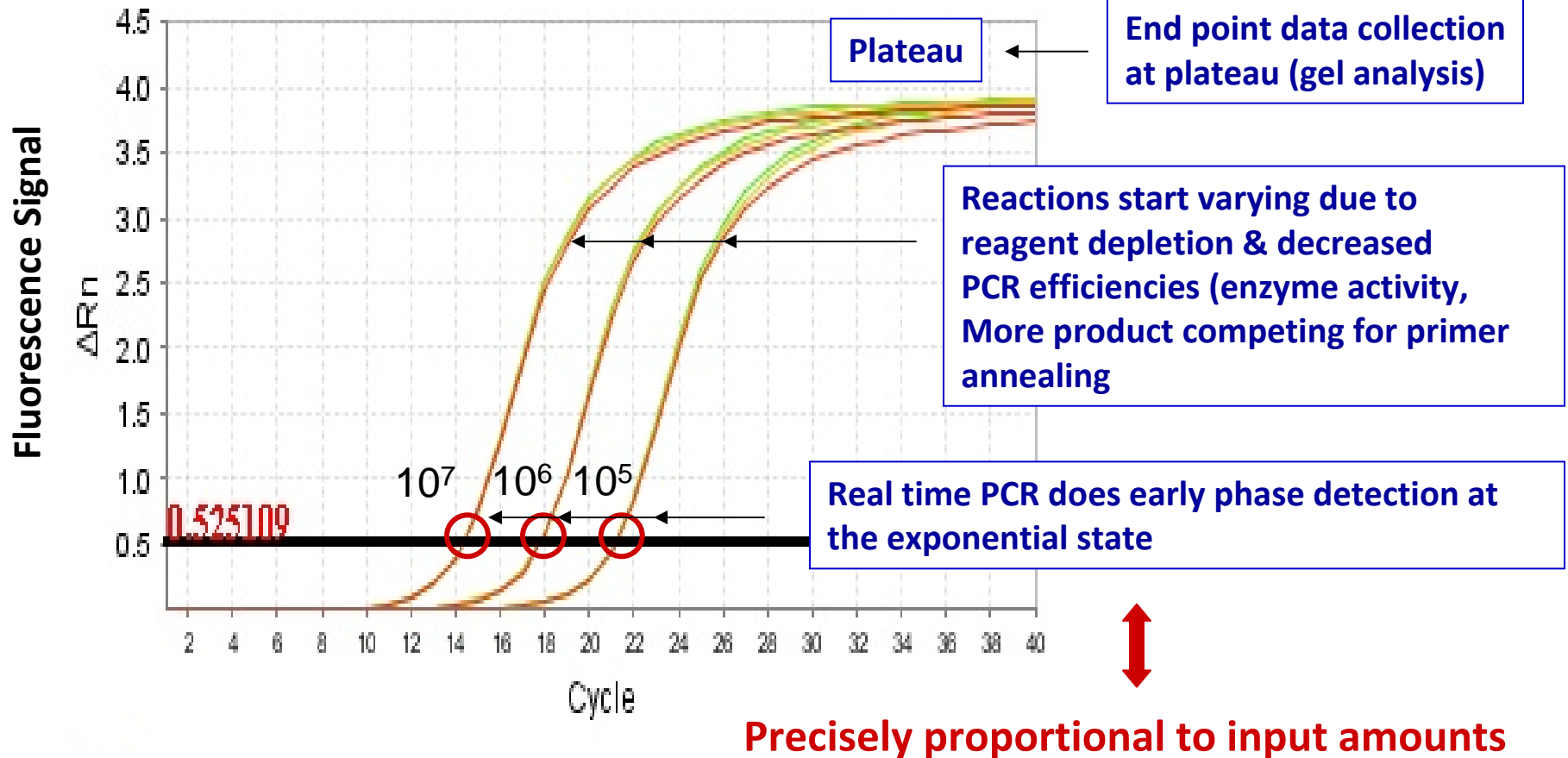
Primer binding followed by extension

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

Amplification Plot (Linear scale)



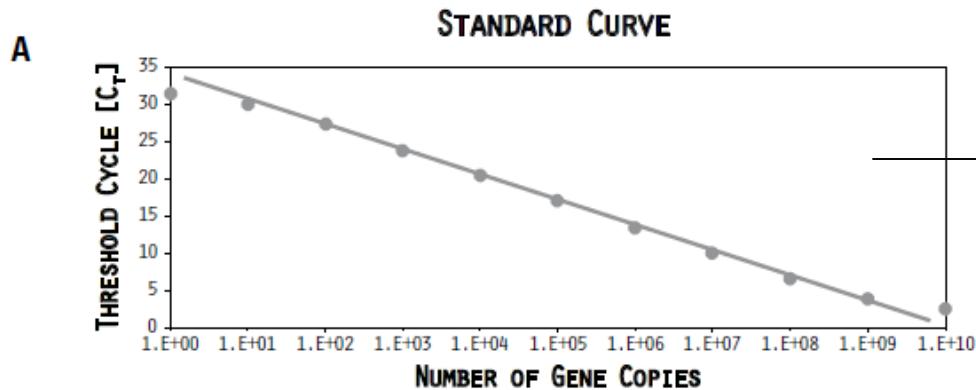
Characteristics of a good qPCR Assay

Amplification efficiency: 100% during exponential phase

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

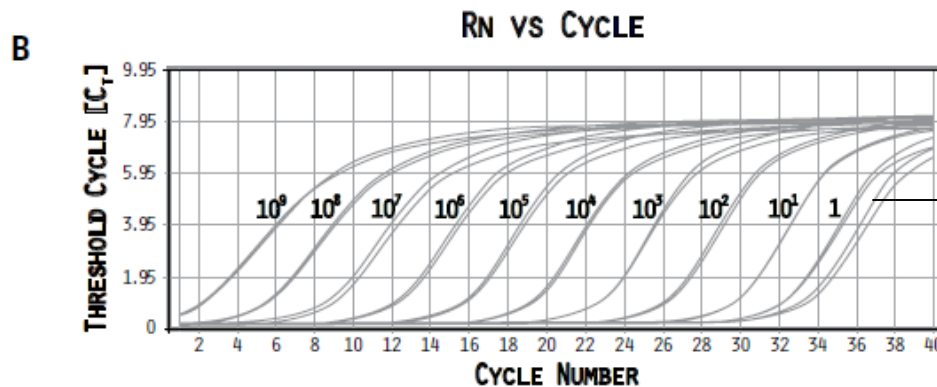
Specificity: 1 assay, 1 target: (no off-target amplification or primer-dimers)

Amplification Analysis: standard curve and single curve analysis



Plot:
x axis dilution
Y axis Ct value

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



Single curve analysis

PCR Miner

<http://miner.ewindup.info/version2>

“DART”

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

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

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)

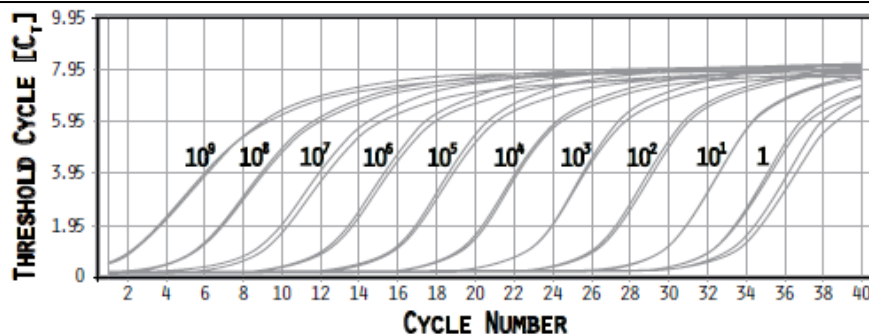
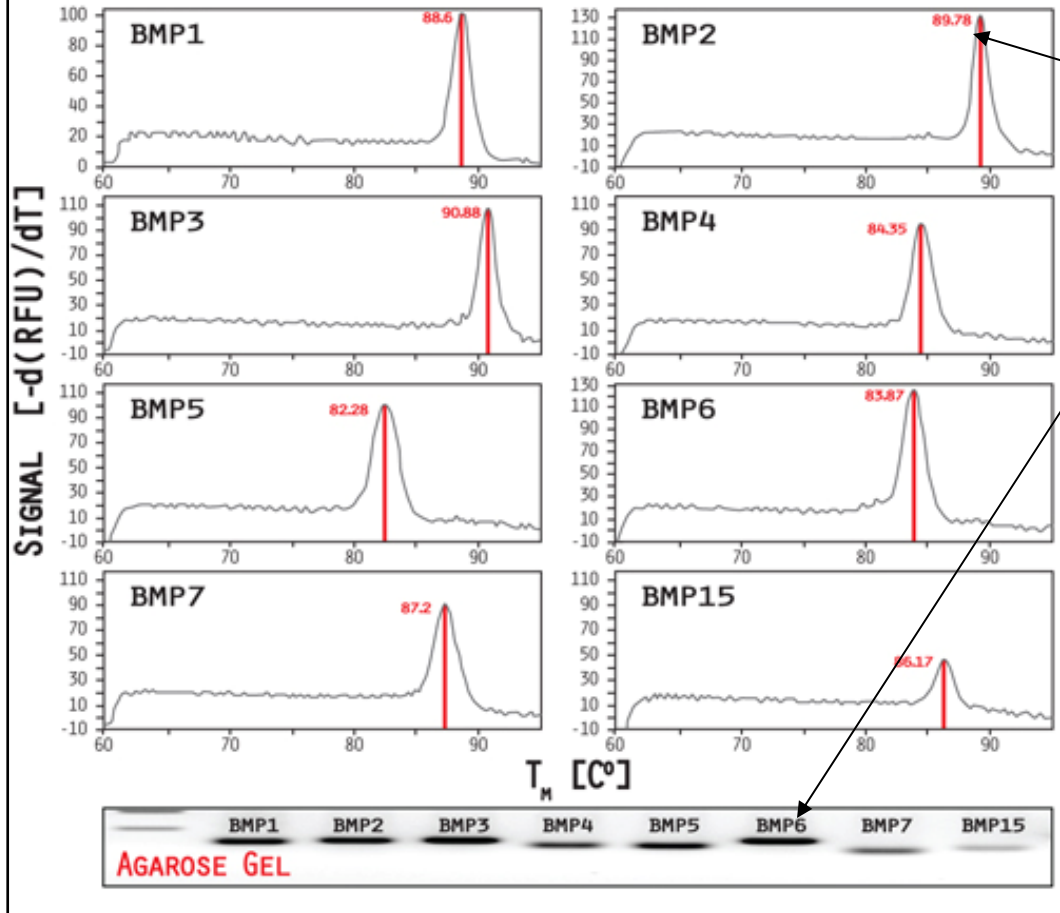


Figure 4: RT² Profiler PCR Arrays and RT² qPCR Assays Have Sufficiently Wide Dynamic Ranges.

Specificity: SYBR Green

SINGLE DISSOCIATION CURVES



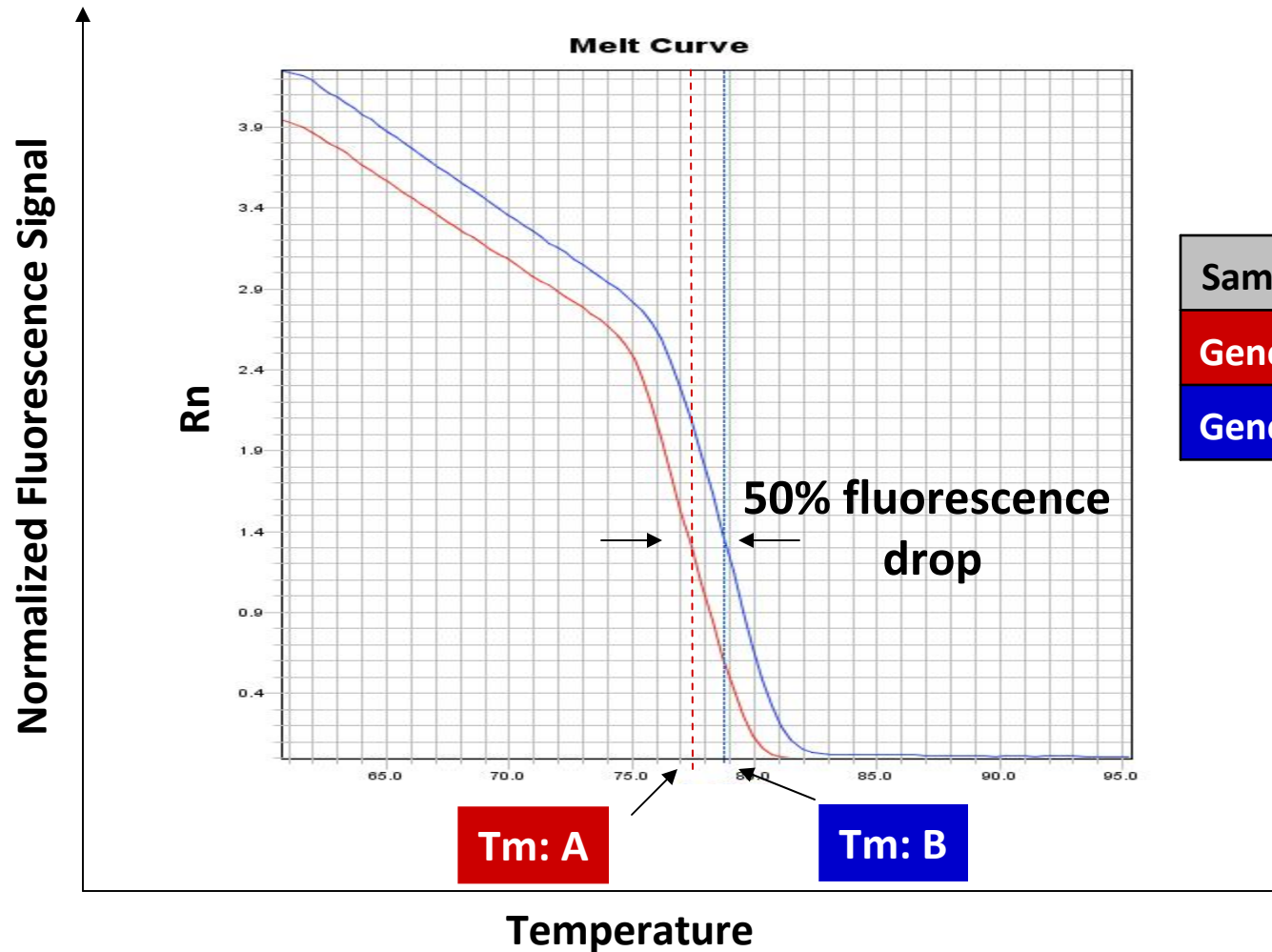
Single peak dissociation curves

Single gel bands of predicted size

Melt Curve Analysis: The General Program Steps

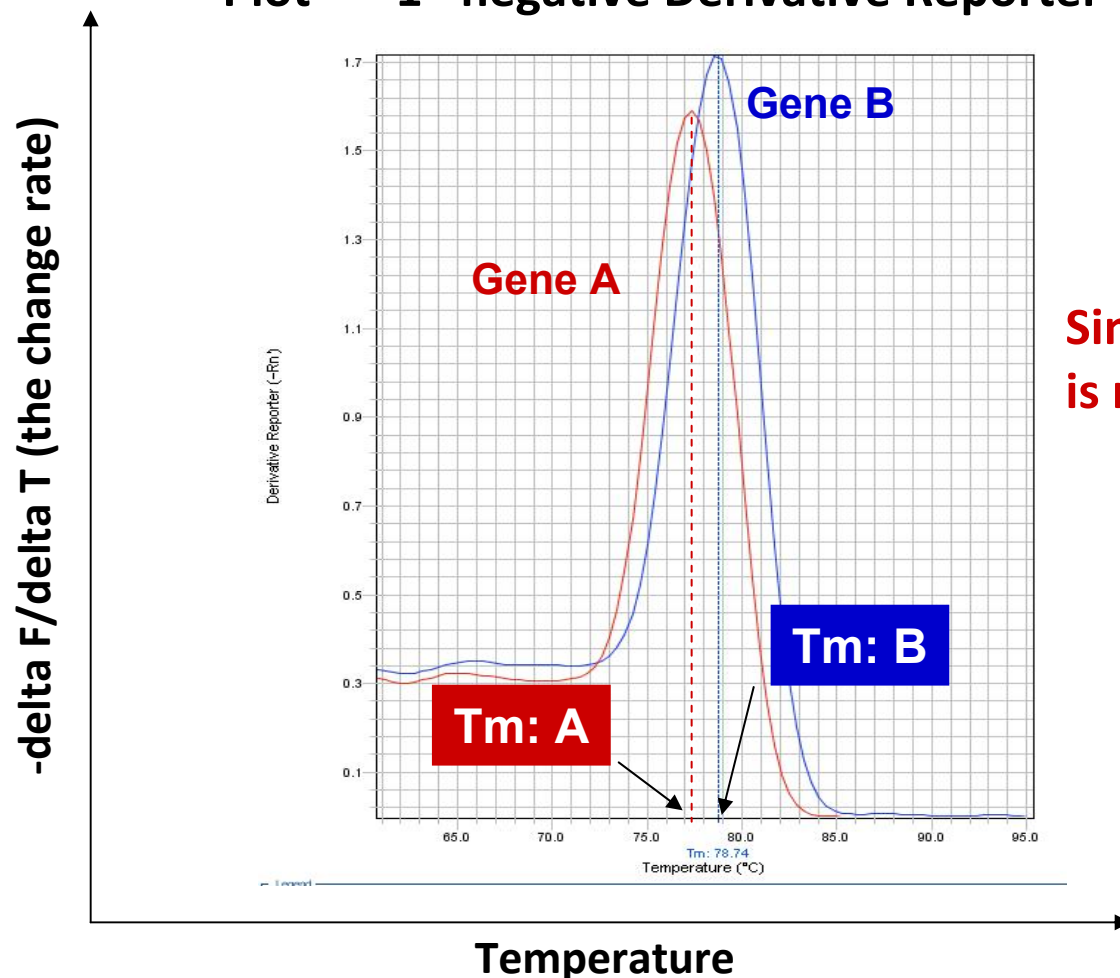
- Rapid heating of amplified samples to 94°C to denature the DNA
- Cooling the sample to 60°C to let DNA double strands anneal
- Slowly heating (by increasing the temperature, usually 0.2°C/sec) the sample while plotting the fluorescent signal versus temperature.
- As the temperature increases, and DNA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

Plot - Normalized Reporter (Fluorescence/Passive dye signal)

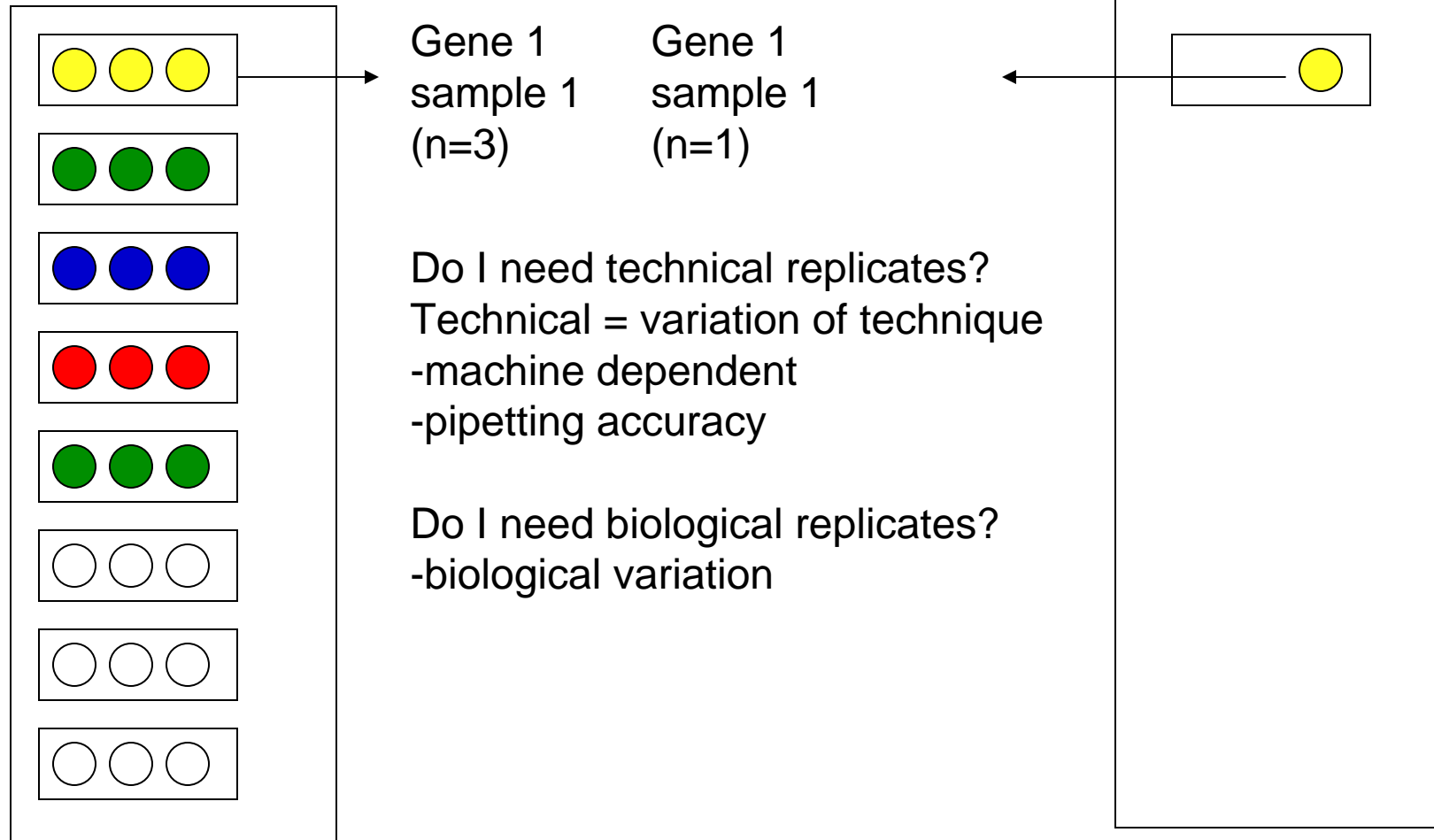


Samples	Tm
Gene A	77.36
Gene B	78.94

Plot - - 1st negative Derivative Reporter



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



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

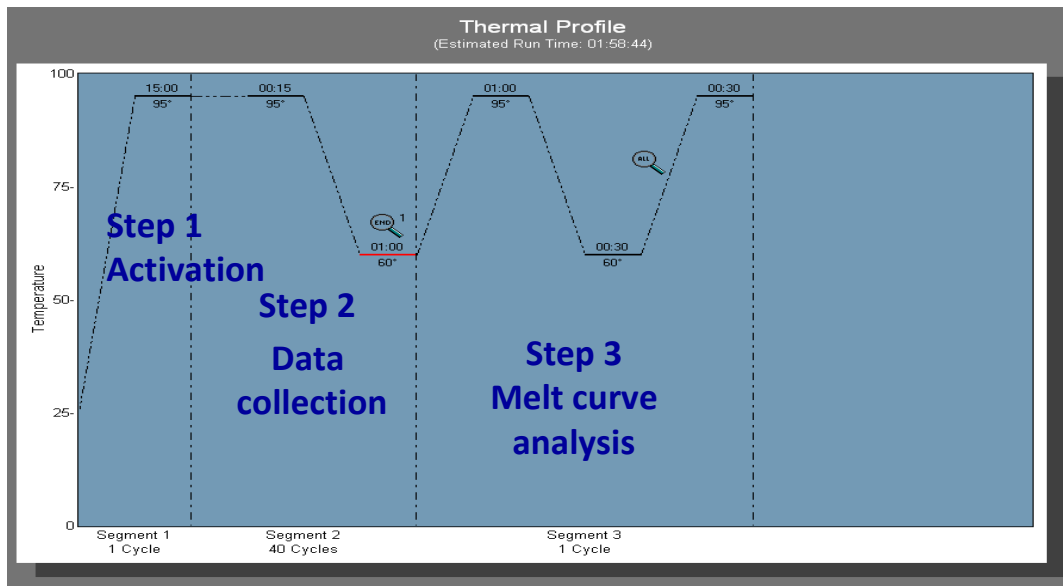
Table 4. Cycling conditions* for Applied Biosystems, Bio-Rad,[†] Stratagene, and Eppendorf[‡] cyclers

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

1

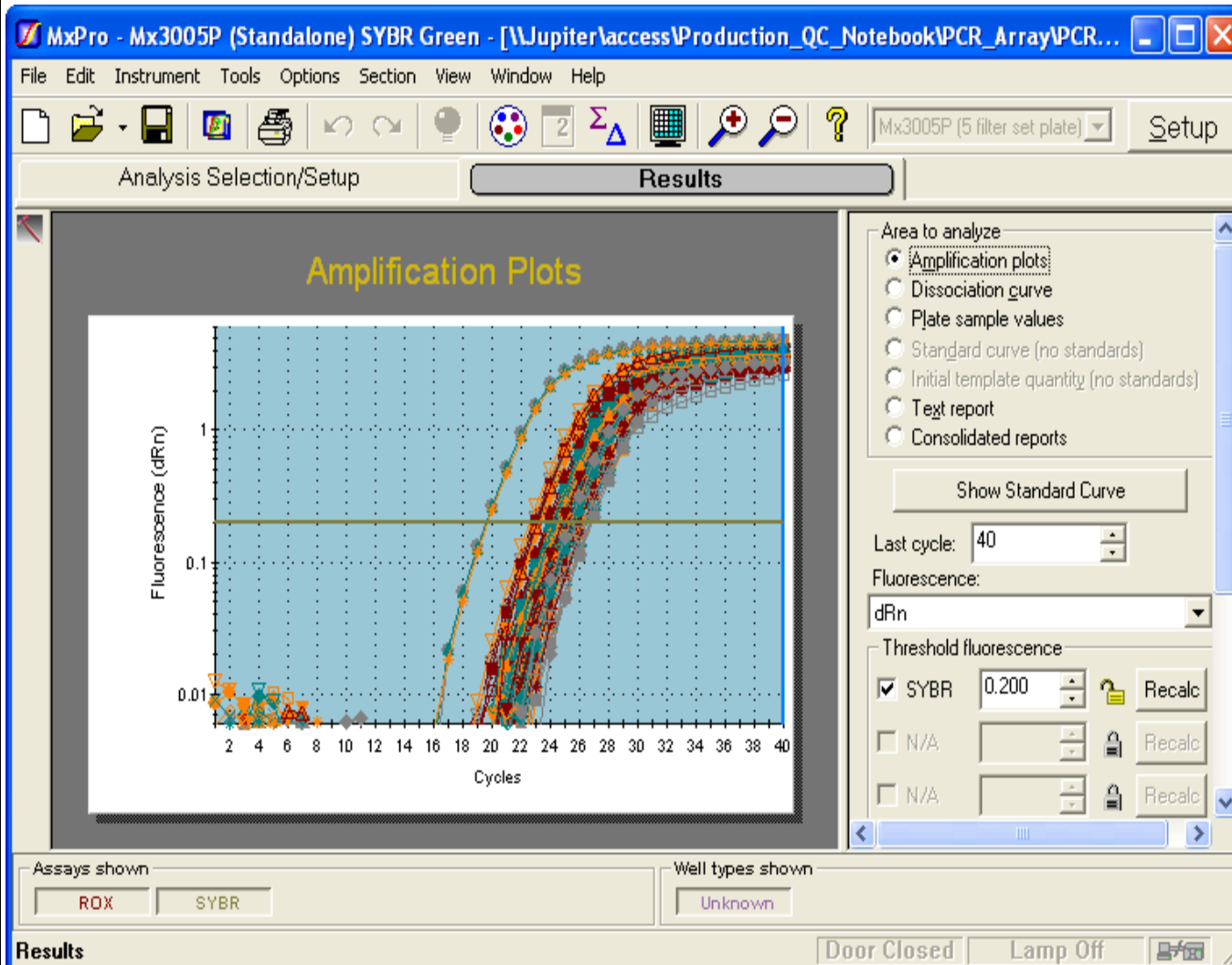
Instrument default melt curve program

Melt curve analysis
(SYBR Only)



Stratagene Mxp3005p

Run qPCR - - Results



Graph Properties

Graph title: Amplification Plots

X axis (affects all charts)

Min: 1 Set to Default

Max: 40 Set to Default

Orientation: ☒ Lo -> Hi ☐ Hi -> Lo

Scale: ☒ Linear ☐ Log

☒ Use Automatic Limits

Y axis (affects all charts)

Min: 0.006 Set to Default

Max: 6 Set to Default

Orientation: ☒ Lo -> Hi ☐ Hi -> Lo

Scale: ☒ Linear ☐ Log

☒ Use Automatic Limits

Legend: ☒ Off ☐ On

Legend position: ☐ Top ☐ Bottom ☐ Left ☒ Right

Bkgd color: Edit

Style: ☒ Plot ☐ Bar

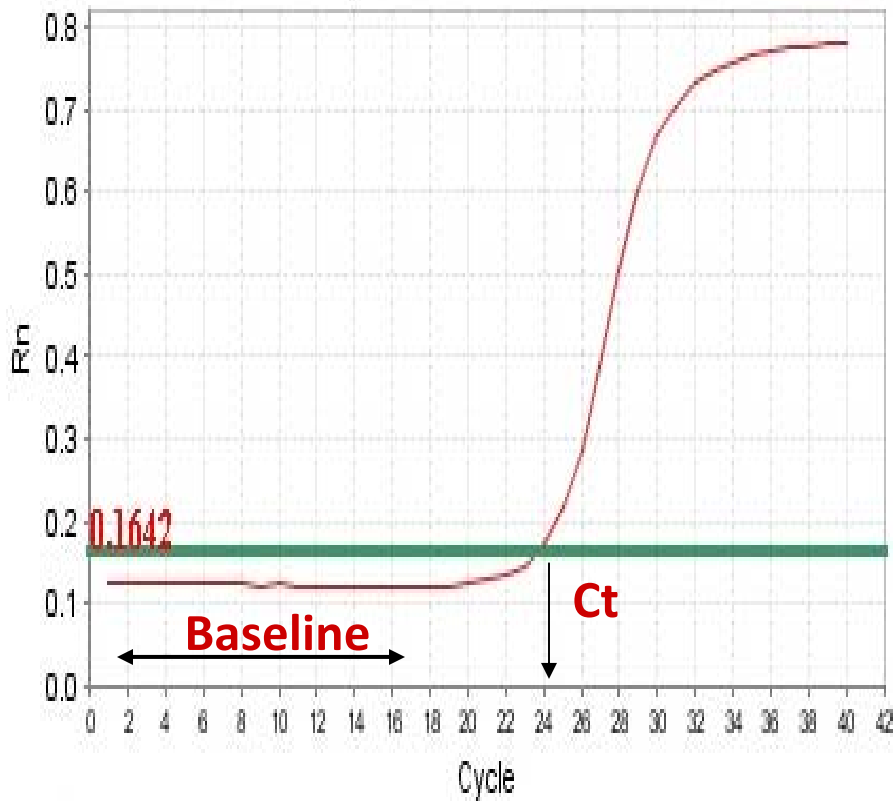
Plot markers: ☒ Show ☐ Hide

X-Axis Label: ☒ Replicate/WellID Assay (e.g. 18 - FAM) ☐ Well Name/WellID Assay (e.g. Sample 1 - FAM)

OK Cancel

How To Define/Set Up The Baseline

Linear Amplification Plot



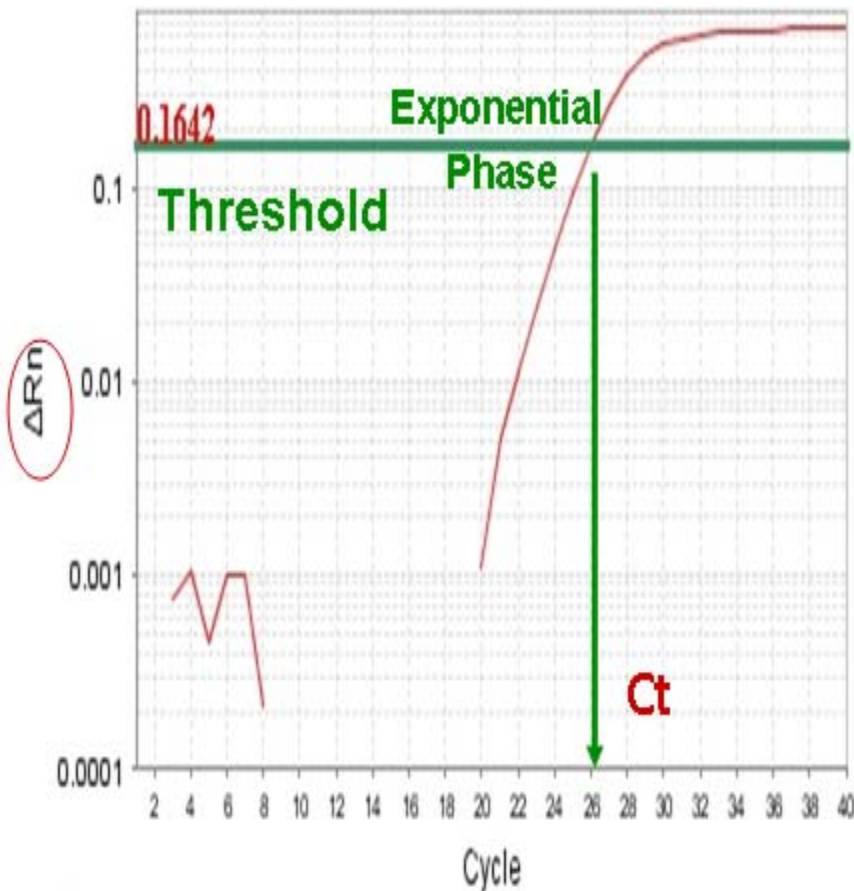
-Automated Baseline Option

if an instrument has a adaptive baseline function

-Define manually:

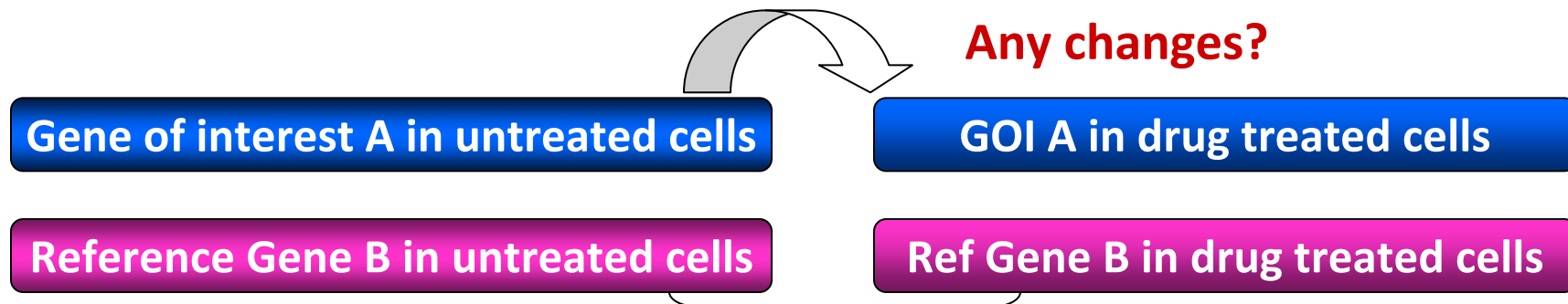
- (1) Use linear view of the plot
- (2) Set up the baseline reading from cycle #2 to the cycle that 2 cycles before the earliest visible amplification
- (3) Usually a baseline falls in **3-15** cycles

Log View Amplification Plot



- Use **log view** of amplification plot
- Threshold should be higher than baseline (higher than the noise level)
- Threshold should be 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

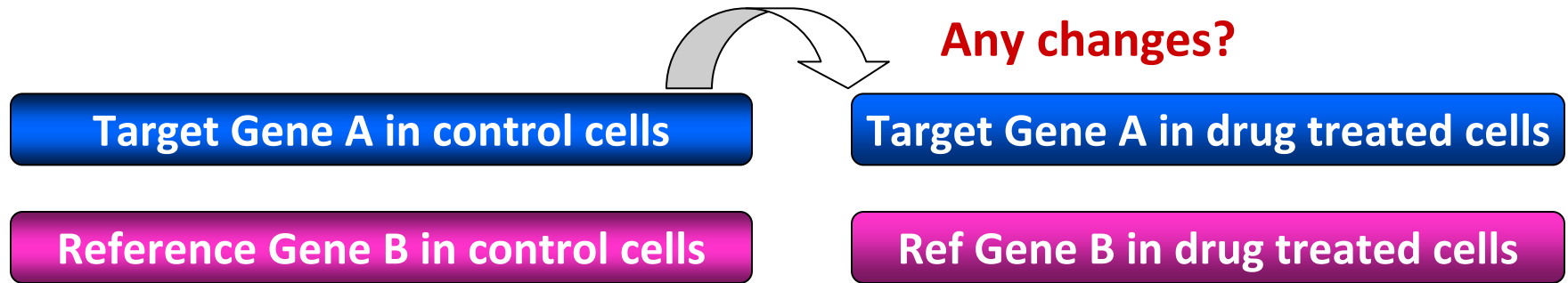
Gene	Gene symbol		Relative expression level*	
	Human	Mouse	Human	Mouse
18S ribosomal RNA	RRN18S	Rn18s	++++	++++
Actin, beta	ACTB	→ Actb	+++	+++
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	→ Gapdh	+++	+++
Phosphoglycerate kinase 1	PGK1	Pgk1	+++	++
Peptidylprolyl isomerase A	PPIA	Ppia	+++	+++
Ribosomal 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

- 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



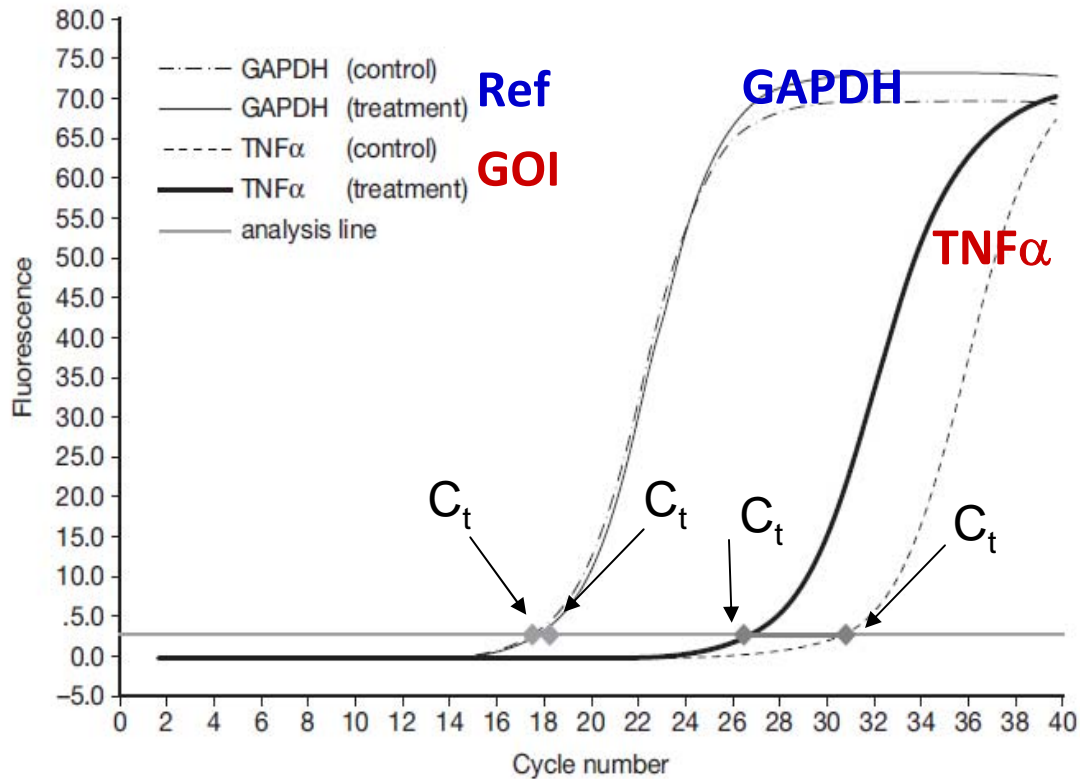
→ $\Delta Ct = Ct (\text{Target A -treated}) - Ct (\text{Ref B-treated})$

→ $\Delta Ct = Ct (\text{Target A-control}) - Ct (\text{Ref B-control})$

→ $\Delta\Delta Ct = \Delta Ct (\text{treated}) - Ct (\text{control})$

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

Delta Delta Ct Method: A Look of Amplification Plots



$$\Delta\Delta C_t = \Delta C_t (\text{TNF}\alpha_{\text{treat}} - \text{GAPDH}_{\text{treat}}) - \Delta C_t (\text{TNF}\alpha_{\text{control}} - \text{GAPDH}_{\text{control}})$$

$$\text{The fold change} = 2^{(-\Delta\Delta C_t)}$$

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
TNF α	31	32.3	33.4	26	27.2	26.8

2.) Calculate Delta Ct value: GOI-HKG

TNF α -GAPDH	13.8	14.3	14.4	9	9.2	9.3
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3.) Average Delta Ct values between experiments (replicates)

Average	14.17			9.17
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4.) Calculate Delta-Delta Ct values (Delta Ct experiment- Delta Ct control)

DD Ct	-5.00
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5.) Calculate Fold Change $2^{(-\text{Delta Delta Ct})}$

Fold Change	32.00
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TNF α is up-regulated 32 fold in the treated cells versus the control

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


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Data Analysis Center



SABiosciences, a QIAGEN company, offers FREE data analysis software and templates for all of the arrays and assays. Click on hyperlinks below to access the software/download the analysis templates. Do you have questions about how to use these analysis tools? Email us at support@sabiosciences.com

RT² Profiler PCR Array
[Web-based software for Cataloged and Custom Arrays](#)
[Templates for Data Formatting and Upload](#)
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miScript miRNA PCR Array
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Tutorials

- PCR Array Data Analysis
- miRNA Expression Profiling
- DNA Methylation
- DNA Mutation Analysis
- Chromatin IP (ChIP)
- Cell-based Reporters
- RNAi Experiments



Upload Data

Analysis Setup

Analysis

Plots & Charts

Export Data

What's Next

Experiment Performed Using:

☐ Standard RT² PCR Array

☐ Custom RT² PCR Array

☒ Single or Multi-Gene qPCR Assays

File:

* File must be a MS Excel Sheet (in .XLS format, not .XLSX).

Excel Templates for Formatting your Experimental Data:

[Cataloged PCR Array](#)

[Custom PCR Array](#)

[Single/Multi-Gene Assays](#)

For Custom PCR Arrays - Data Analysis Patches

Convert your custom array raw data into the compatible format for your customized Data Analysis Template.

96-well	384-well
8 genes x 12 samples	8 genes x 48 samples
12 genes x 8 samples	12 genes x 32 samples
16 genes x 6 samples	16 genes x 24 samples
24 genes x 4 samples	24 genes x 16 samples
32 genes x 3 samples	32 genes x 12 samples
48 genes x 2 samples	48 genes x 8 samples

New to RT² Data Analysis? Learn more by:

- [Taking a test run](#)
- [Playing a movie guide \(Flash\)](#)
- [Attending a webinar](#)

Notes:

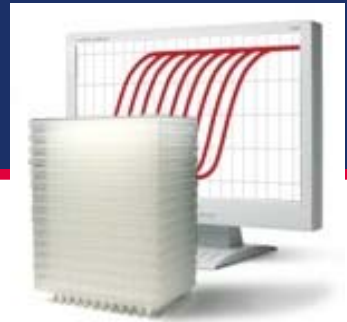
- Please note that you must complete all of your work with the PCR Array Data Analysis Web Portal in the same session. Your data is not stored on a server, so all work is lost once the session (or your web browser) is closed. Be sure to export all processed data and results to an Excel file saved on your local computer.
- Please set your screen resolution to **1024 X 768** or greater, if possible.
- Turn off any window pop-up blockers. The software will launch separate windows for viewing the plots and charts.

Instructions

- Choose the experiment that was performed: Standard/Cataloged RT² Profiler PCR Array, Custom RT² PCR Array or individual assays
 - If you selected Standard RT² PCR Array then Enter the PCR Array Pathway Number from the drop-down list.
 - If you selected Custom RT² PCR Array then Enter the Custom Array ID (ex. CAPX###) in the text field.
 - If your experiment used individual primer assays then select Single or Multi-Gene qPCR Assays.
- Browse and select the MS Excel file containing your PCR data with a maximum number of 100 samples. Click "Upload".
- Analysis Setup page:
 - In the "Basic Setup" section, assign samples to different groups. At least two groups are needed, where one of those groups must be the control group. Click "Update" when finished. You may exclude samples from the analysis by selecting "Exclude" on the drop down menu.
 - Review the "Data QC" section to assess each groups' PCR reproducibility, reverse transcription efficiency, and the presence of genomic DNA contamination.
 - The "Select Housekeeping Genes" section allows you to remove or add preferred housekeeping genes for data normalization by clicking the appropriate checkboxes. Click "Update" when finished.
 - Review the "Data Overview" section to see each group's distribution of threshold cycle values and the average of the raw data in each group.



Questions?



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

Telephone: (888) 503-3187

Email: support@SABiosciences.com

Thank you!