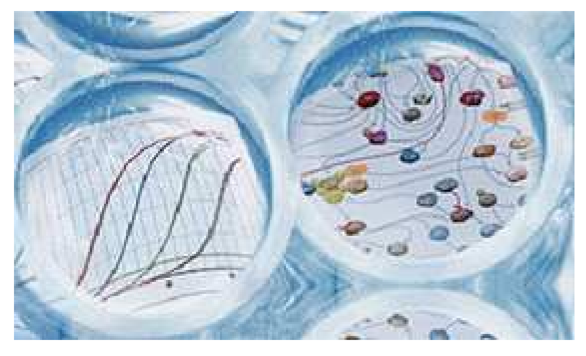
Introduction To Real-Time Quantitative PCR (qPCR)

Webinar-related questions:
QIAwebinars@QIAGEN.com

Technical Support:
BRCsupport@qiagen.com



qPCR technology overview, applications, data analysis and interpretation

- ❑ Part 1: Introduction to Real Time PCR (Q-PCR/qPCR/qrt-PCR)
- ❑ Part 2: Pathway-focused Gene Expression Analysis - Advanced Real-Time PCR Array Technology
- ❑ Part 3: PCR Array Data Analysis Tutorial

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How does qPCR work?

Question: How far apart are the 2 cars?

- Cars race at same speed to finish line
- As car 1 crosses finish line, calculate time for car 2 to finish
- Calculate difference in starting position mathematically (d = rate x time)

How does qPCR work?

Car 2
Finish Line
Car
Car
Car 1
Car
Car

Question: How far apart are the 2 cars?

- Many cars; how to differentiate cars of interest

How does qPCR work?

Car 2

Finish Line

Car 1

Question: How far apart are the 2 cars?

- Cars race at same speed to finish line
- As car 1 crosses finish line, calculate time for car 2 to finish
- Calculate difference in starting position mathematically (d = rate x time)

Seminar Topics

1. What is qPCR? Applications and workflow
2. qPCR for gene expression: What is the change in gene expression during differentiation?
3. Factors Critical For A Successful qPCR Assay
4. RNA purity and integrity
5. Reverse Transcription
6. qPCR in Action
7. Reporter chemistries
8. Characteristics of a good qPCR assay
9. Analyzing qPCR curves
10. Data & analysis

What does Real-Time qPCR stand for?

- Quantitative Polymerase Chain Reaction (qPCR) is a **sensitive and reliable** method for detection and quantification of nucleic acid (DNA & RNA) levels.

- It is based on **detection and quantification of fluorescence** emitted from a reporter molecule **at real time**.

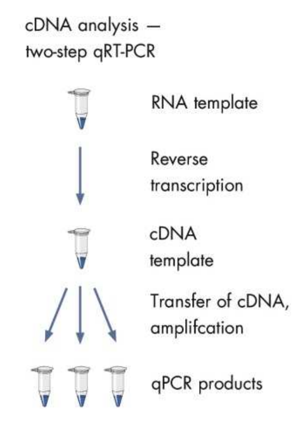
- This detection occurs during the accumulation of the PCR product **with each cycle of amplification**, thus allows monitoring the **PCR reaction during early & exponential phase** where the first significant increase in the amount of PCR product correlates to the initial amount of target template.

What is qPCR? Applications and workflow

Applications for qPCR

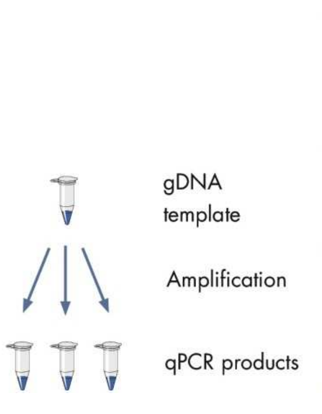
RNA

- Gene Expression Profiling Analysis
- miRNA Expression Profiling Analysis

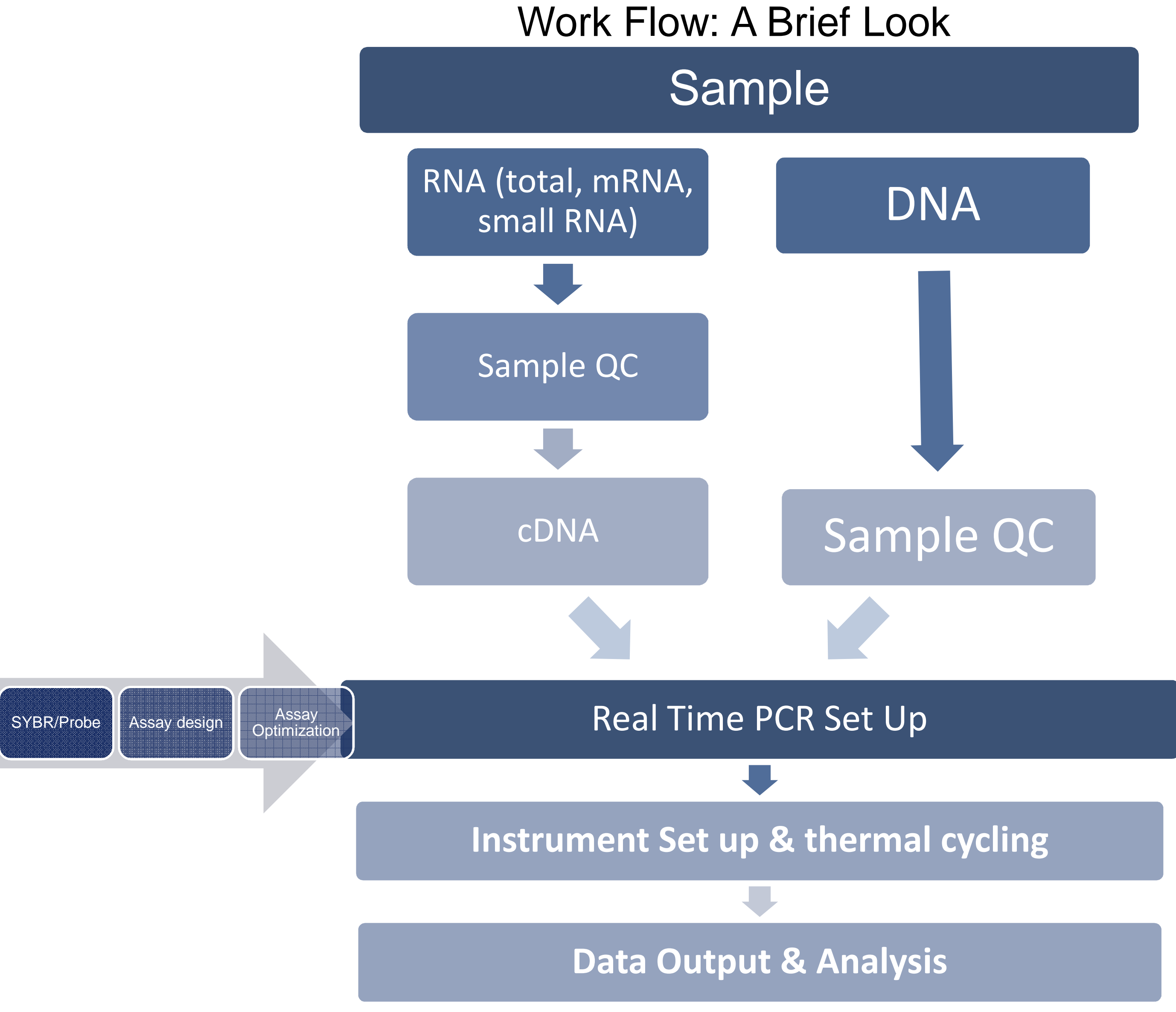


DNA

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



What is qPCR? Applications and workflow



What is qPCR? Applications and workflow

Applications for qPCR

RNA

- Gene Expression Profiling Analysis
- miRNA Expression Profiling Analysis

DNA

- SNP Genotyping & allelic discrimination
- Somatic Mutation Analysis
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- DNA Methylation Detection
- Pathogen Detection
- Viral Quantification

qPCR for gene expression: Application example

hMSC

Osteogenesis – Day 16

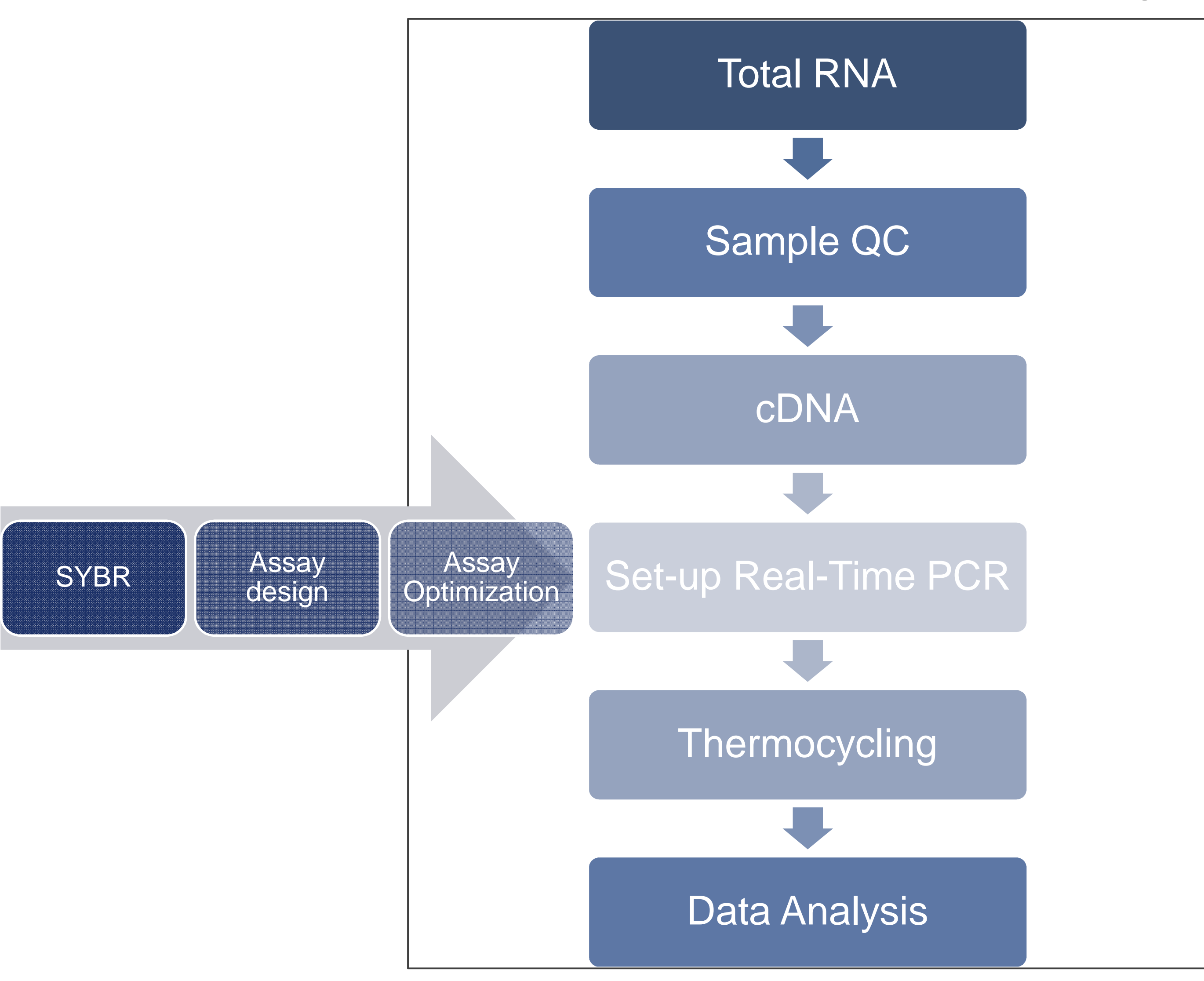
T1 T2 T3 T4

Neurogenesis – 72 hr

T1 T2 T3 T4

Gene expression changes during differentiation

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

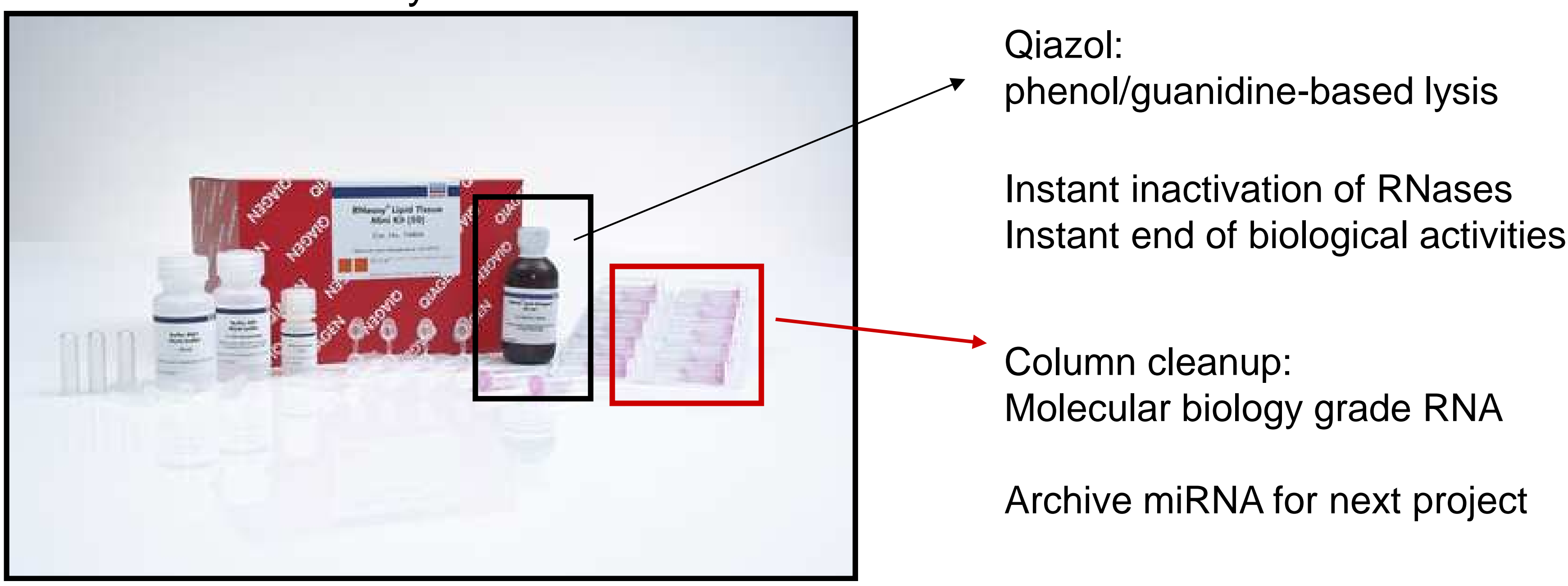

Work Flow: Gene expression profiling
Total RNA
Sample QC
cDNA
Set-up Real-Time PCR
Thermocycling
Data Analysis
SYBR
Assay design
Assay Optimization

Factors Critical For A Successful qPCR Assay

- DNA or RNA sample preparation - Template quality
  - Appropriate sample prep kits/reagents
  - Inhibitors can compromise RT or PCR
- Reverse transcription to convert 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
  - Commercial mastermix or make own (primer, probe, master mix)
- Data analysis tool
  - User friendly
  - Streamlined data analysis module

RNA purity and integrity

- 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



RNA purity and integrity

Purity/ Quantity:

Spectroscopic: measure 260/280 and 260/230

•OD260 is used to calculate amount of nucleic acid

•260/280 ratio (typical minimum value 1.8-2.0)

•260/230 ratio (typical minimum value 1.7)

- Low ratio may indicate a contaminant; protein, QIAzol, Carbohydrates, Glycogen
- Absorbance measurements do not show integrity of RNA

Integrity:

Denaturing RNA Agarose Gel

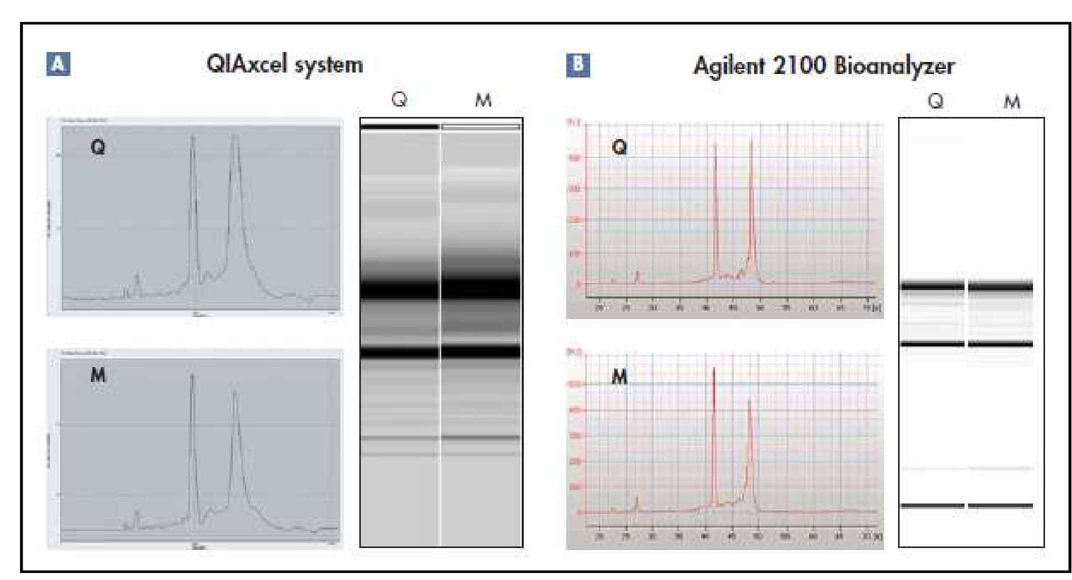
•Usually through ribosomal bands

QIAxcel/ Bioanalyzer

•Capillary electrophoresis

•Automate RNA integrity analysis

•RNA integrity analysis number



Factors Critical For A Successful qPCR Assay

qPCR Components

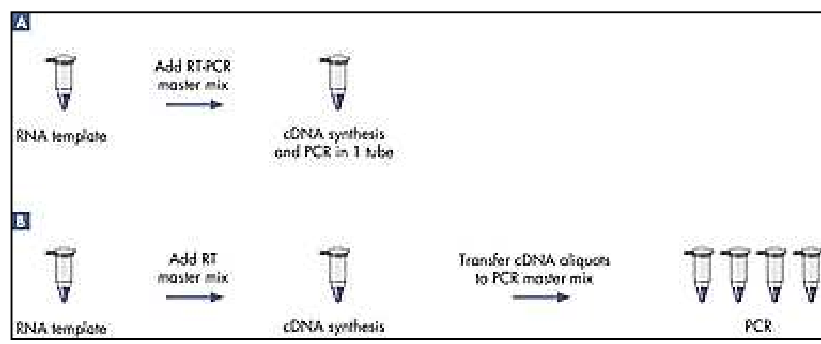
A. Templates:
- RNA
  - Starting amount ~10-1000 copies of NA per qPCR assay
  - For a low-expressed gene, need 10ng equivalent of RNA per reaction
  - Want to start with about 100pg to 1ug RNA
- Reverse Transcription
  - One-Step or Two-Step Reaction

B. Primers/Probes

C. Master Mix
- DNA polymerase
- Mg++
- dNTPs
- Buffer
- Passive reference dye

One-Step PCR
- 1 Tube Reaction

Two-Step PCR
- 2 separate reactions
- RT Reaction
- qPCR Reaction



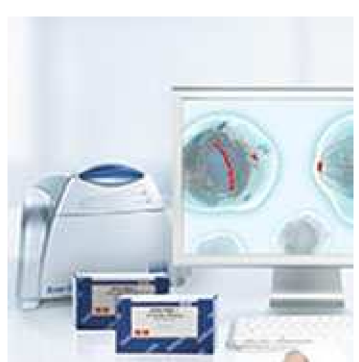
Reverse Transcription: Used to make cDNA copy of RNA

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?

Important Notes:

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



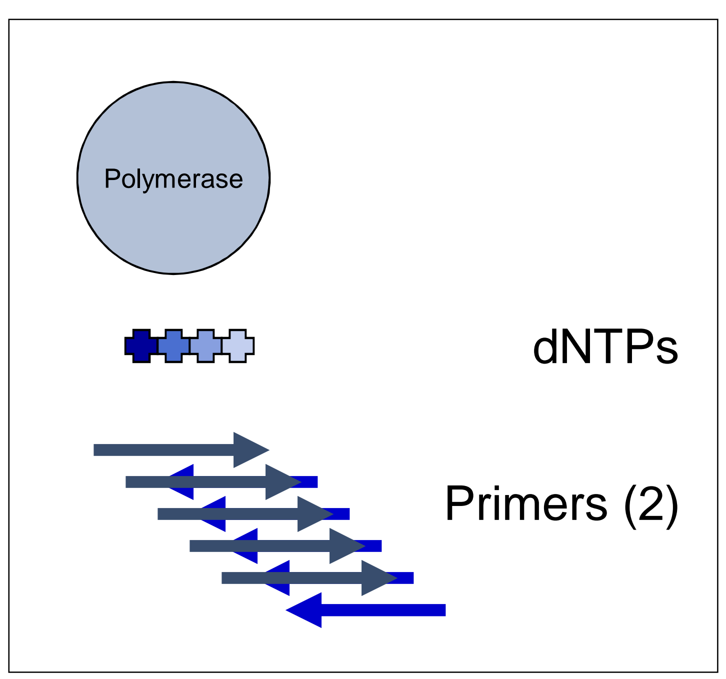
qPCR in Action

DNA Template
(ss or ds)

What is in a PCR Reaction?

PCR= **P**olymerase **C**hain **R**eaction
Exponential Amplification of DNA in single tube
All reagents in excess (non-limiting)

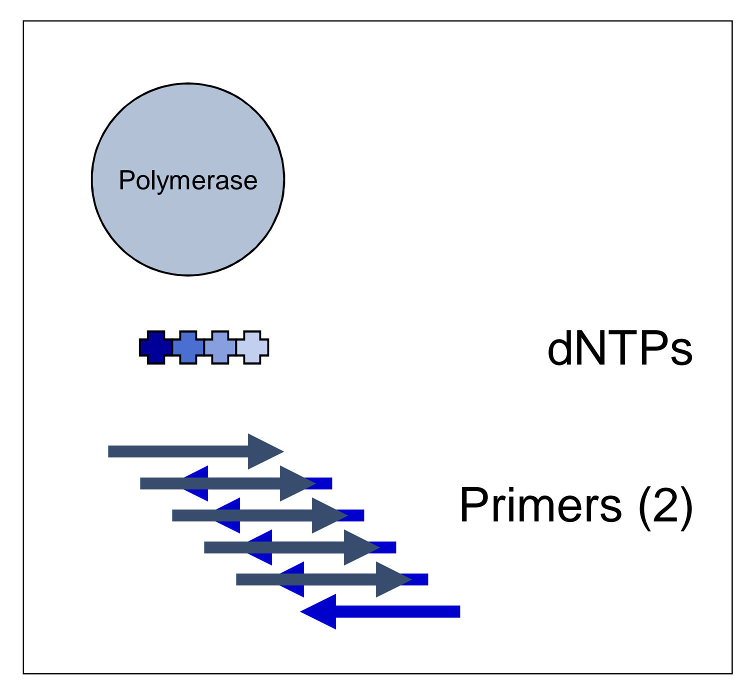
Components:
•Thermostable polymerase
•dNTPs
•Primers
•Template



qPCR in Action

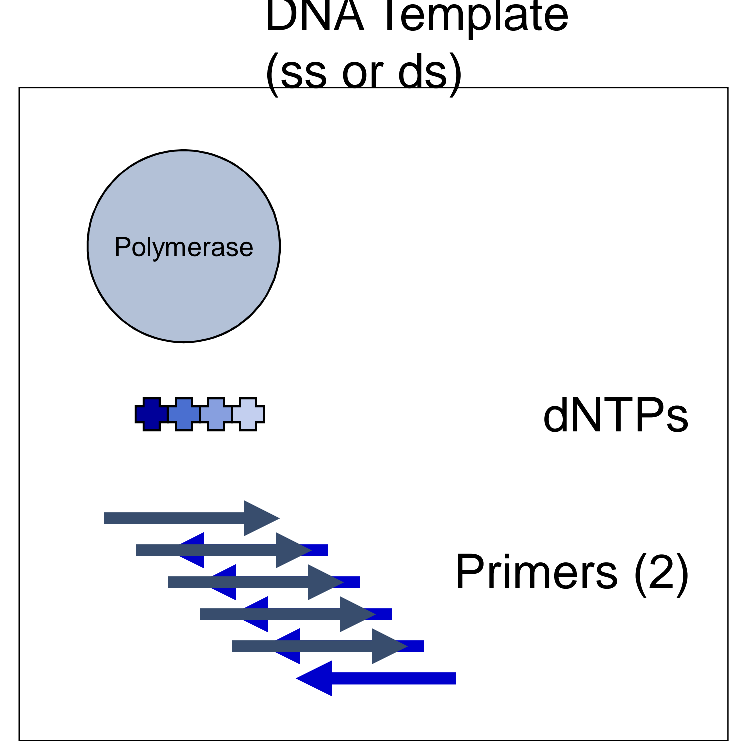
DNA Template
(ss or ds)

1. Heat denature template (~95C)
2. Annealing (~60C)
3. Extension (~60C)
4. Repeat (~95C)



Heat denature

DNA Template
(ss or ds)



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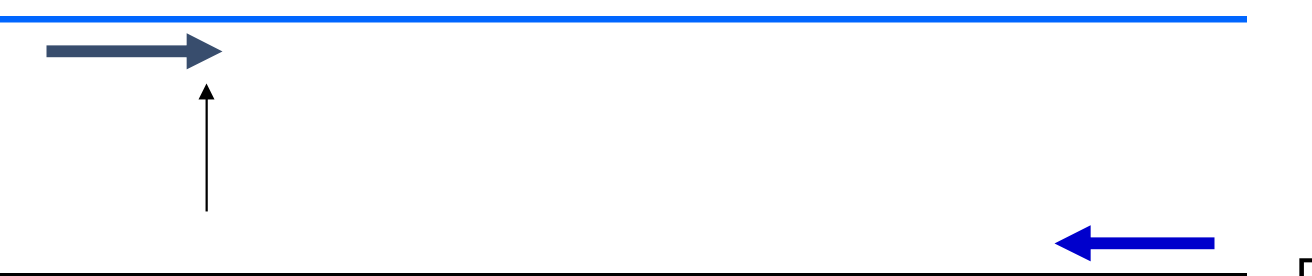
qPCR in Action



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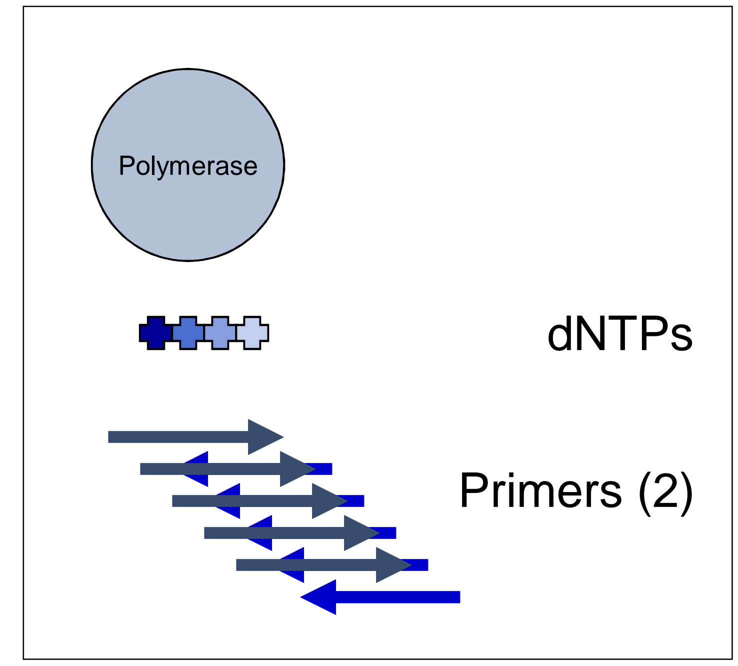
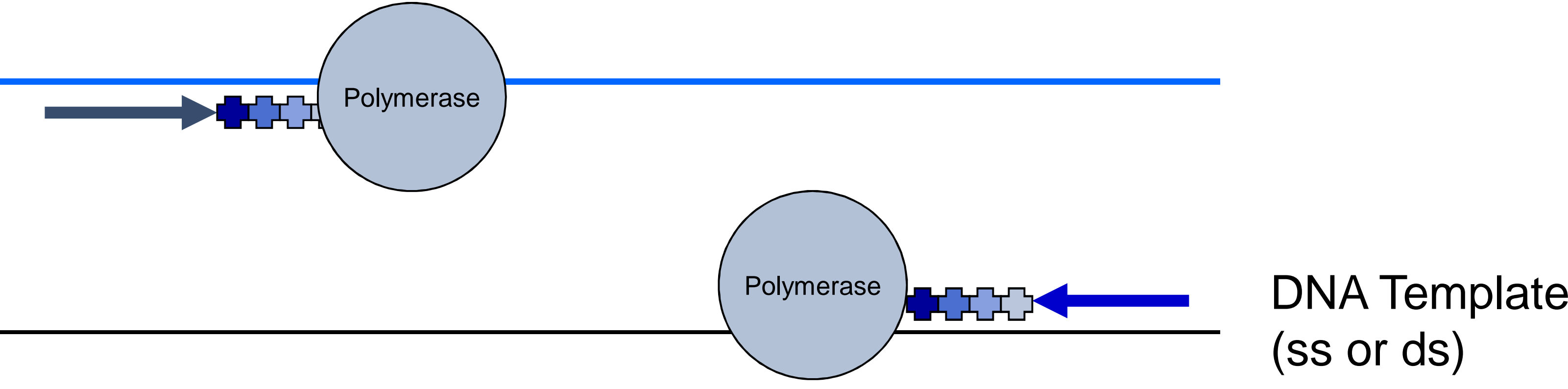
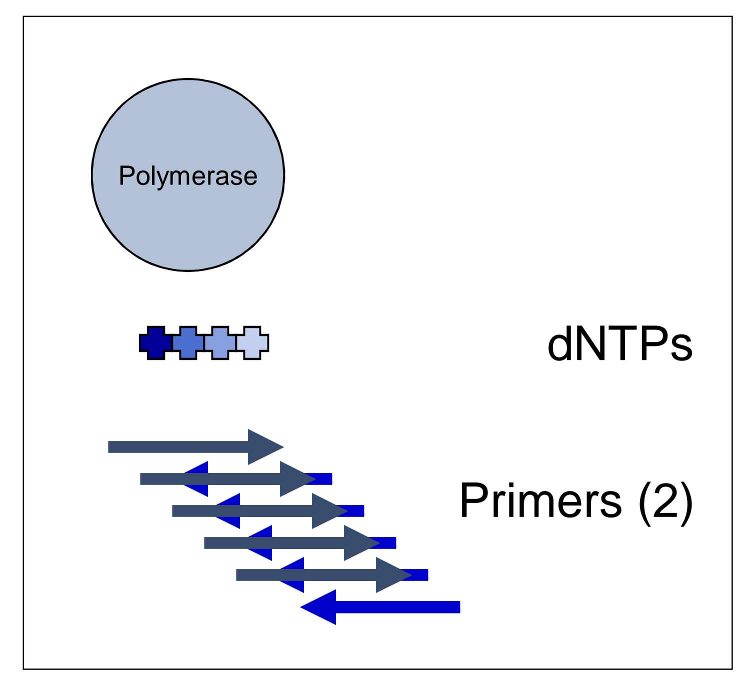


qPCR in Action





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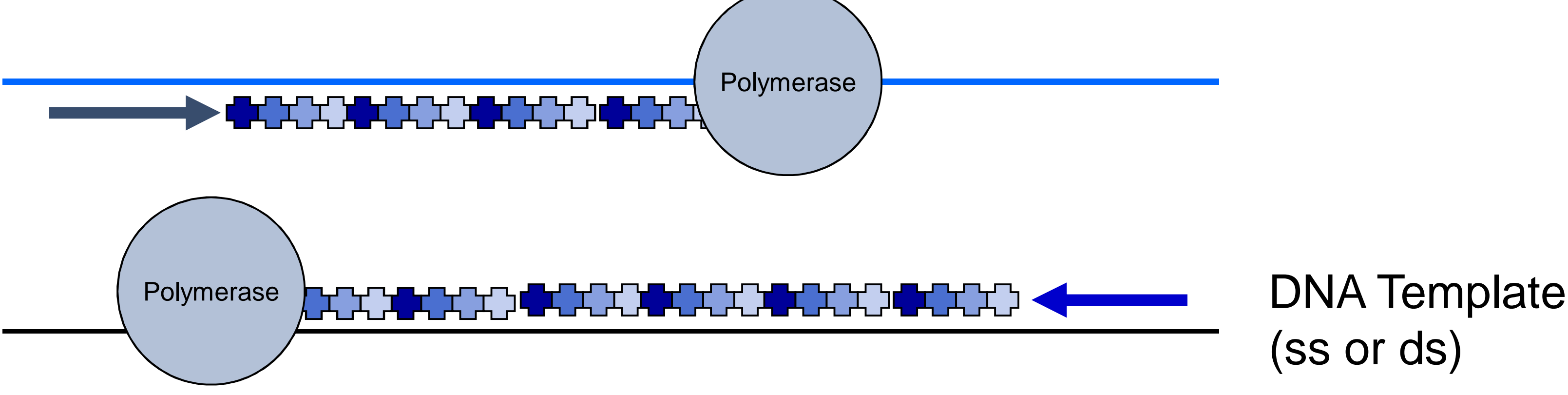
qPCR in Action



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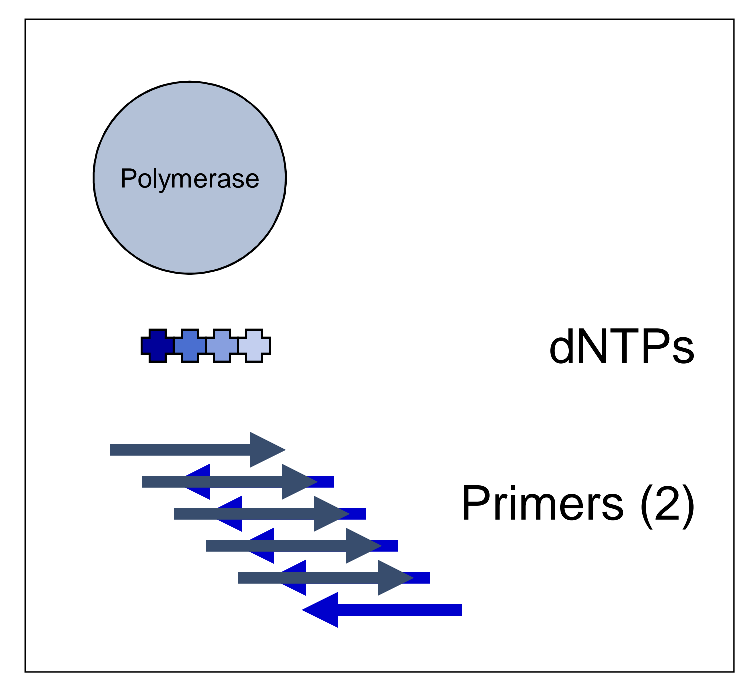
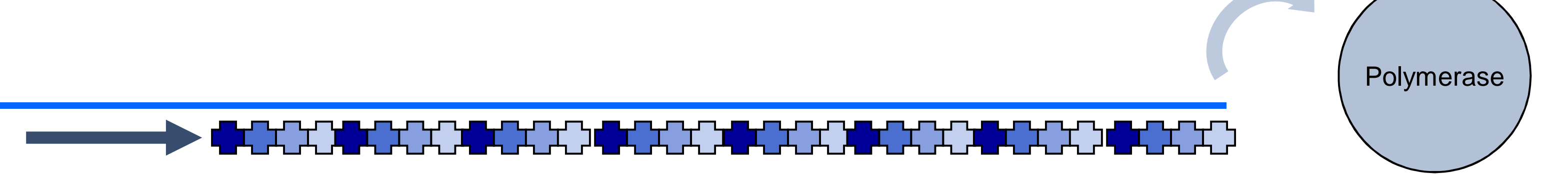
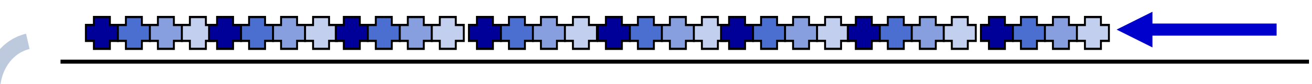


qPCR in Action





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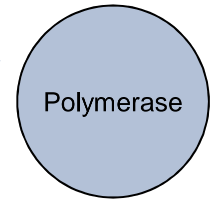

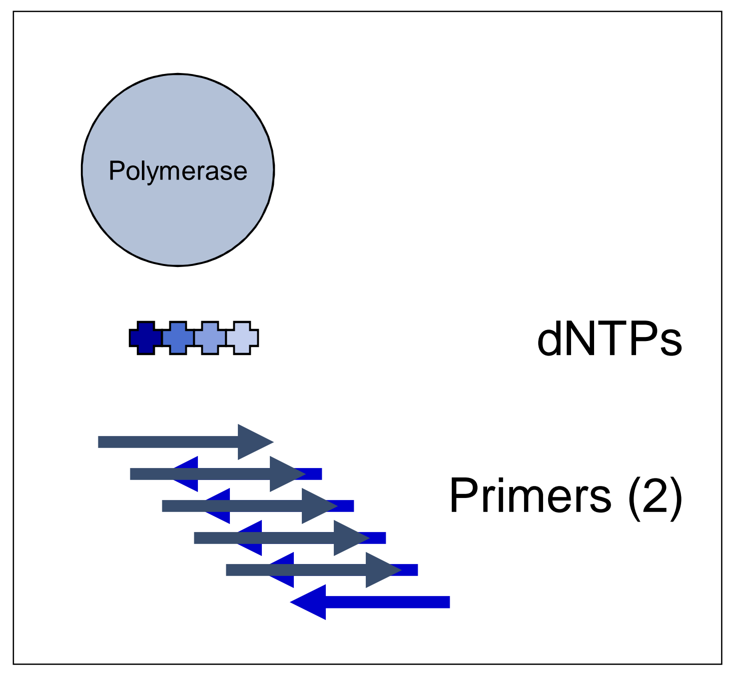

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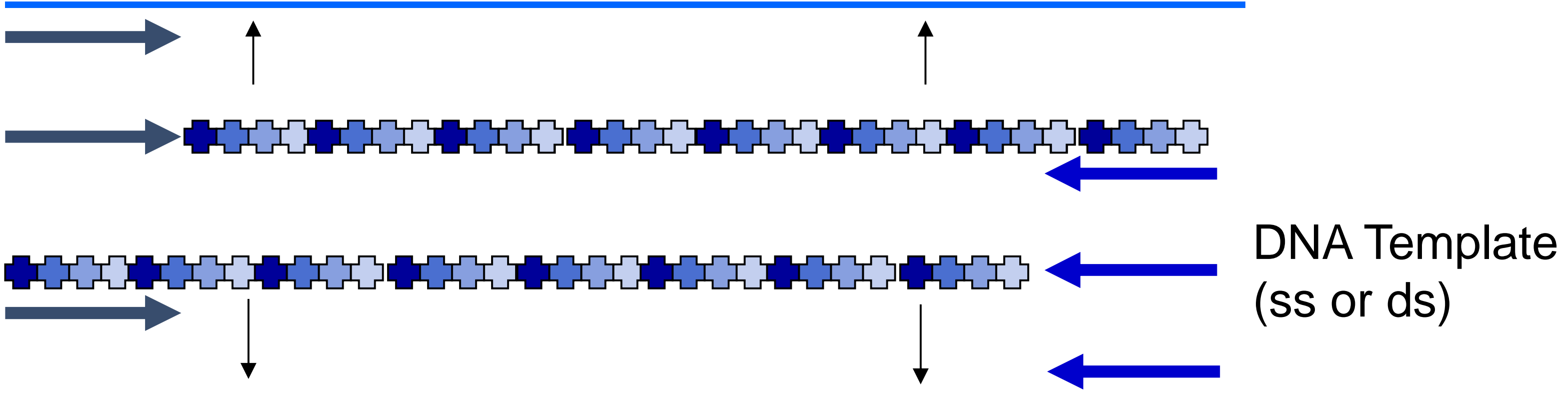

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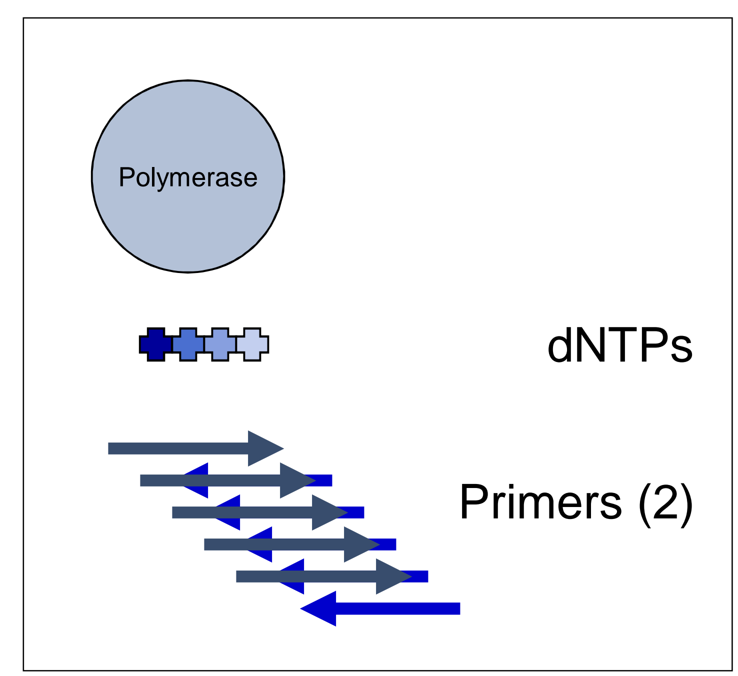
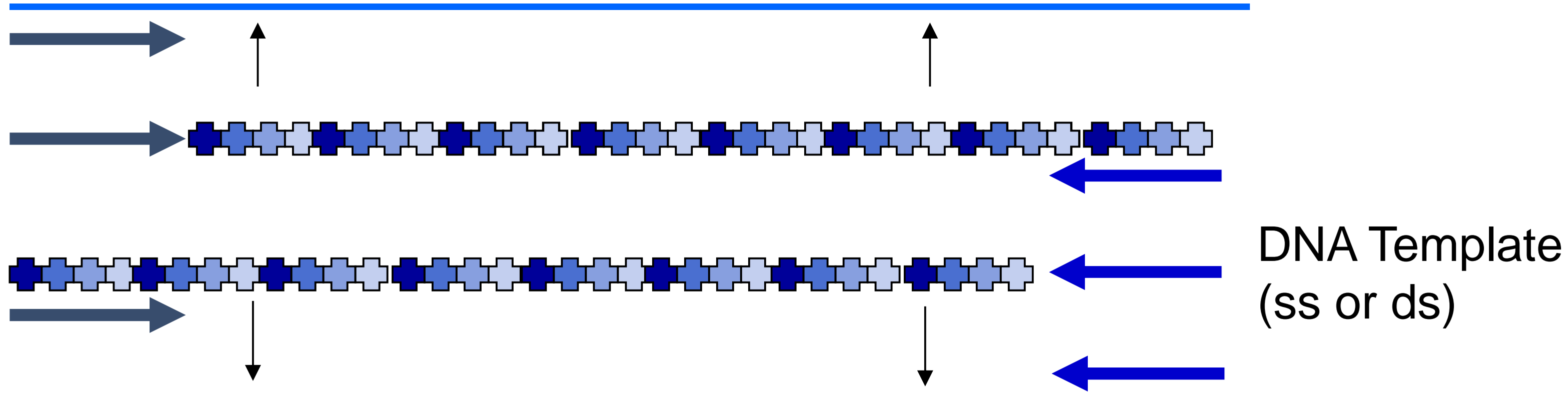


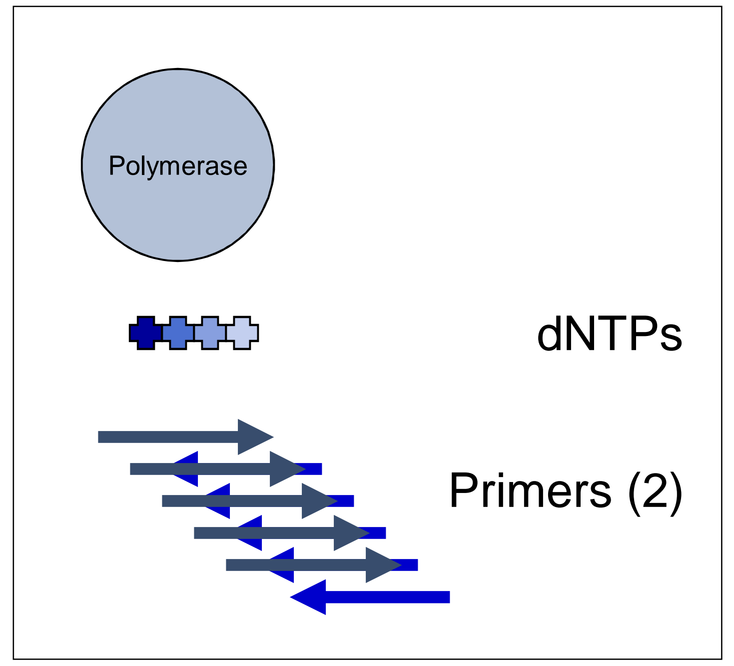
qPCR in Action



How do you make this a quantitative PCR?

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

1. Heat denature template (~95C)
2. Annealing (~60C)
3. Extension (~60C)
4. **Measure amount of PCR Product**
5. Repeat (~95C)

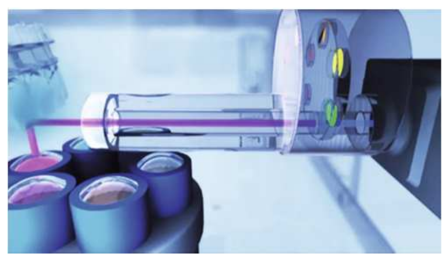


Real-Time qPCR Fluorescence Chemistry

- DNA binding agents
  - SYBR® I Dye

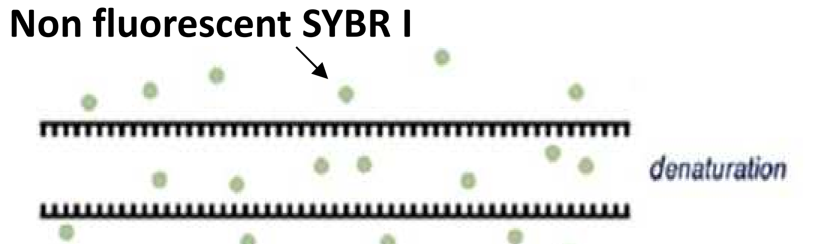
- Hydrolysis Probes
  - Dual-labeled Hydrolysis (Taqman®) probe

- Others, such as hybridization probes
  - Molecular beacon and scorpion probes

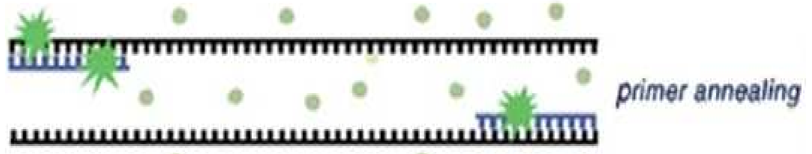


Reporter chemistries: SYBR® Green I Assay

Non fluorescent SYBR I

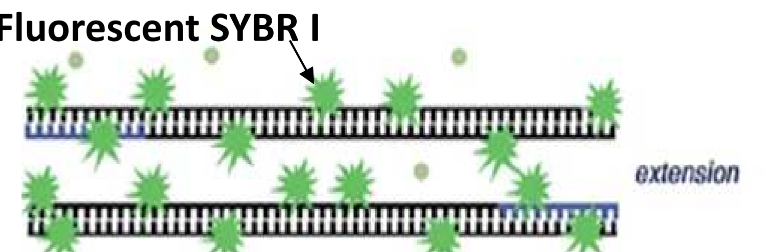


SYBR I binds to double-strand DNA but not single strand DNA. Little fluorescence emitted from SYBR I in solution.



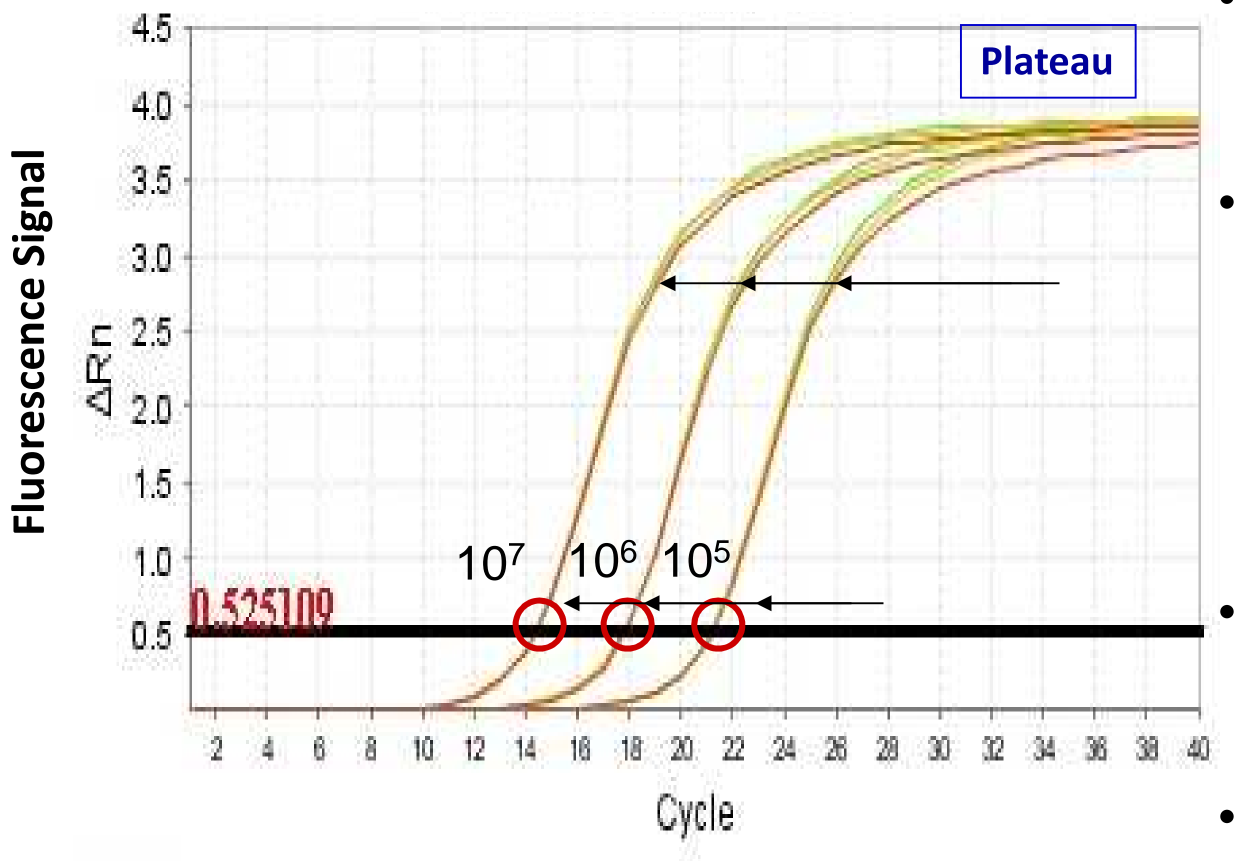
SYBR I upon binding to double-strand DNA emits fluorescence very brightly

Fluorescent SYBR I



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

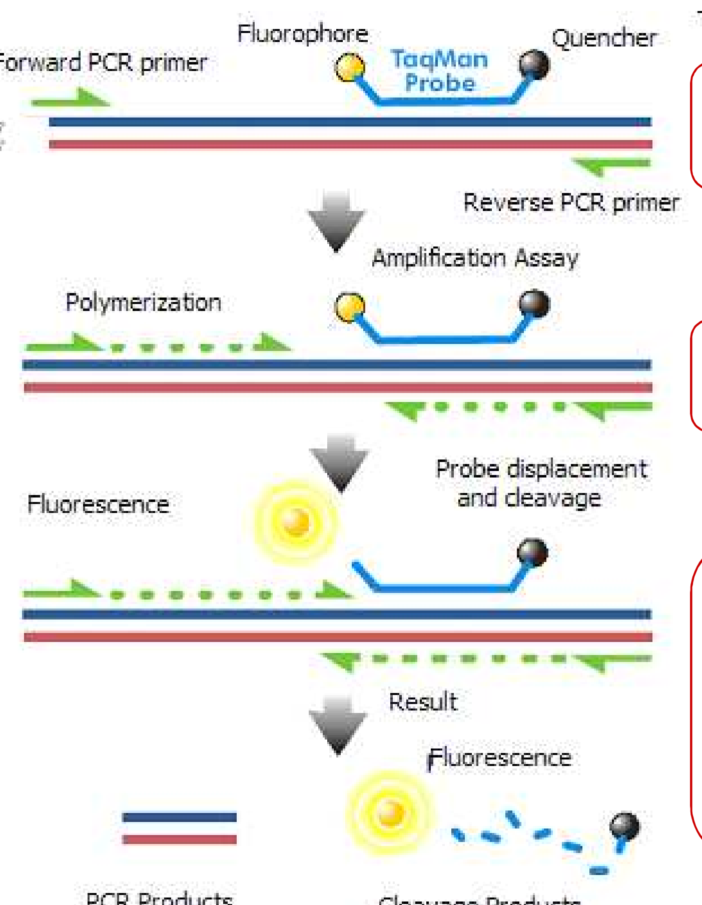
- Simple & cost saving
- **High Specificity Is Required** when using SYBR Green since SYBR I binds all double-strand DNA (non-specific or primer dimer).



- End-point PCR data collection at plateau (gel analysis)
- Reactions start varying due to reagent depletion & decreased PCR efficiencies (enzyme activity, more product competing for primer annealing
- Real time PCR does early phase detection at the exponential state
- Precisely proportional to input amounts

Reporter Chemistries

Hydrolysis Based Probe - - - Taqman® Probe Assay



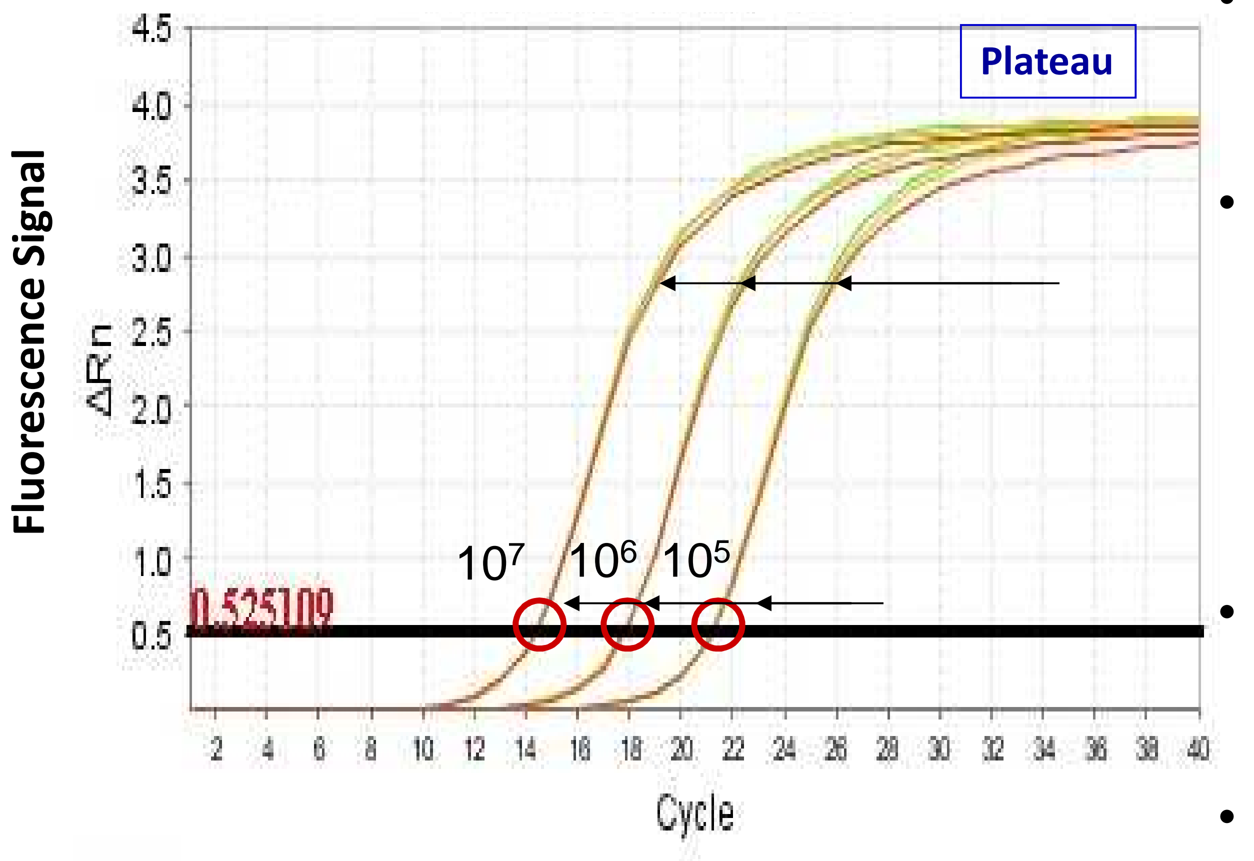
The fluorescence of the reporter dye is suppressed by the quencher

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)

Reporter Chemistries: Understanding Kinetics in PCR



- End-point PCR data collection at plateau (gel analysis)
- Reactions start varying due to reagent depletion & decreased PCR efficiencies (enzyme activity, more product competing for primer annealing
- Real time PCR does early phase detection at the exponential state
- Precisely proportional to input amounts

Characteristics of a good qPCR Assay

What factors do you need to address to create a good PCR Assay?

Amplification efficiency: 100% during exponential phase (template product doubles with each cycle)

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)

- Melt-curve analysis - 1 peak, 1 product
- Agarose gel

Dynamic Range: Ability to detect genes with varied expression levels, another judge of sensitivity

- 10 to 10^9 copies is ideal

Reproducibility: Confidence in your results, enables profiling of multiple genes in the same sample

- All lab members get the same results
- Technical reproducibility ensures changes seen in results are due to the biology and not the technology itself or sample handling

Characteristics of a good qPCR Assay: Amplification Efficiency

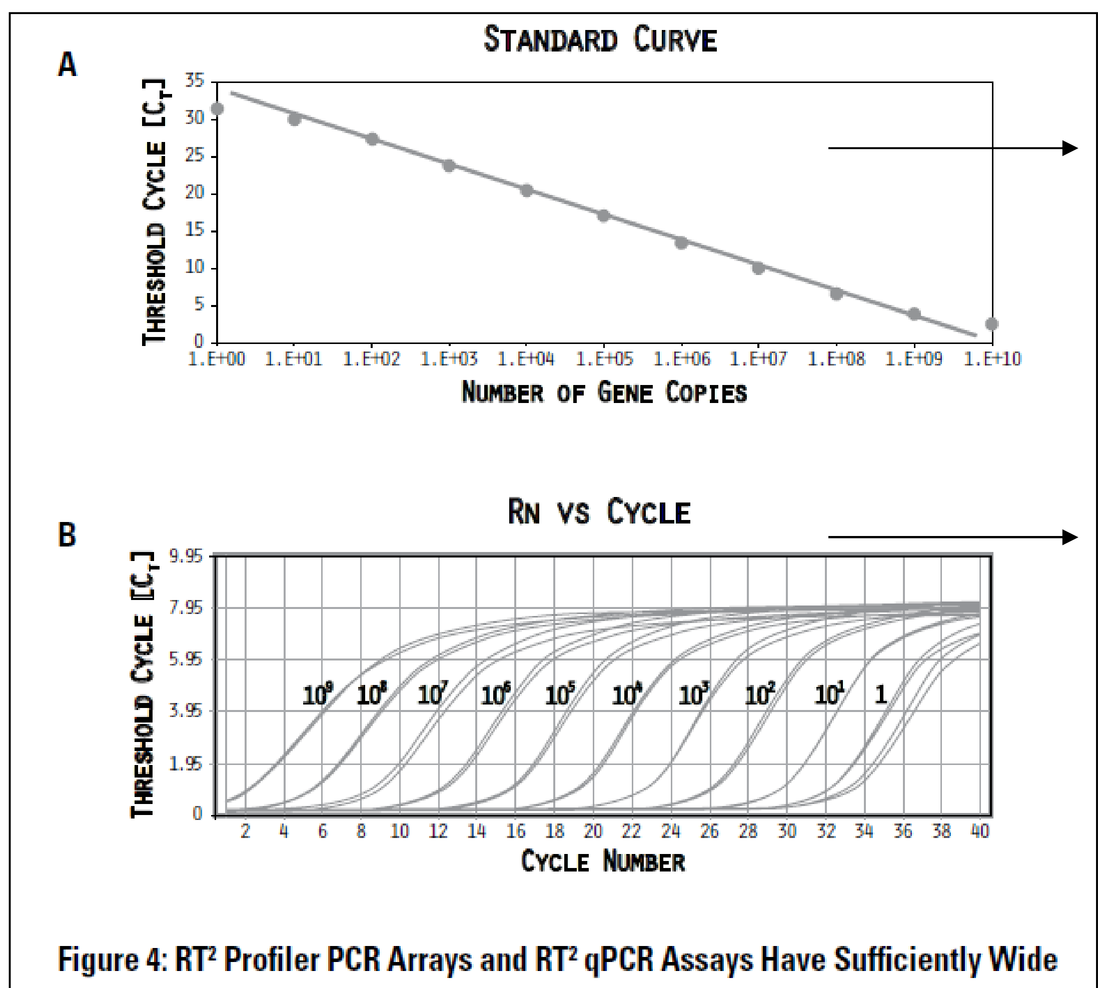


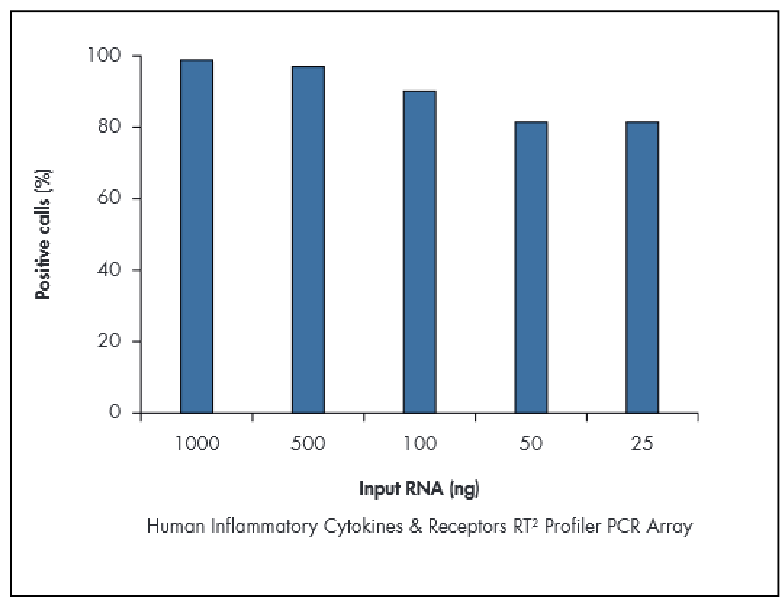
Figure 4: RT² Profiler PCR Arrays and RT² qPCR Assays Have Sufficiently Wide Dynamic Ranges.

Amplification Efficiency: reliable and accurate experiment

Two Methods:

- Standard curve
  - X axis - dilution
  - Y axis - Ct value
  - Amp efficiency = $10^{(-1/slope)}$ -1 *100
- Single curve analysis
  - PCR Miner: http://miner.ewindup.info/version2
  - “DART”: www.gene-quantification.de/DART_PCR_version_1.0.xls

Characteristics of a good qPCR Assay: Sensitivity



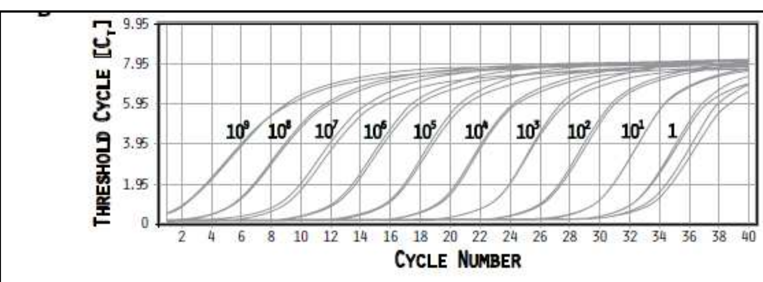


Figure 4: RT² Profiler PCR Arrays and RT² qPCR Assays Have Sufficiently Wide Dynamic Ranges.

Sensitivity: How many copies can my assay detect?

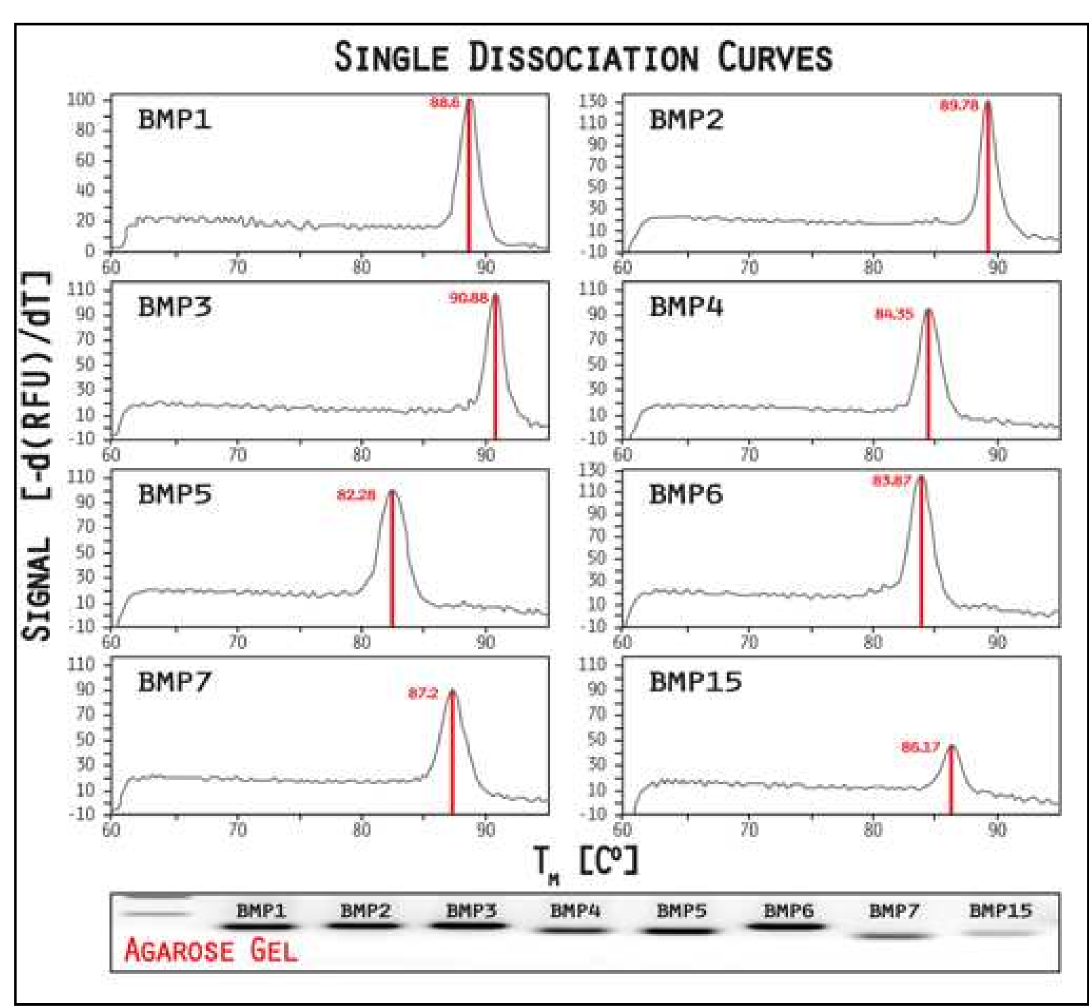
- Important for low expressed genes or where there is limited sample

Two Methods:

- 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)

Characteristics of a good qPCR Assay: Specificity



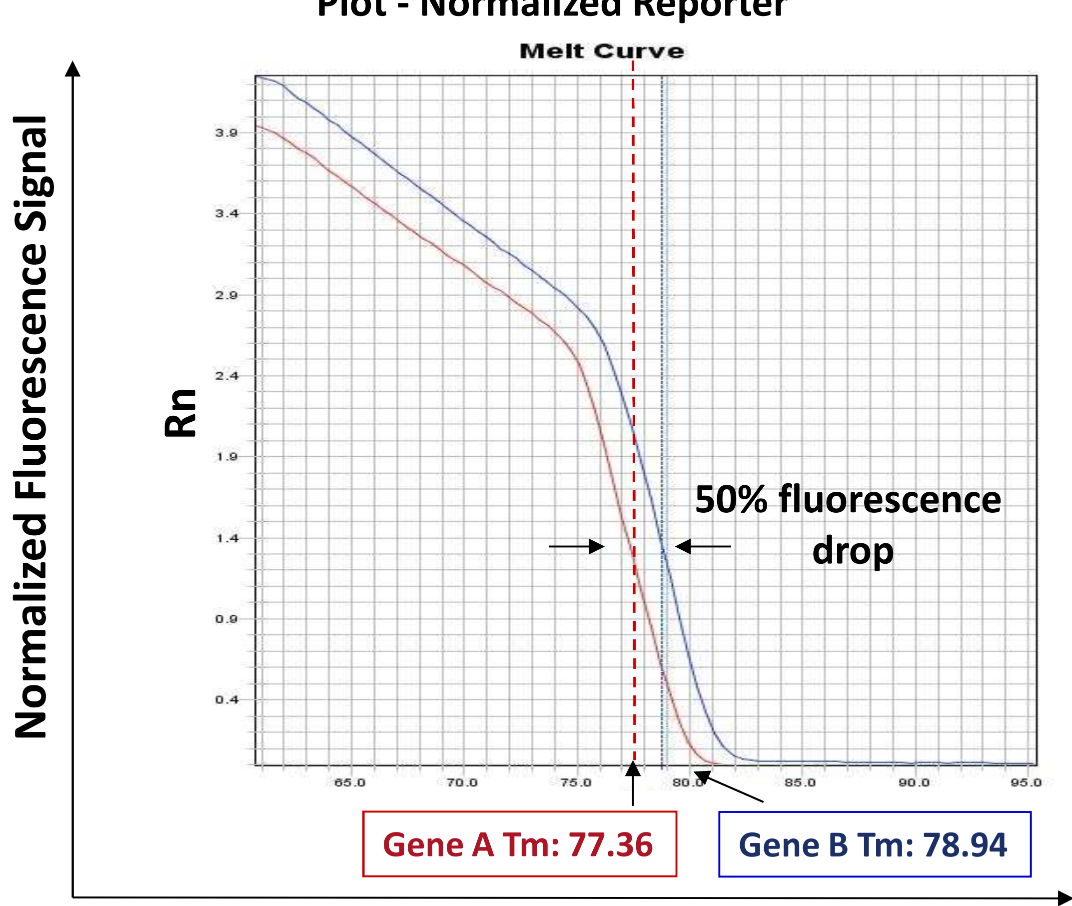
Specificity: 1 target amplified

Two Methods:

- Melt Curve analysis
  - 1 peak, 1 product
- Agarose gel
  - Band at correct size

Characteristics of a good qPCR Assay: Specificity

Melting Curve Analysis

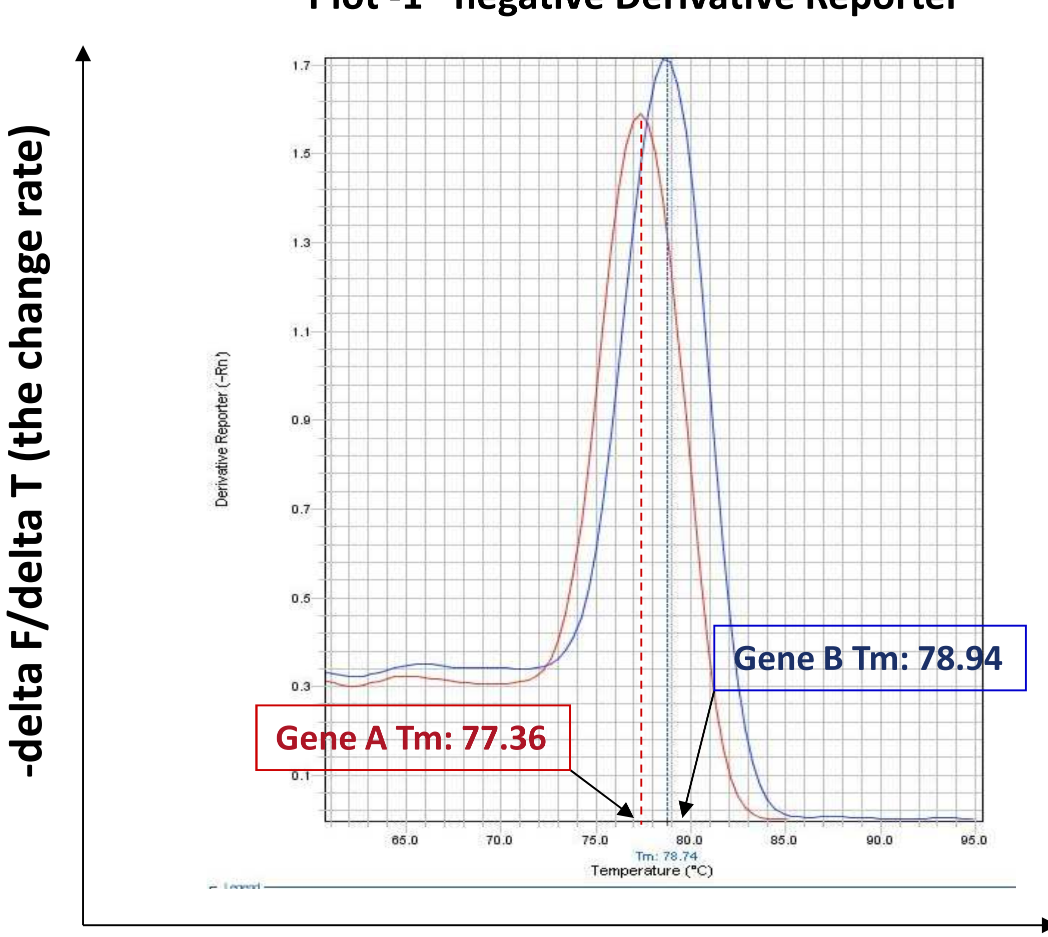


The General Program Steps

- Heat to 94℃ to denature DNA
- Cooling to 60℃ to let DNA double strands anneal
- Slowly heat (increase temp. to 0.2℃/sec) while plotting the fluorescent signal vs. temperature.
- As the temp increases, DNA melts, fluorescent signal should decrease.
- Significant drop in signal when 50% DNA melts.

Characteristics of a good qPCR Assay: Specificity

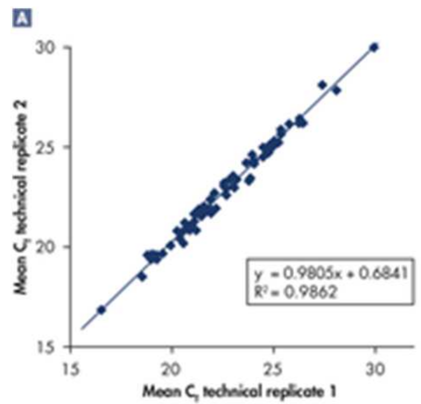
Melting Curve Analysis



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

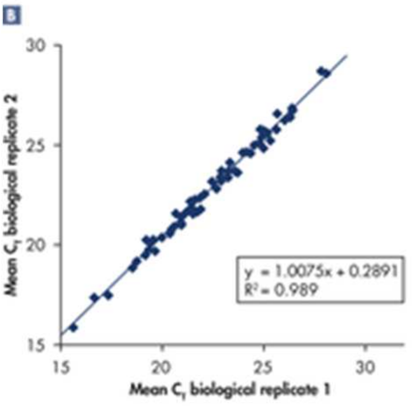
Biological Replicates: 3 different experiments

- Shows variability due to experiment



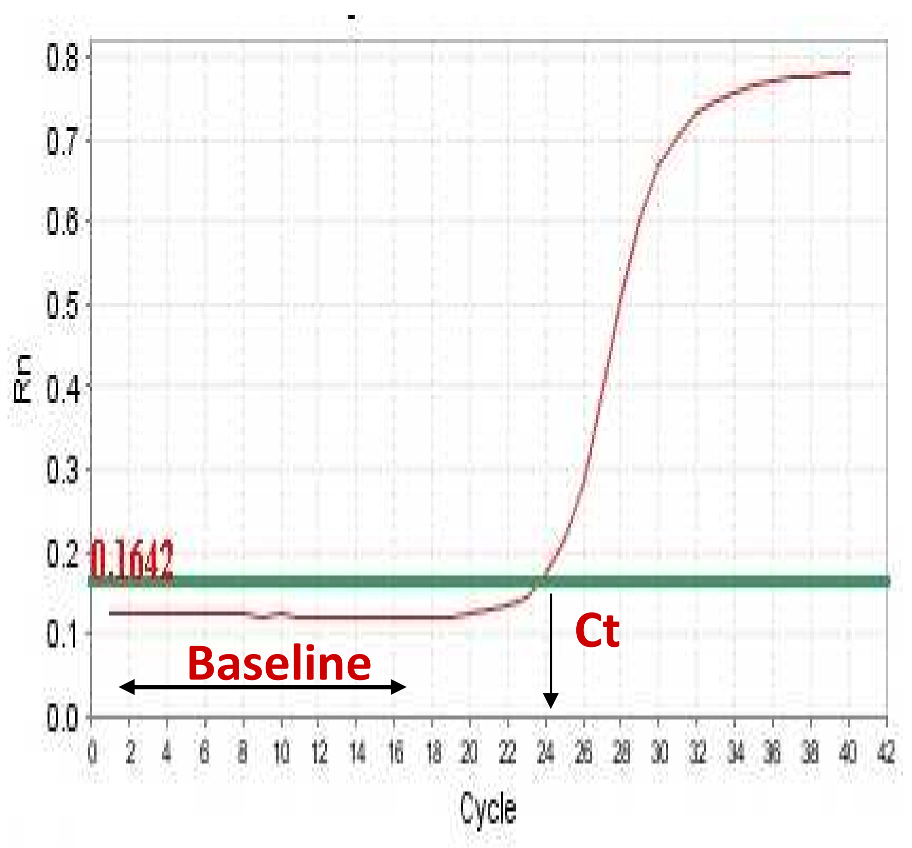
Technical replicates: 3 different measurements for same step

- Shows variability due to pipetting, machine, enzymes, etc.



Analyzing qPCR curves: How to Define Baseline

Linear Amplification Plot



- **Automated Baseline Option**

if an instrument has a adaptive baseline function

- **Manual Baseline Option**

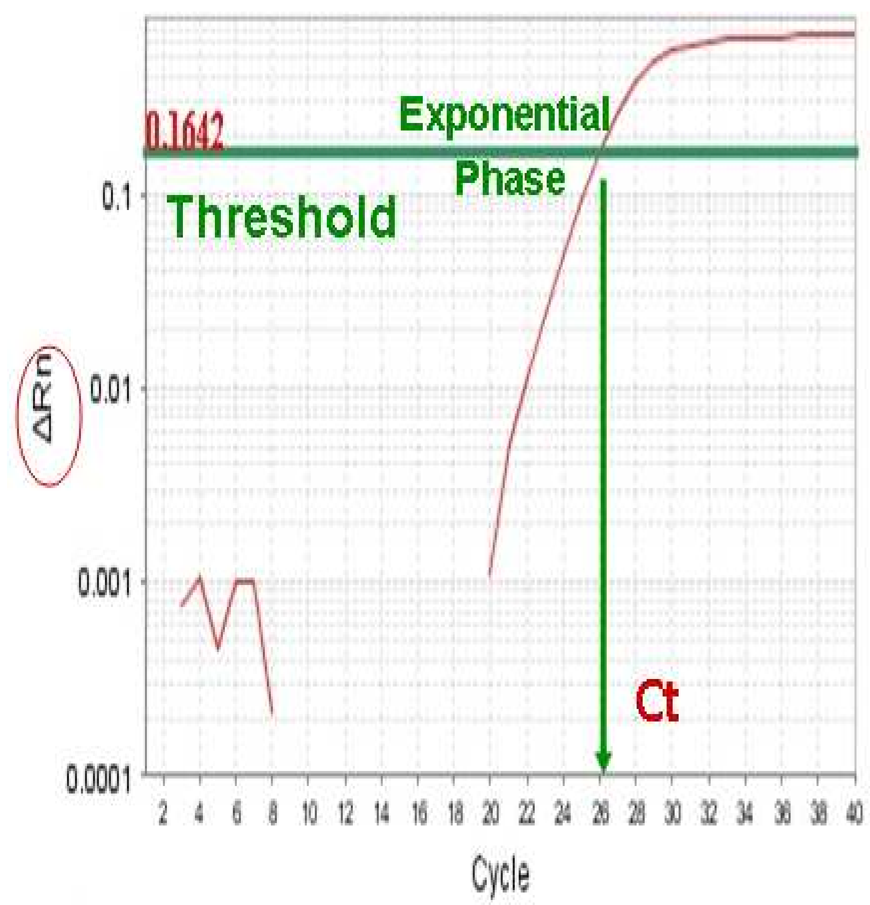
(1) Use linear view of the plot

(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

Analyzing qPCR curves: 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

Data Analysis: Housekeeping/Reference Genes

Any changes?

GOI A in control cells	GOI A in drug treated cells
Ref Gene B in control cells	Ref Gene B in drug treated cells

Reference gene

- Expression level remains consistent under experimental conditions/different tissues
- 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

Data Analysis: Commonly Used Housekeeping Genes

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Table 7. Housekeeping genes commonly used as endogenous references

Gene	Gene symbol Human	Gene symbol Mouse	Relative expression level* Human	Relative expression level* 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 PO		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.

Data & analysis

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

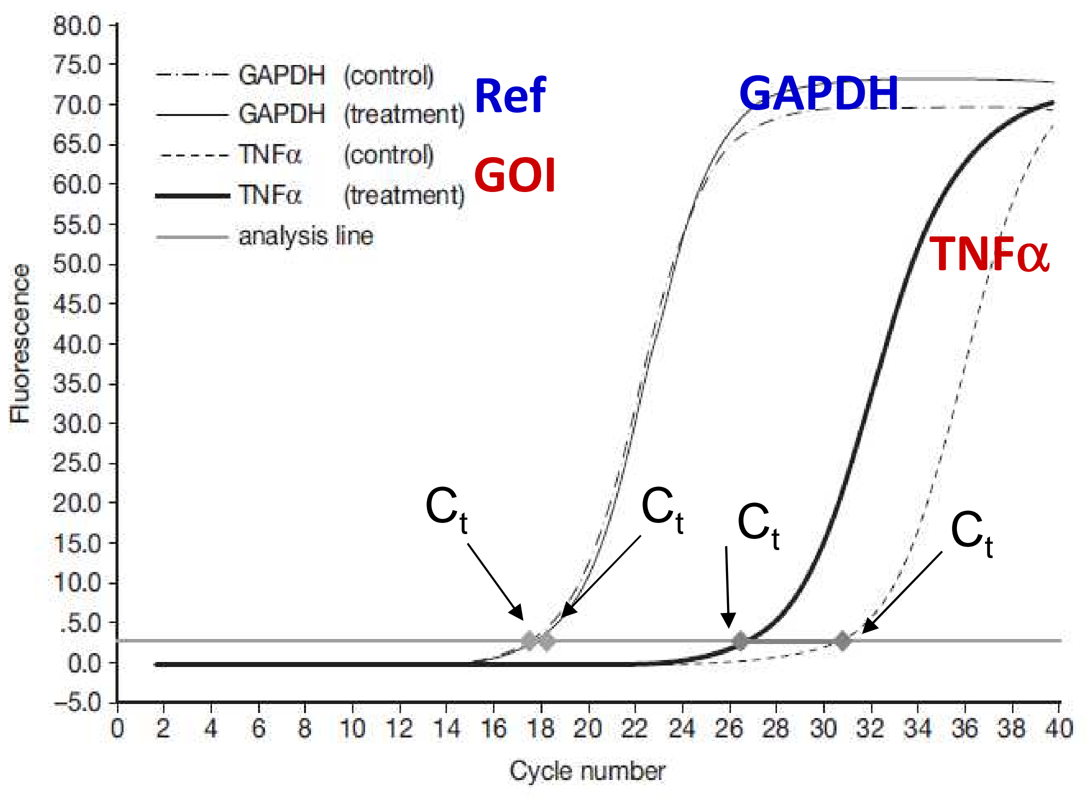
Any changes?

Target Gene A in control cells	Target Gene A in drug treated cells
Reference Gene B in control cells	Ref Gene B in drug treated cells

- 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 = $2^{(-\Delta\Delta Ct)}$

Data & analysis: Delta Delta Ct Method - 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

17.1, 17.2, 17.2 ← qPCR replicates

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

TNFα is up-regulated 32 fold in the treated cells versus the control

Data & analysis

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The GeneGlobe Data Analysis Center is a web resource for scientists analyzing their real-time PCR or NGS data. The real-time PCR modules transform threshold cycle (C_T) values to calculated results for gene and miRNA expression, somatic mutation detection and copy number measurements. The NGS module supports the analysis of QIAGEN's GeneRead target enrichment panels. Register on QIAGEN.com to access this complimentary suite of tools to accelerate your data analysis and interpretation.

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You Selected:

Technology: RT² Profiler PCR Array

Catalog Number: PAHS-021Z

Plate Format: 96-well

Want to analyze data from a different product?
Return to GeneGlobe Data Analysis Center

File: Browse... No file selected. Upload

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

Excel Templates for Formatting your Experimental Data:

96-Well Cataloged PCR Array

Instructions:

Does your data look like this?
If not, please download the appropriate Excel template to format your data correctly.

	A	B	C	D	E	F
1		Resting 6 h	Resting 6 h	Resting 6 h	6-h Stimulation	6-h Stimulation
2		Control Group	Control Group	Control Group	Test Group 1	Test Group 1
3	A01	29.08	29.02	29.27	29.89	29.56
4	A02	32.02	32.13	31.96	31.15	31.27
5	A03	33.83	34.22	33.09	31.57	31.24
6	A04	33.95	33.26	32.65	31.3	32.24
7	A05	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
8	A06	29	28.84	28.53	26.67	26.27
9	A07	Undetermined	37.05	Undetermined	37.28	35.35
10	A08	27.33	27.11	27.31	29.54	29.45
11	A09	25.52	25.6	25.81	21.26	21.29
12	A10	27.12	27.21	27.12	16.77	16.86
13	A11	35.53	36.21	37.66	33.49	36.02
14	A12	23.03	23.28	23.16	21	20.94
15	B01	34.1	34.36	32.92	35.17	35.06
16	B02	33.13	36.08	34.1	33.1	33.11
17	B03	25.3	25.36	25.3	25.02	25

Topics Covered Today

1. What is qPCR? Applications and workflow
2. qPCR for gene expression: What is the change in gene expression during differentiation?
3. Factors Critical For A Successful qPCR Assay
4. RNA purity and integrity
5. Reverse Transcription
6. qPCR in Action
7. Reporter chemistries
8. Characteristics of a good qPCR assay
9. Analyzing qPCR curves
10. Data & analysis

Upcoming webinars

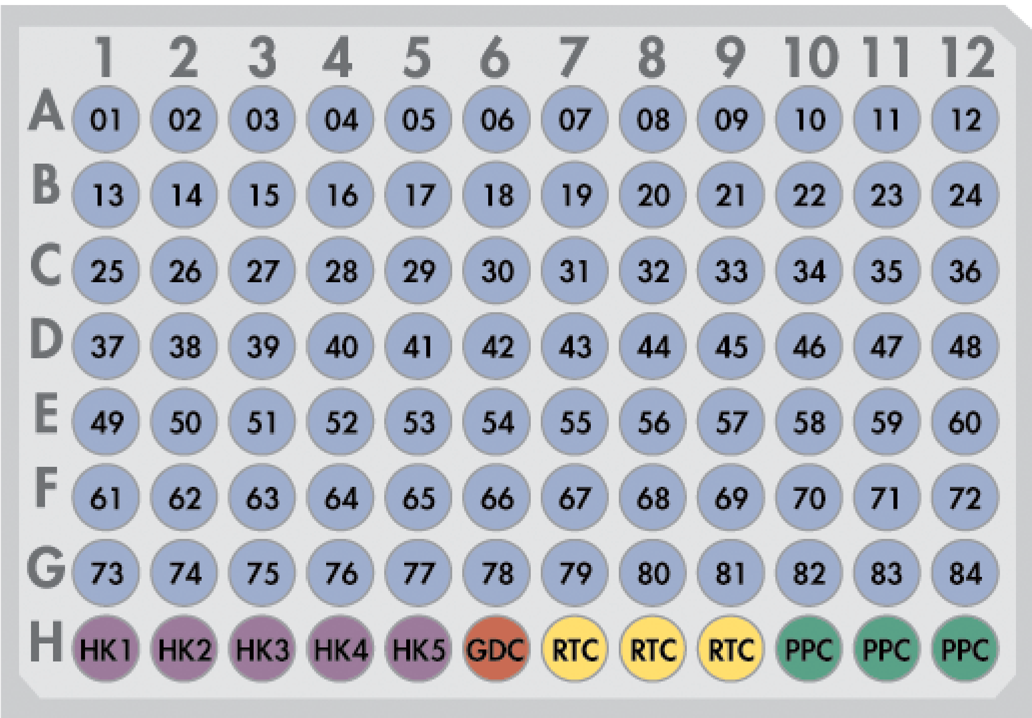
Monday	Tuesday	Wednesday	Thursday	Friday
2 Analysis somatic BRCA1 and BRCA2 variants*	3 Cancer stem cells	4 Exosomes in liquid biopsy*	5 Research solutions for liquid biopsies	6
9 Host-pathogen interactions*	10 NGS: introduction to technology and applications	11 Maximize quantity and purity of exosomal RNA*	12 Genomic biomarker discovery	13 New advances in lncRNA research† qPCR introduction
16 Microbiome: from identification to characterization*	17 Addressing the challenges of NGS workflows	18 Biomarker discovery in biofluids*	19 Circulating biomarkers: New solutions for DNA and RNA	20 PCR arrays for pathway analysis
23 Innate immune system*	24 Targeted NGS for cancer research	25 Meeting the challenges of biomarker research*	26 Critical factors for successful RT-PCR* lncRNAs in cancer & miRNA regulation	27 Optimize NGS conditions based on your DNA sample† PCR array data analysis tutorial
30 Toll-like receptors in inflammation*	31 NGS data analysis for genetic profiling			

- 9:30 am EST (2:30pm GMT)*
- 1:00 pm EST (6:00pm GMT)
- 11:00 am CST (Beijing Time)†

Register:
http://www.qiagen.com/Knowledge-and-Support/Webinars/

Learn about RT² Profiler PCR Arrays

Catalogued RT² Profiler by pathway & disease



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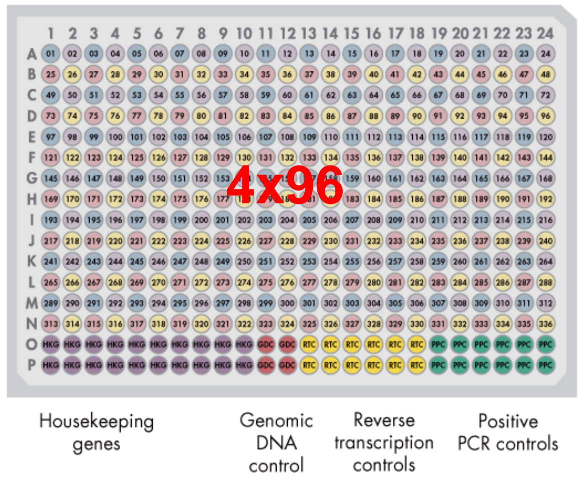

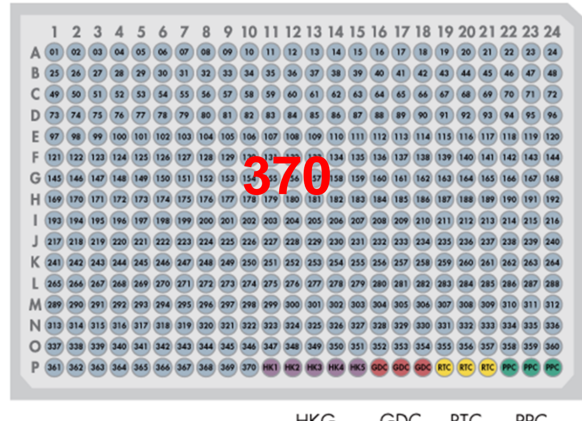

Pre-validated qPCR assays with controls

Thank You for Attending

Questions? Ask now or contact Technical Support

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