iQ5 & MYiQ Real-Time PCR

CFX-96 and CFX Connect Real-Time PCR

CFX96/CFX Connect

iQ5/MYQ

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What is Quantitative PCR?

The monitoring of PCR reactions in real time for the estimation of relative or absolute gene expression

Quantitative PCR

Through the use of fluorescent dyes (SYBR Green, fluorescent probes) that detect PCR products, real-time PCR measures the reaction directly during amplification

Why Use Quantitative PCR?

More accurate than gel-based reverse transcriptase PCR

Publications are requiring real time PCR data rather than RT PCRs

Ease and speed of quantification

Can do many technical replicates without exhausting cDNA template

Real-Time PCR: Applications

Real-Time qPCR provides information for **relative** or **absolute** measurements of starting material.

- Gene Expression Studies
- NextGen sequencing/Microarray Validation
- Chromatin Immunoprecipitation (ChIP)
- Allelic Discrimination/SNP
- Transgene Analysis/GMO Testing
- Viral/Bacterial Load Studies

Limitations of Conventional PCR

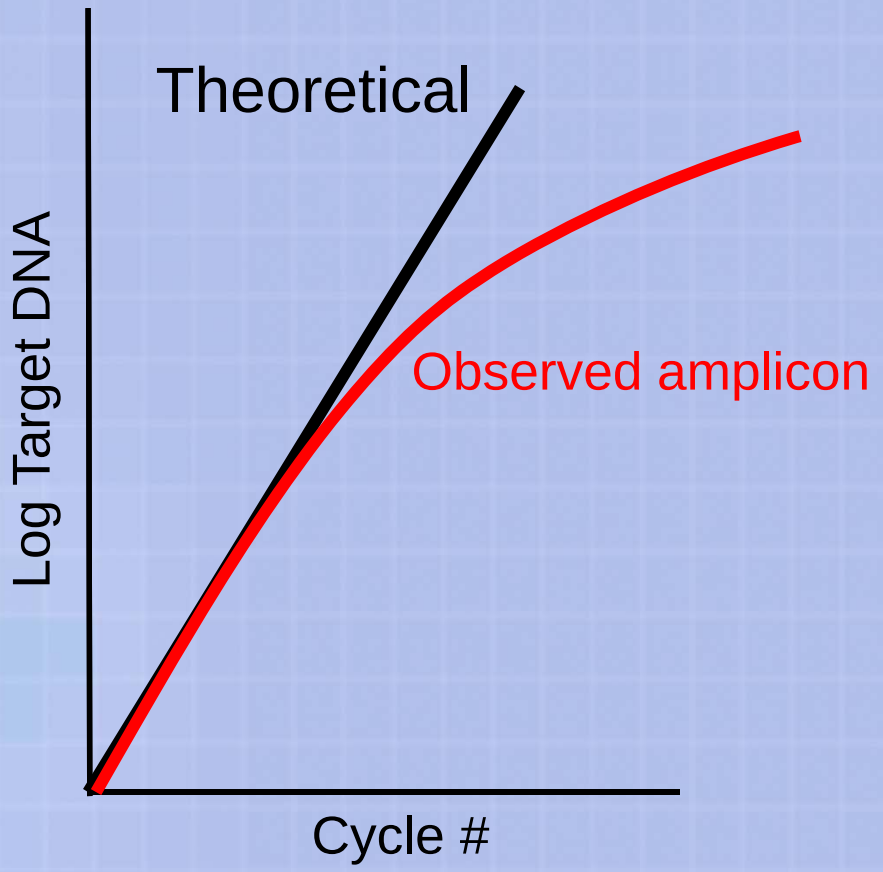
Amplification is exponential initially

In theory, the amount of DNA produced at every cycle should double,

Product = Template quantity x 2^n

(n = # of cycles)

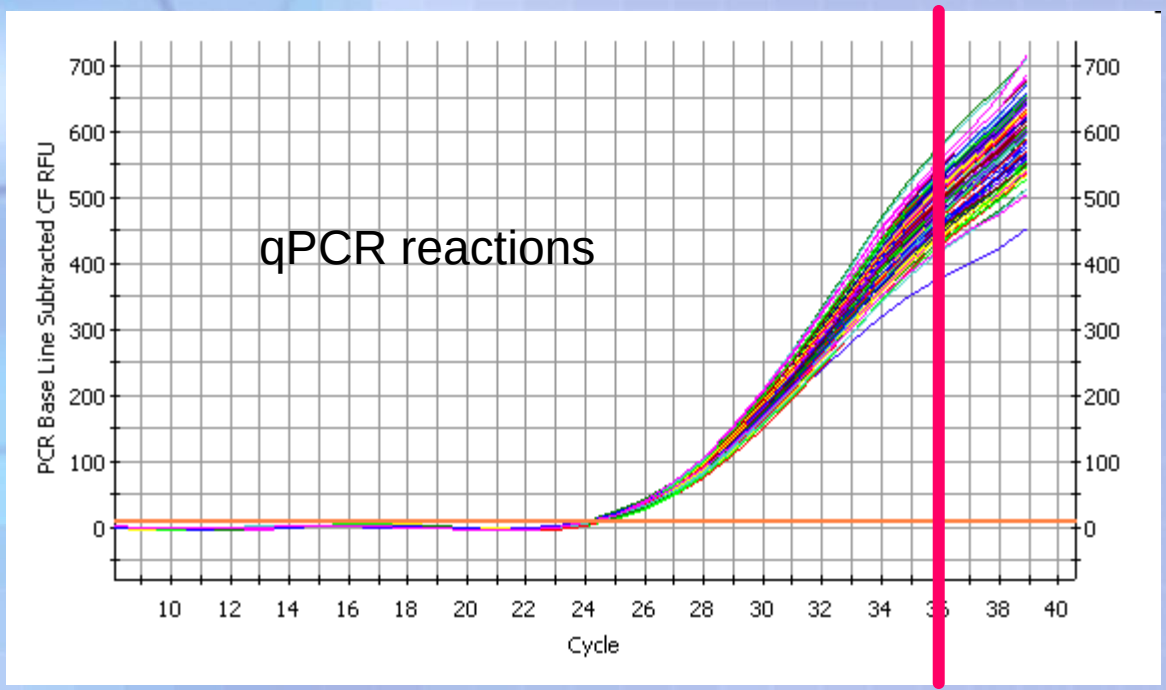
But the exponential phase becomes linear as reactants become rate-limiting



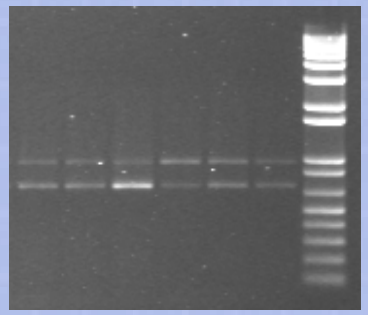
Gel-based RT PCR is an End-Point Analysis

96 identical reactions will have very different final amounts of fluorescence at endpoint due to differences in kinetics and pipetting

Relative fluorescence
(DNA amount)



Gel-based RT PCR
to measure gene expression

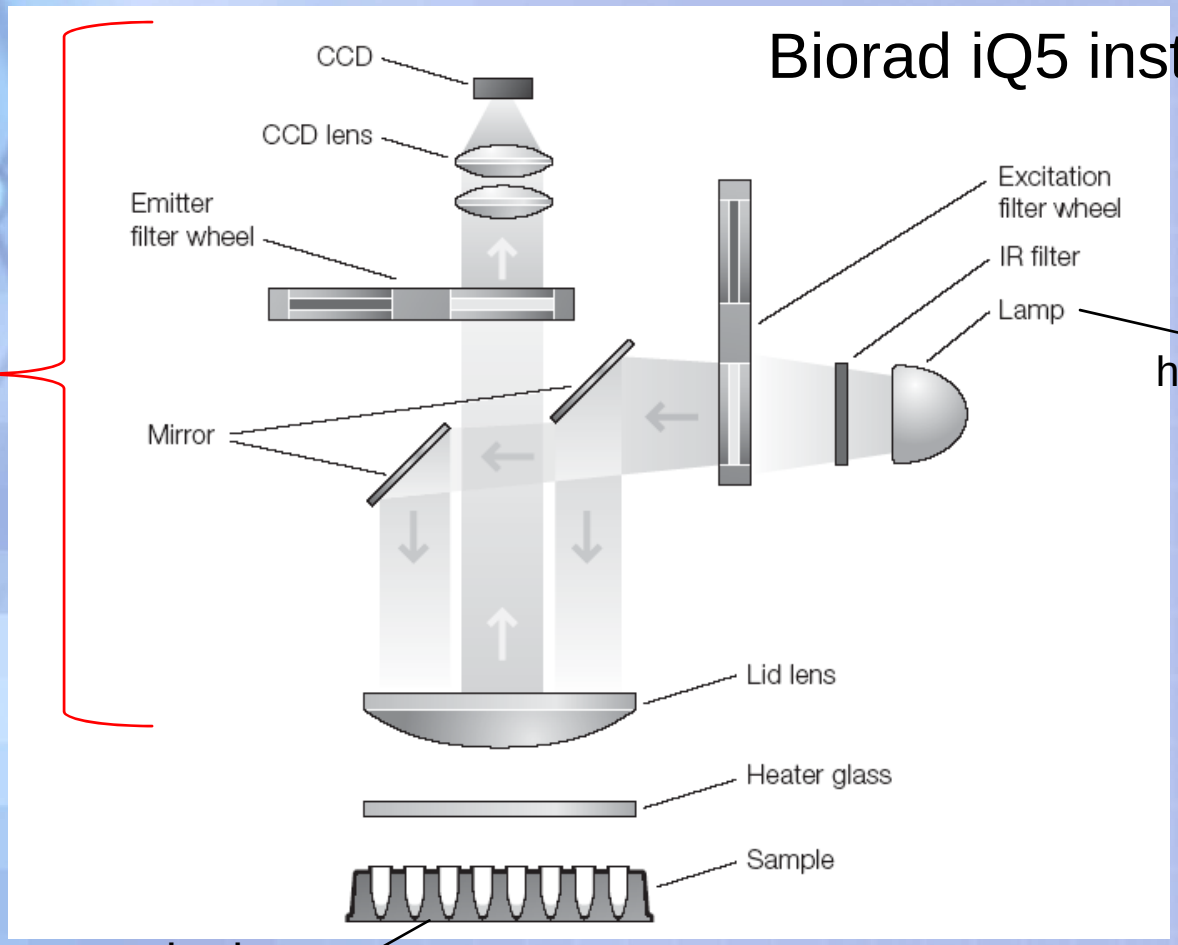


How do real-time PCR instruments work?

Lamp Based-Optical System

Biorad iQ5 instrument

Optical unit

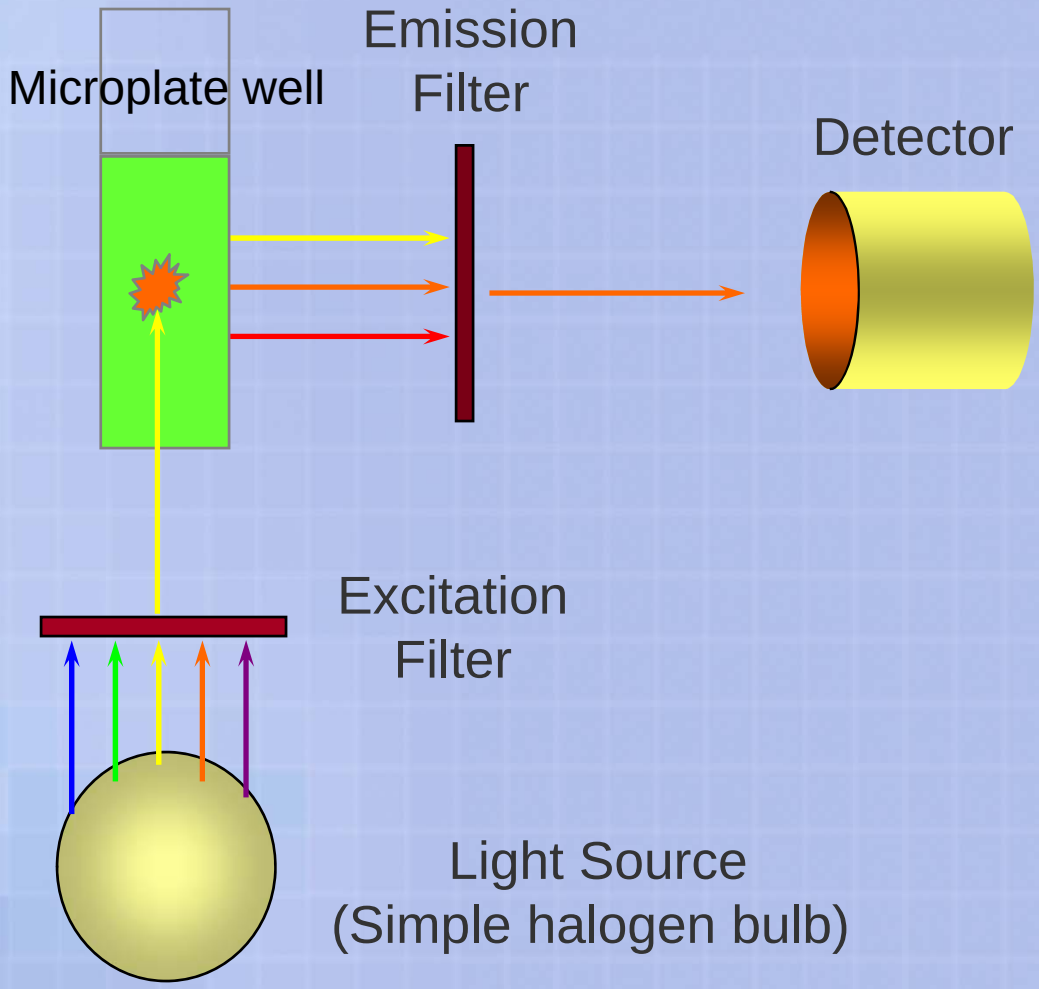


Simple halogen lamp

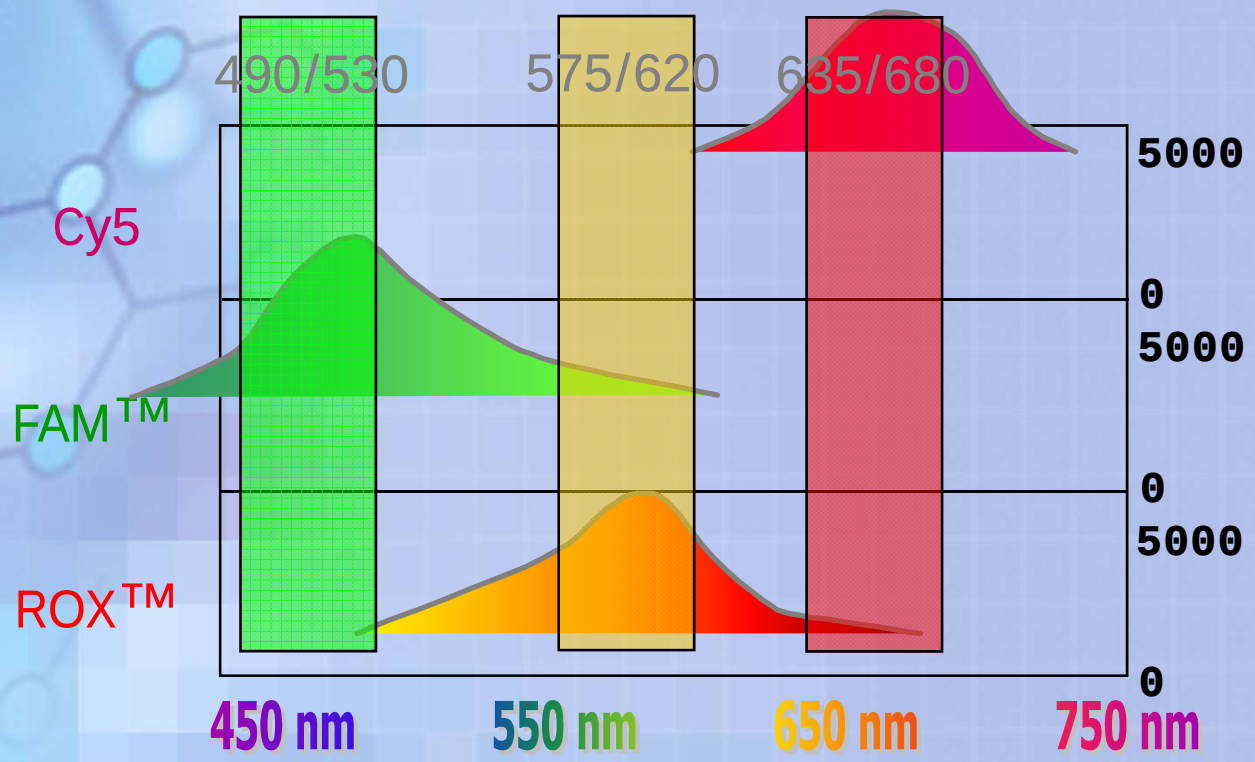
Thermocycler base

Optics Utilize Filter Wheels

Biorad iQ5 instrument



Emission Filter Windows



- Some (iQ5, ABI) instruments detect up to 5 dyes colors
- Simpler instruments (MYQ) detect SYBR Green only

Fast Thermal Cycling Instruments

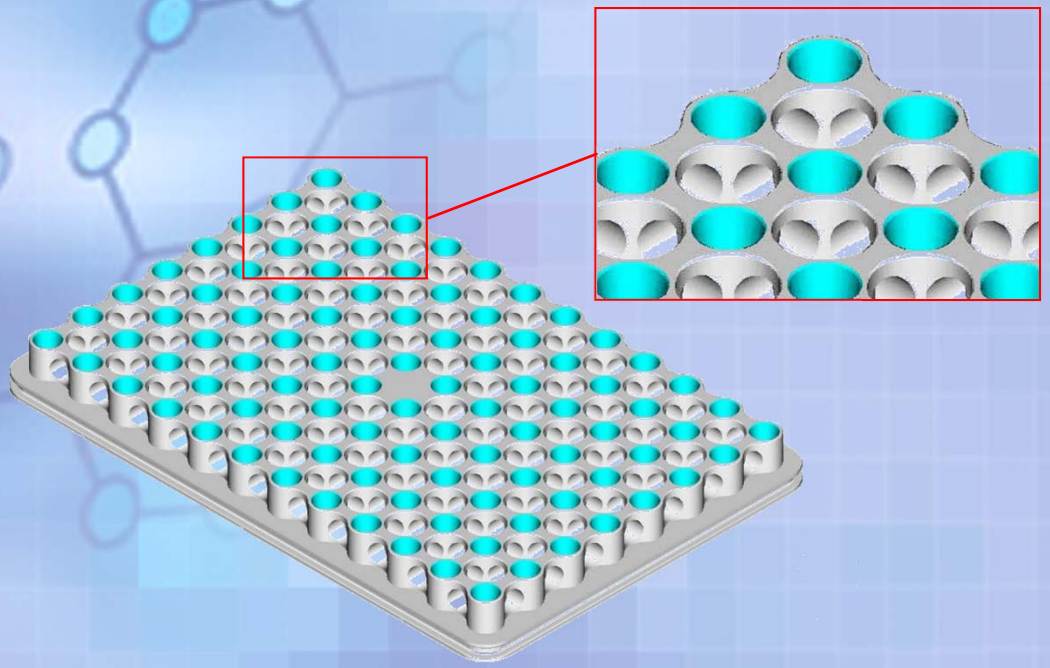
- Some instruments have thermocyclers that can ramp very rapidly to save time
- These instruments have more advanced blocks
- Examples include Biorad CFX96, ABI 7500 and others

Max ramp rate	5°C/sec
Average ramp rate	3.3°C/sec
Temp Accuracy	± 0.2°C
Temp Uniformity	± 0.4°C in 10 sec
Temp Range	0-100°C

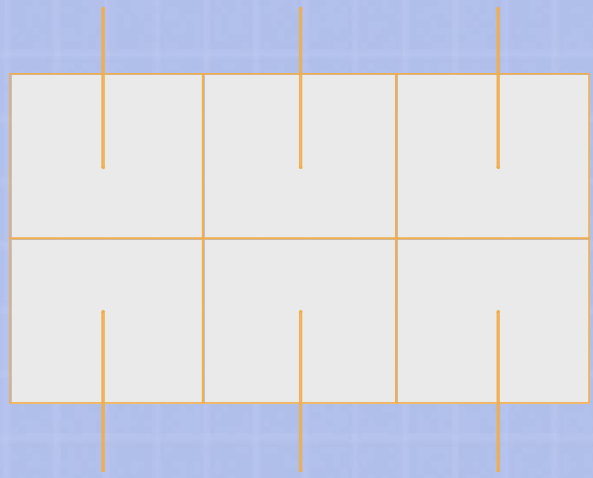
Biorad CFX96 instrument

Fast Block Design

Honeycomb architecture produces more uniform heating and cooling



Bio-Rad CFX96 block



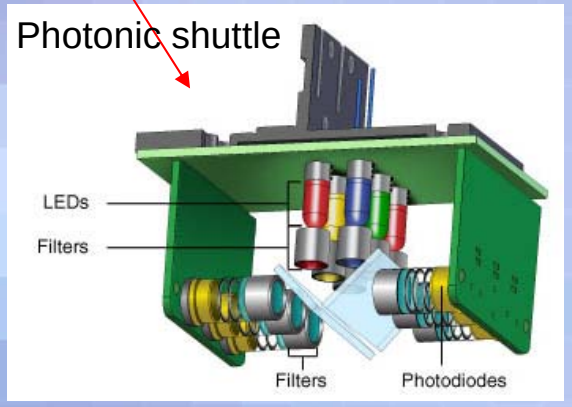
6 independent zones of thermal control

LED Optical Technology


CFX96

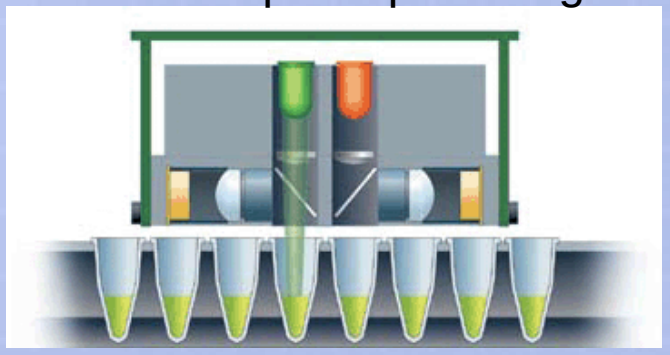
- CFX96 uses a scanning shuttle
  - 6 filtered LEDs for excitation
  - 6 filtered photodiodes for detection
  - LEDs fire sequentially
- Multiplex up to 5 targets
- Fixed optical path length for all wells
- Reduce cross talk between wells

Photonic shuttle



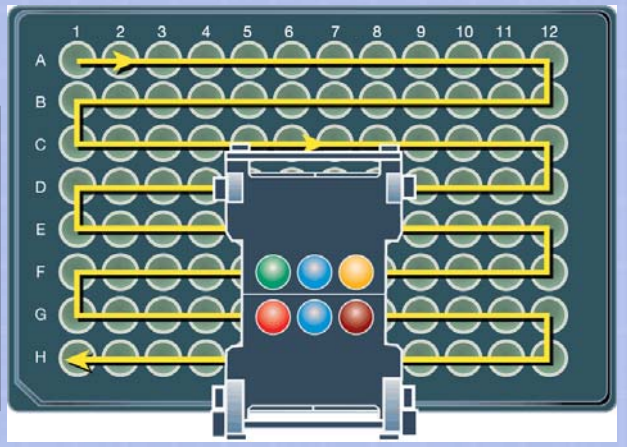
Once detector/filter
for each color detected

Fixed optical path length



LED Optical Technology

Shuttle moves in zig-zag pattern



- LEDs are long lasting
- Factory calibrated.
  - Does not require periodic calibration.
- No need for Passive Reference (Rox/Fluoroscein)

- LED instrument always acquires data from all wells in all channels (CFX)
- Laser Homing of shuttle at every scan

Fast Scanning on CFX

- Flexibility of 3 data acquisition scan modes
- Shorten run times using the SYBR/FAM Only fast scan mode

Mode	Channel(s)	Scan Time (sec)
All Channels	1-5	12
SYBR/FAM Only	1	3
FRET (Fluorescence resonance energy transfer	6	3

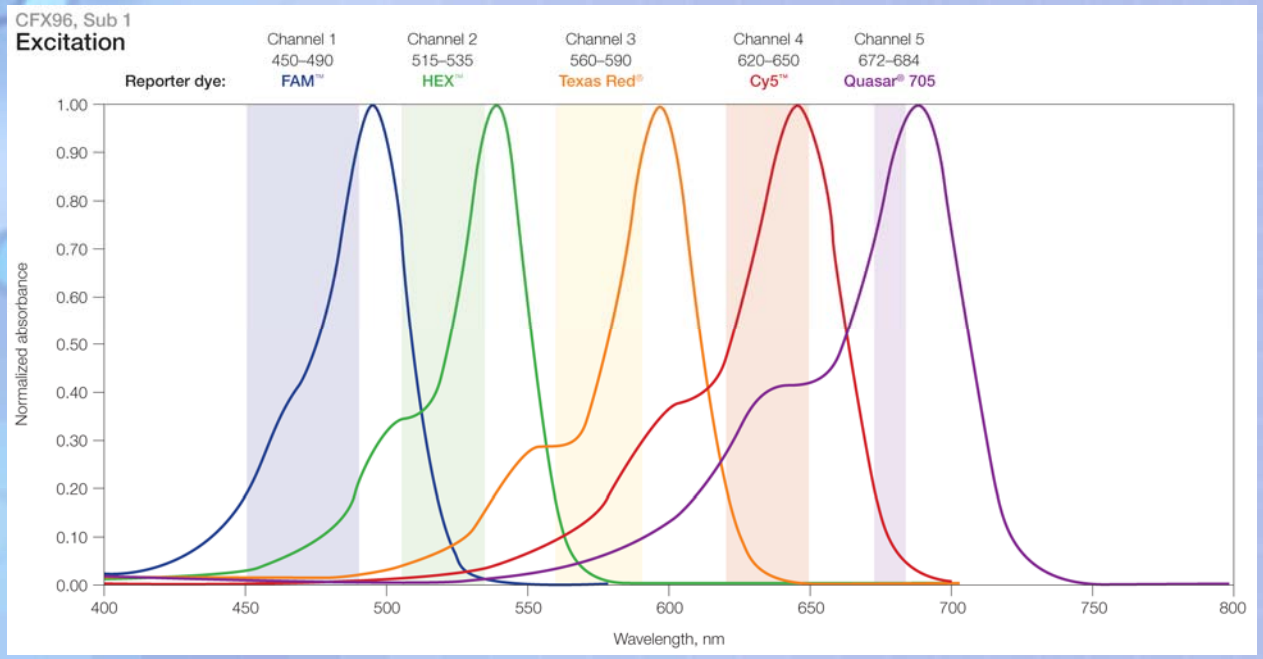
FRET useful for examining protein-protein interactions

Multiple Filter Sets for Multiplex Assays

Channel	Excitation (nm)	Detection (nm)	Calibrated Fluorophores
1	450-490	515-530	FAM™, SYBR Green I™
2	515-535	560-580	VIC®, HEX™, TET™, Cal Gold 540™
3	560-590	610-650	ROX™, TEXAS RED®, Cal Red 610™
4	620-650	675-690	CY5, Quasar 670™
5	672-684	705-730	Quasar 705™
6	450-490	560-580	Accommodates FRET Chemistry

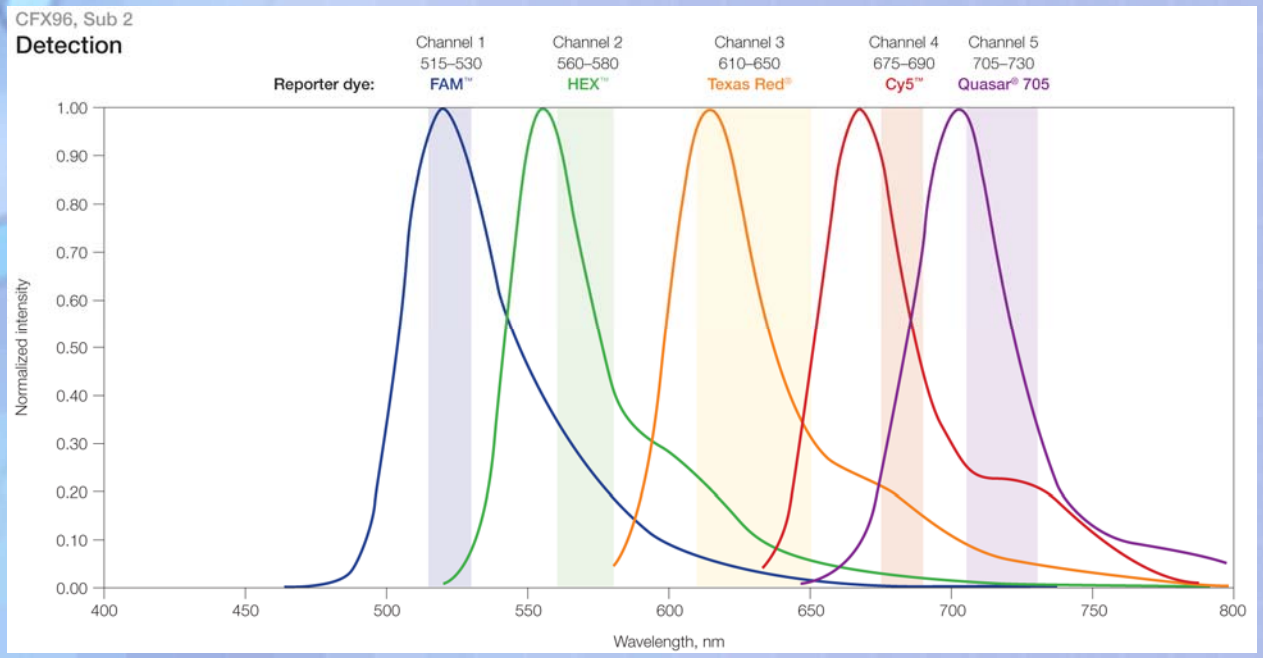
Excitation Wavelengths

The LED-filter combinations permit excitation of multiple specific dyes



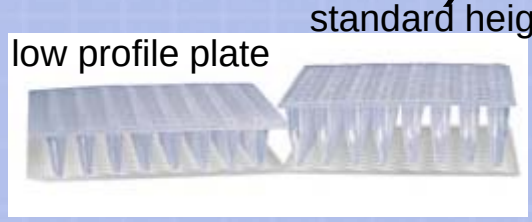
Detection Wavelengths

The detection filters can detect fluorescence from multiple dyes



Practical Differences (iQ5/MYiQ vs CFX96)

- CFX96 requires low profile plate (Biorad MLL 9601)



- All instruments can use SYBRGreen Supermix or other qPCR reagents with fluoroscein. However,
  - CFX96 does not require any normalization dye
  - CFX96 can use SooAdvanced reagents (about 45min run)
  - even using SYBRGreen Supermix will take <1.5hrs for run vs 2.5 hrs for iQ5/MYiQ
- CFX96 and iQ software are sharable
  - CFX96 software will be somewhat familiar to iQ users and offers a few additional features

Quantitative PCR workflow

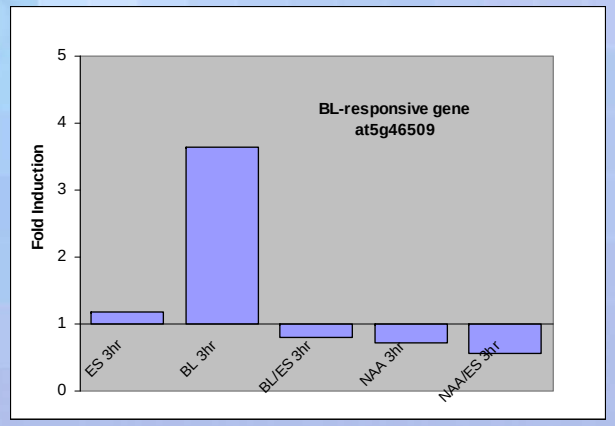
Purified total RNA

Purified DNA

Convert to 1st strand cDNA

cDNA template, primers, Taq polymerase, dNTPs, SYBR Green

Determine relative gene expression (ddCt method)

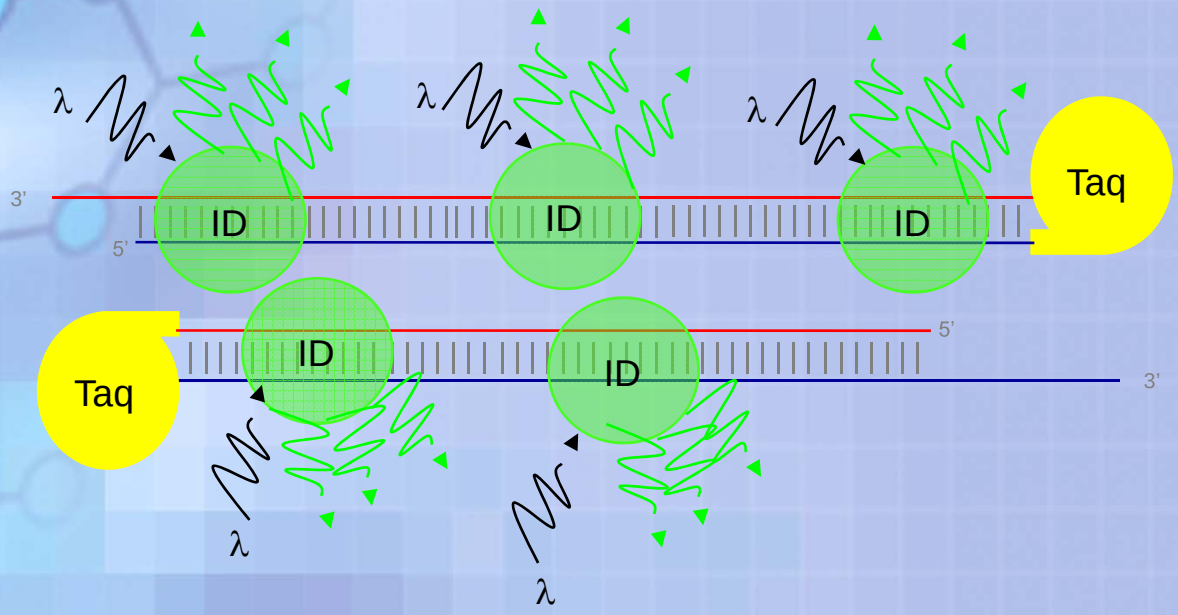


	Well	Fluor	Type	Identifier	Replicate #	Threshold Cycle (Ct)	Ct Mean	Ct Std. Dev
30 mins	A01	SYBR	Unkn	1A_DMSO30_Ub	1	21.65	22.19	0.487
	A02	SYBR	Unkn	1A_DMSO30_Ub	1	22.58		
	A03	SYBR	Unkn	1A_DMSO30_Ub	1	22.35		
	A04	SYBR	Unkn	1B_DMSO30_BL	2	30.56	30.42	0.147
	A05	SYBR	Unkn	1B_DMSO30_BL	2	30.27		
	A06	SYBR	Unkn	1B_DMSO30_BL	2	30.44		
	A07	SYBR	Unkn	2A_Pru30_Ub	3	21.64	21.83	0.175
	A08	SYBR	Unkn	2A_Pru30_Ub	3	21.86		
	A09	SYBR	Unkn	2A_Pru30_Ub	3	21.99		
	A10	SYBR	Unkn	2B_PrU30_BL	4	29.22	29.65	0.436
	A11	SYBR	Unkn	2B_PrU30_BL	4	30.09		
	A12	SYBR	Unkn	2B_PrU30_BL	4	29.63		
	B01	SYBR	Unkn	3A_BL30_Ub	5	21.5	21.34	0.14
	B02	SYBR	Unkn	3A_BL30_Ub	5	21.32		
	B03	SYBR	Unkn	3A_BL30_Ub	5	21.22		
	B04	SYBR	Unkn	3B_BL30_BL	6	27.67	27.32	0.324
	B05	SYBR	Unkn	3B_BL30_BL	6	27.25		
	B06	SYBR	Unkn	3B_BL30_BL	6	27.04		
	B07	SYBR	Unkn	4A_NAA30_Ub	7	21.35	21.53	0.172
	B08	SYBR	Unkn	4A_NAA30_Ub	7	21.57		
	B09	SYBR	Unkn	4A_NAA30_Ub	7	21.69		
	B10	SYBR	Unkn	4B_NAA30_BL	8	30.43	29.98	0.402
	B11	SYBR	Unkn	4B_NAA30_BL	8	29.68		
	B12	SYBR	Unkn	4B_NAA30_BL	8	29.82		
	C01	SYBR	Unkn	5A_BLPrU30_Ub	9	21.62	21.39	0.197
	C02	SYBR	Unkn	5A_BLPrU30_Ub	9	21.29		
	C03	SYBR	Unkn	5A_BLPrU30_Ub	9	21.26		
	C04	SYBR	Unkn	5B_BLPrU30_BL	10	28.15	28.48	0.309
	C05	SYBR	Unkn	5B_BLPrU30_BL	10	28.76		
	C06	SYBR	Unkn	5B_BLPrU30_BL	10	28.52		
	C07	SYBR	Unkn	6A_NAAPrU30_Ub	11	21	21.19	0.19
	C08	SYBR	Unkn	6A_NAAPrU30_Ub	11	21.18		
	C09	SYBR	Unkn	6A_NAAPrU30_Ub	11	21.38		
	C10	SYBR	Unkn	6B_NAAPrU30_BL	12	29.77	29.74	0.242
	C11	SYBR	Unkn	6B_NAAPrU30_BL	12	29.48		
	C12	SYBR	Unkn	6B_NAAPrU30_BL	12	29.96		

Detection Methods

Intercalation Dyes: SYBR Green I

SYBR displays strong fluorescence in the presence of dsDNA



Ethidium bromide is 25x more fluorescent when intercalated into dsDNA
SYBR Green ***125x*** more fluorescent when intercalated into dsDNA

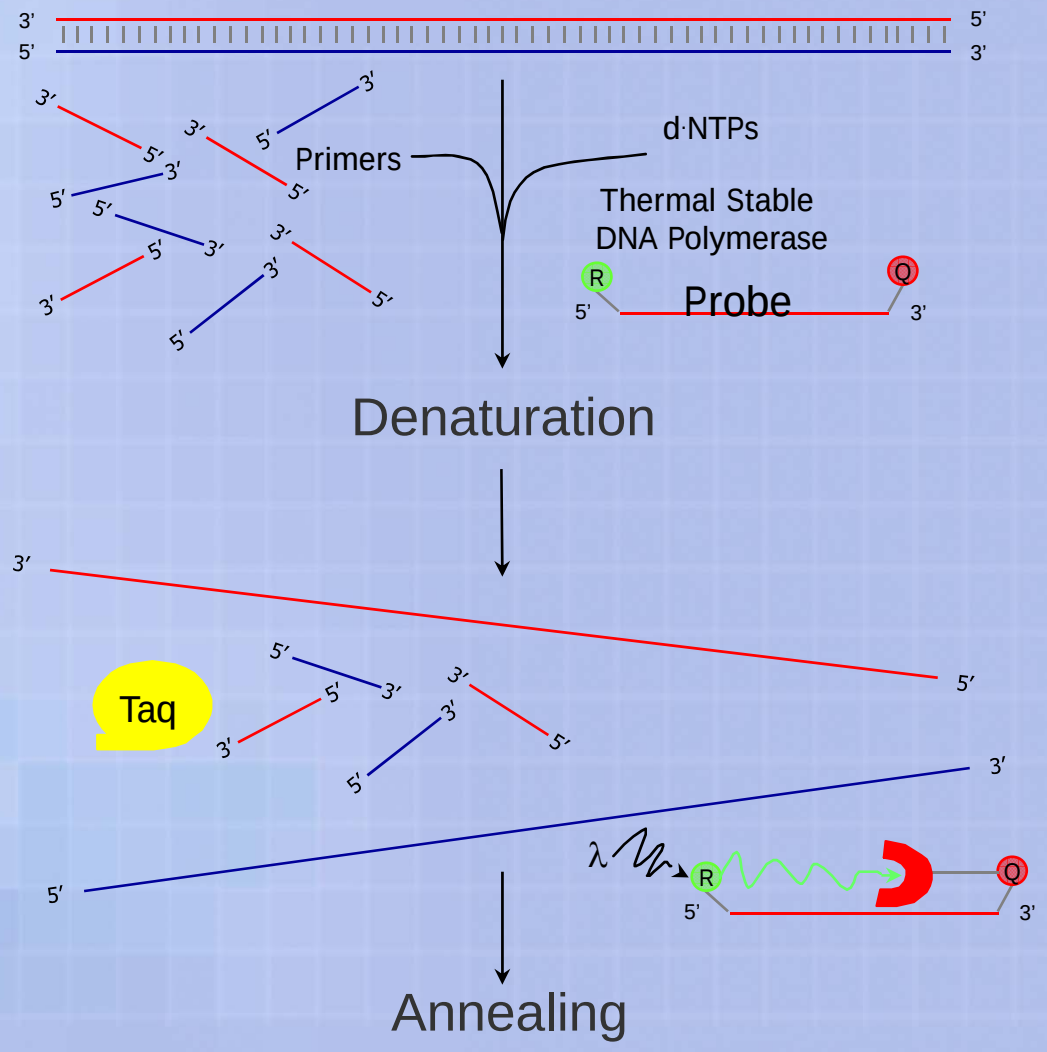
Hybridization Probes

Currently, hybridization probe strategies fall into three main categories

- **Cleavage-based assay**
  - TaqMan™ Assays
  - Locked nucleic acids (LNA)
- **Displaceable probe assays**
  - Molecular Beacons
  - Dual-oligo FRET probes
- **Probes incorporated directly into the primers**
  - Amplifluor & Scorpions

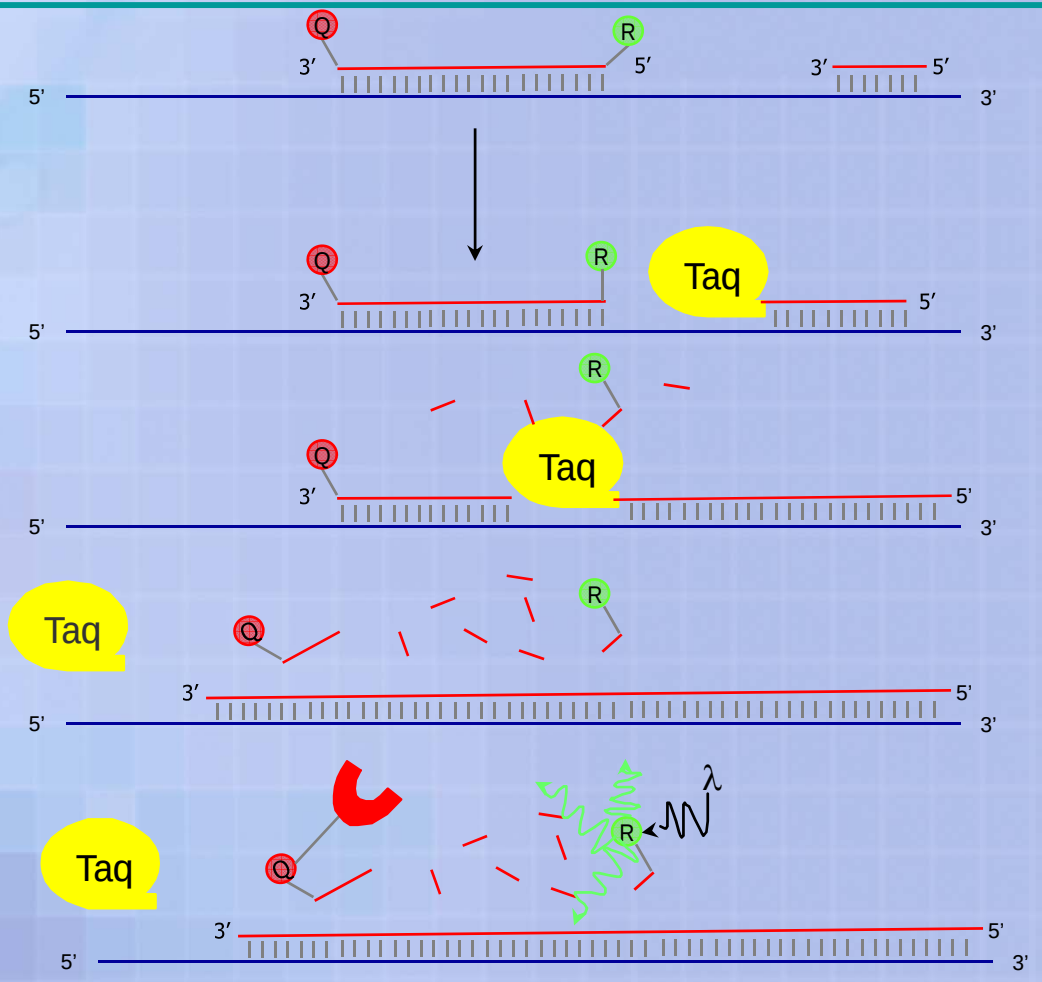
Cleavage-based assay: TaqMan™

Add iQ Supermix, Hybridization Probe and sample



Cleavage-based assay: TaqMan™

Extension Step

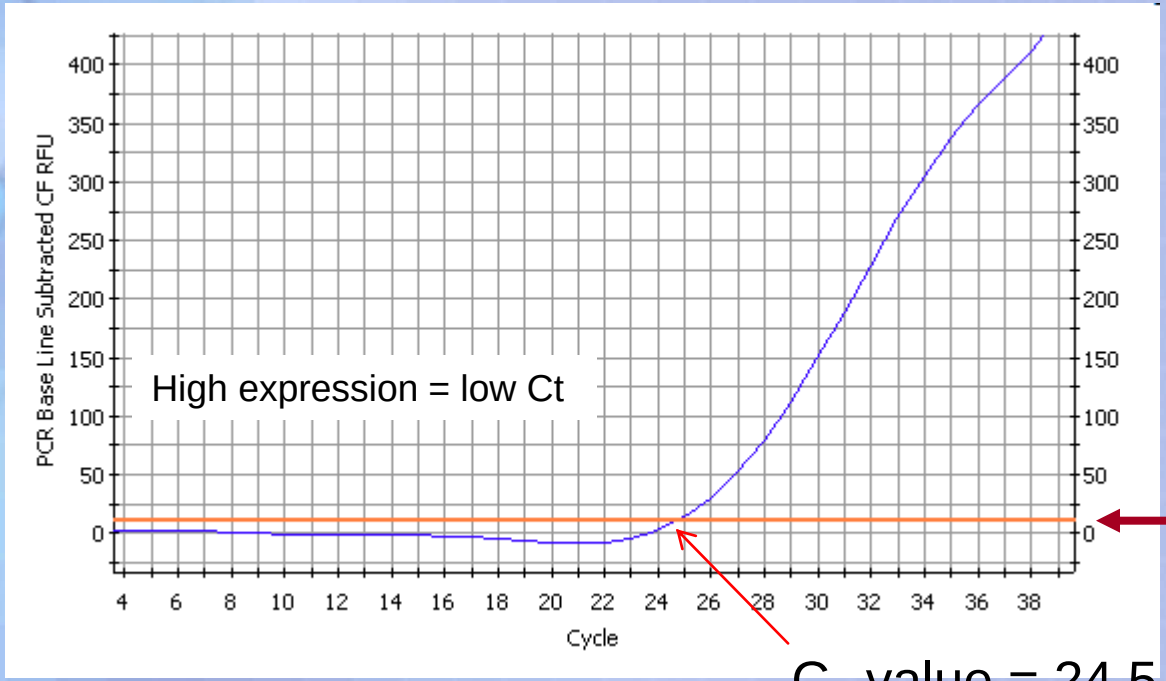


Released signal is proportional to quantity of starting mRNA in the cDNA population

Threshold and Standard Curves for Primer Efficiency

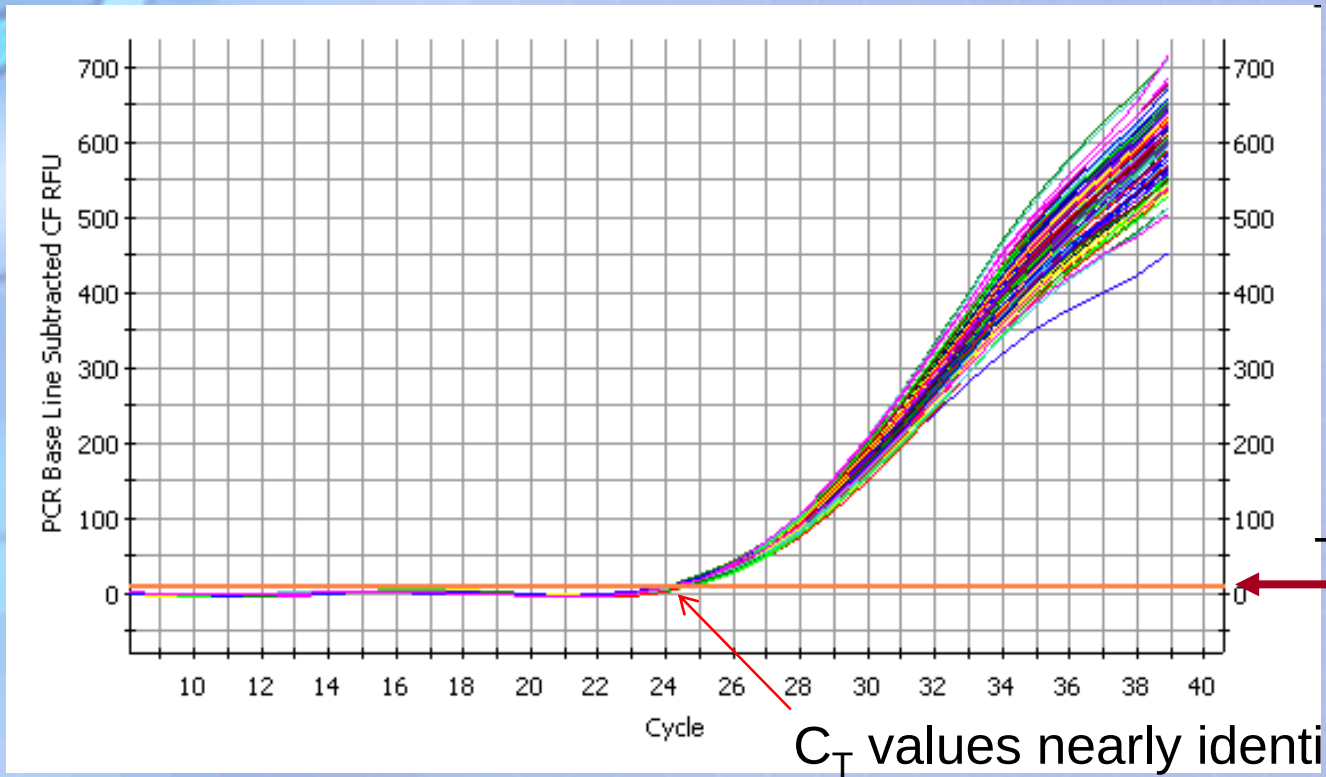
Cycle Threshold (C_T)

- Assigned point at which the fluorescence rises appreciably above background
- The C_T can be placed anywhere in the exponential (log-linear) phase



Results are More Accurate in the Log Phase

96 identical reactions will have nearly identical C_T values when measured at the linear phase of amplification



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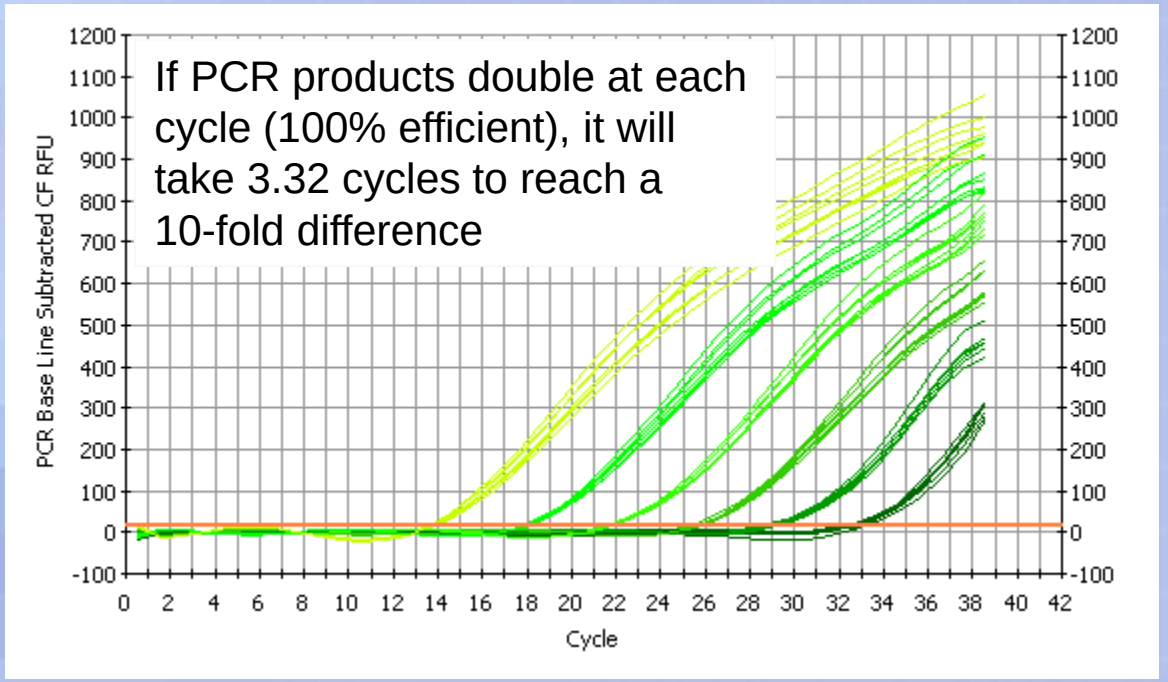
Cycle Threshold (C_T)

- At the C_T, the fluorescence correlates well with the starting copy number
- Instruments have a dynamic range of up to 9 orders of magnitude
- Each 10x dilution of template equals 3.3 cycles. Thus qPCR is relatively *insensitive* to the concentration of the target gene(s).

Assuming exact doubling of amplicon at each cycle then

$$2^n = 10\text{-fold}$$

$$n = 3.32$$



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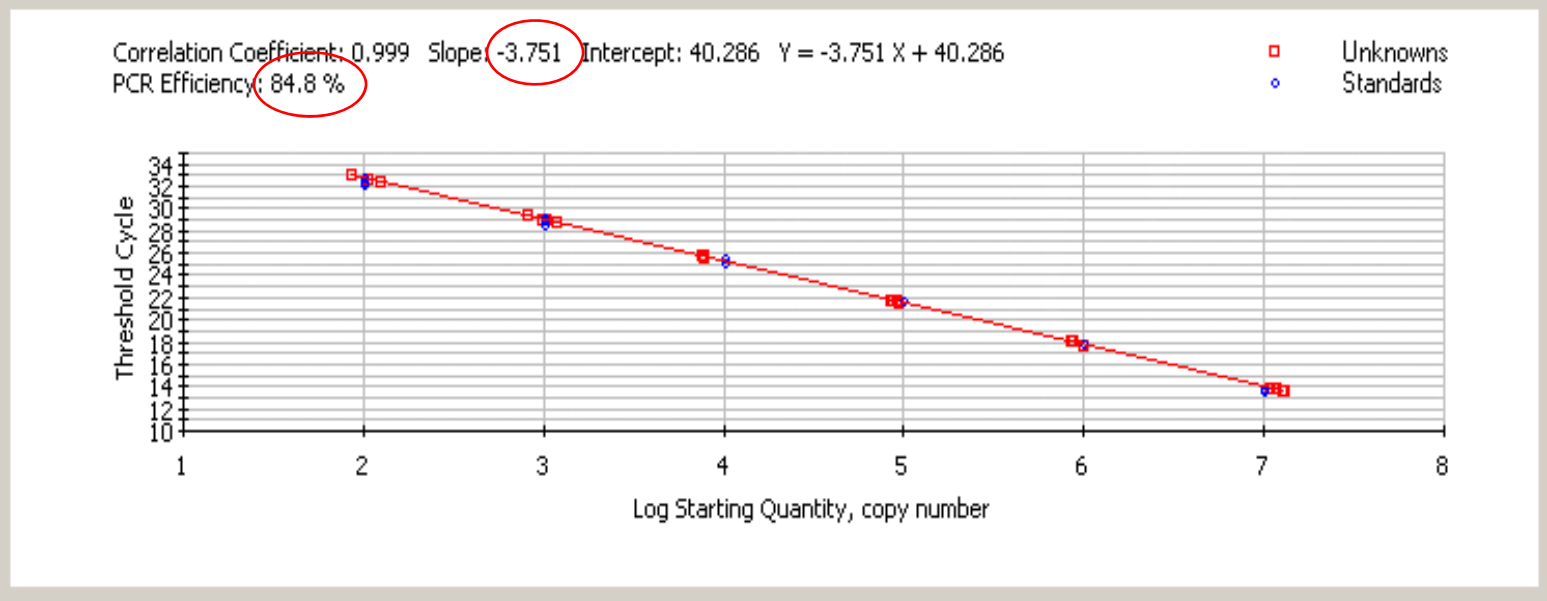
Standard Dilution Series

1) To perform absolute quantification
2) To examine the efficiency of the qPCR reaction (This can influence the results of your experiment significantly; -3.32 slope is 100% efficiency)



Efficiency = $10^{(-1/\text{slope})}$ = 1.85

% Efficiency = $10^{(-1/\text{slope})}$ - 1 X 100 = 85%

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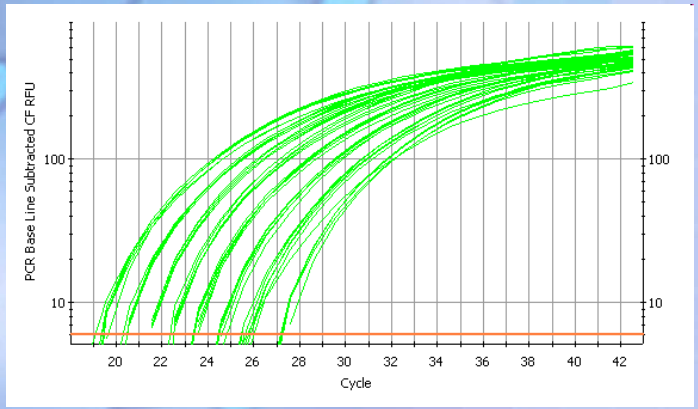
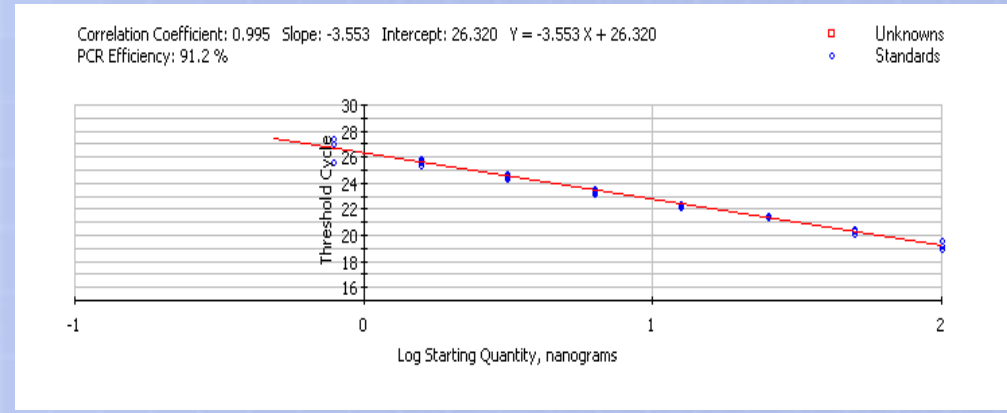
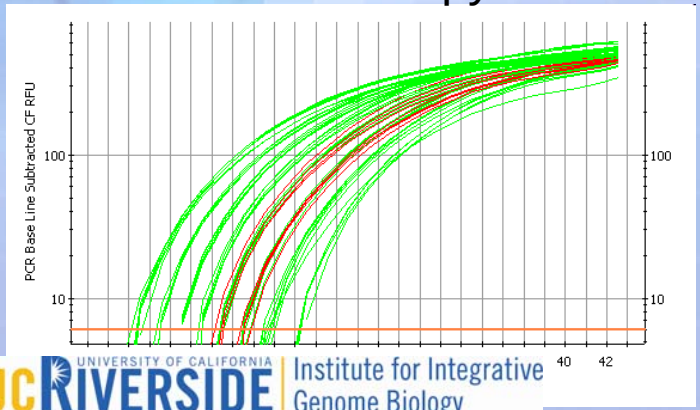
Standard Efficiency Curves

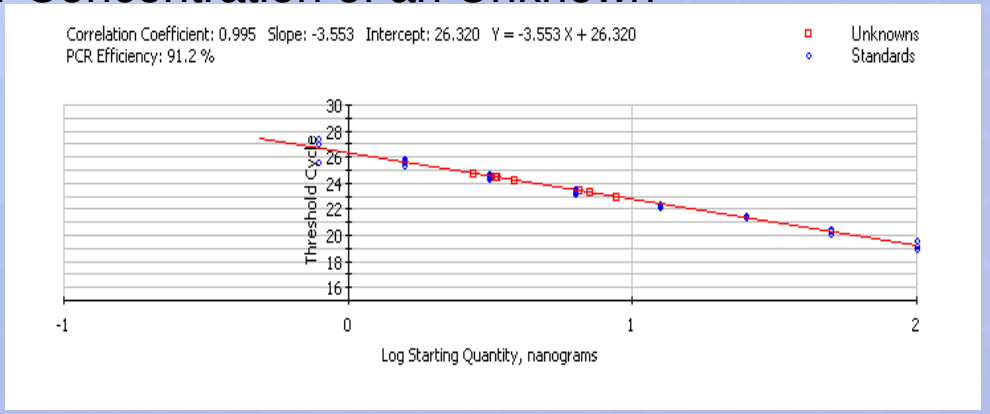
Standard Dilution Series can be used to……





…determine the Copy Number or Concentration of an Unknown





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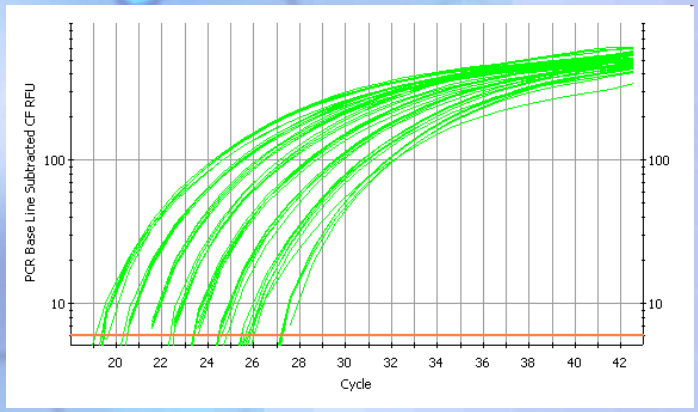
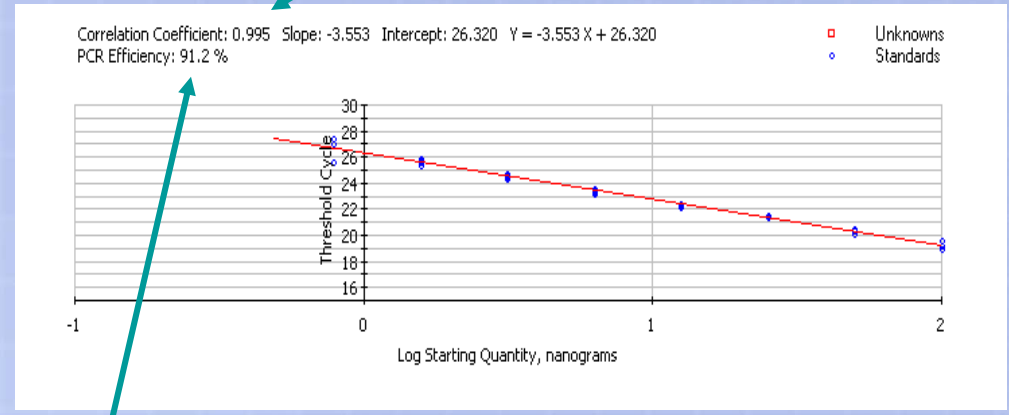
Example of Good Efficiency

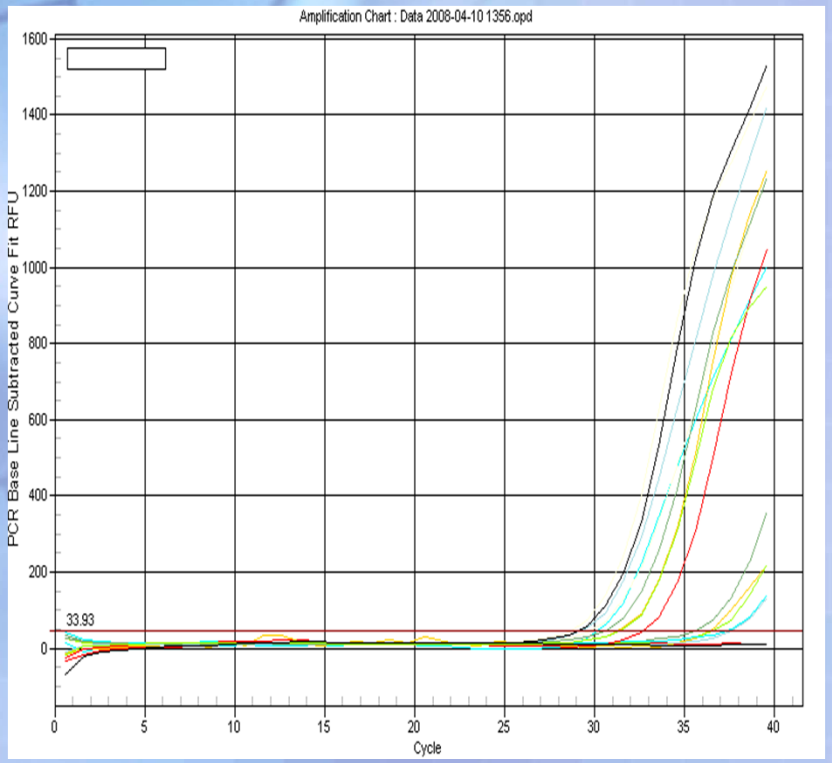
Correlation Coefficient >0.9

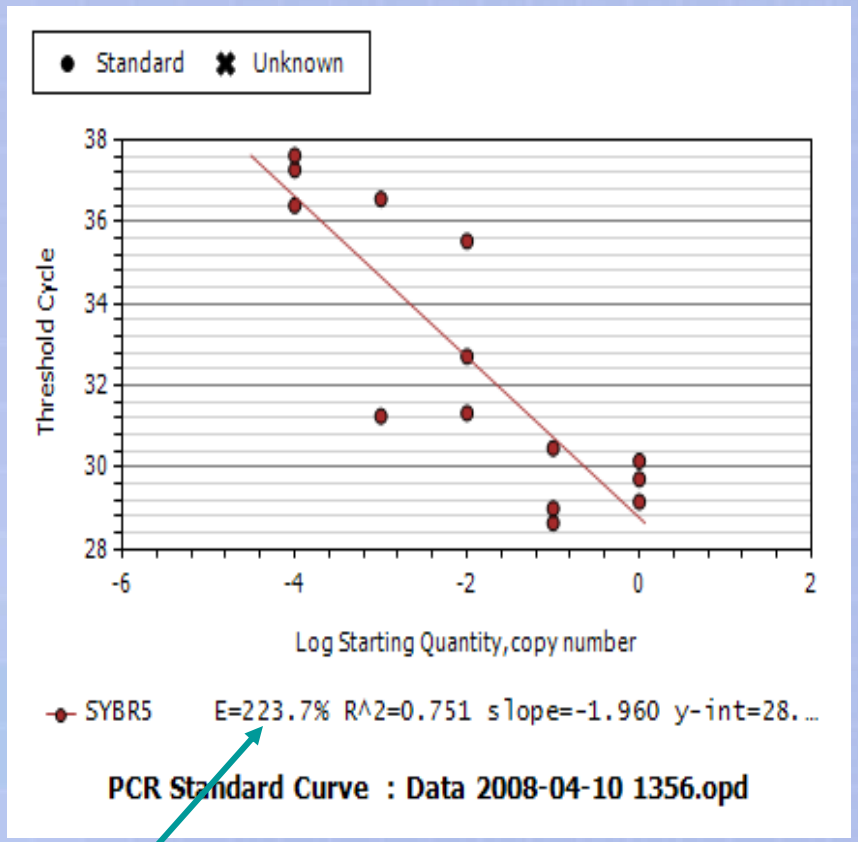




Efficiency >90%

Example of Unreasonably High Efficiency





How do you get an efficiency of >200%?

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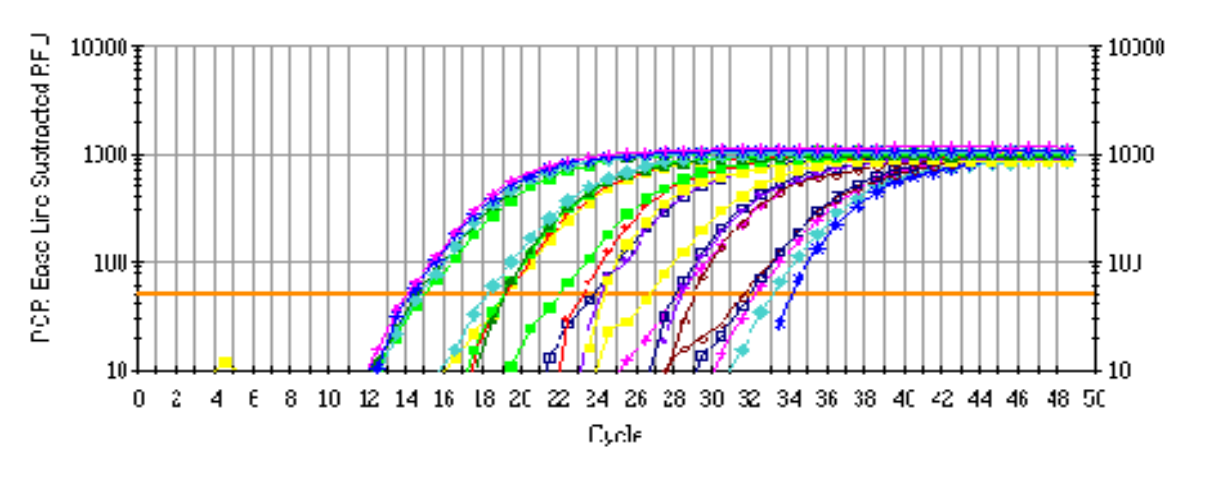
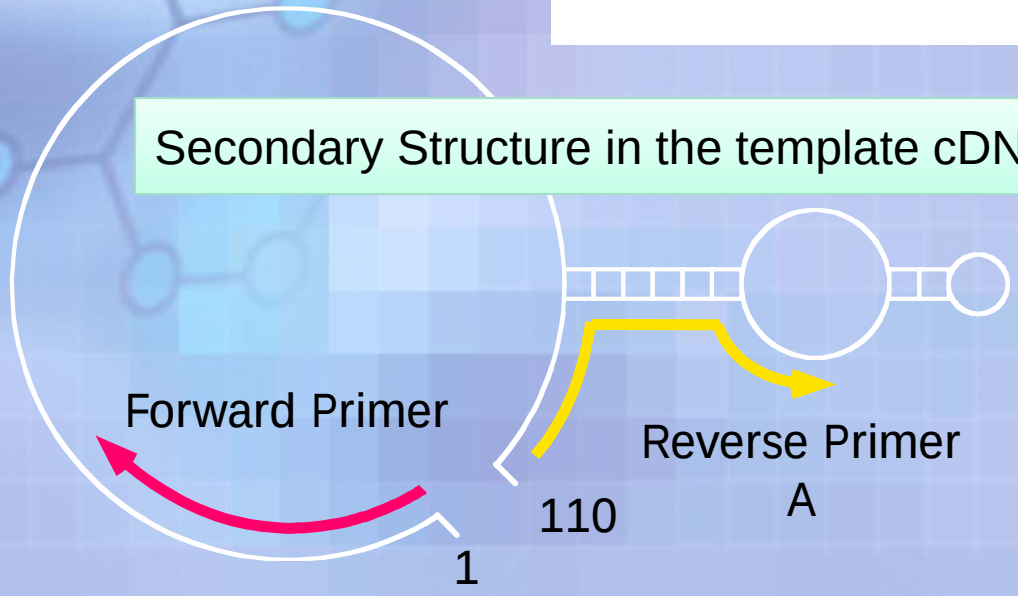
Unreasonably Low Efficiency



Secondary Structure in the template cDNA



Primer efficiency = 66.3%

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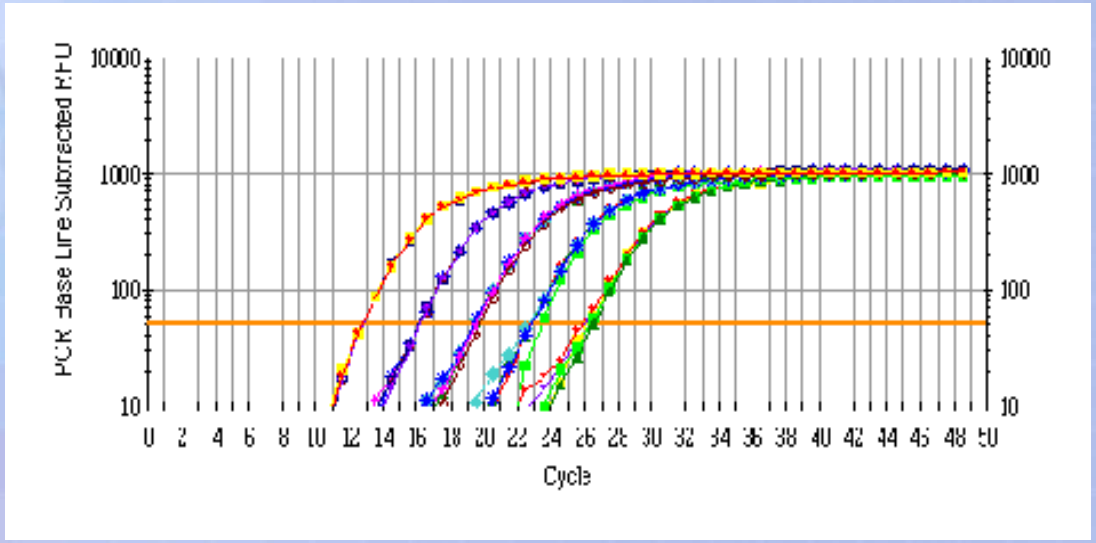
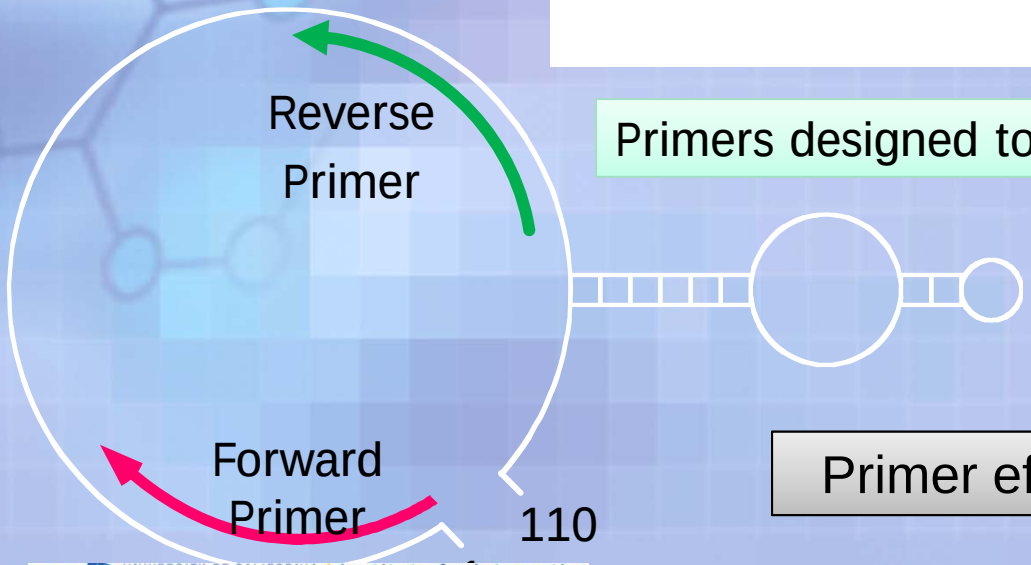
Efficiency is Now Good





Primers designed to avoid secondary structure

Primer efficiency = 95.8 %

Real-time PCR Efficiencies

Efficiency $= 10^{-1/slope}$

% Efficiency $= 10^{-1/slope} - 1 \times 100$

- ***Primer-dimer!***
- Pipette calibration
- Dilution Errors

110%

90%

Target Range

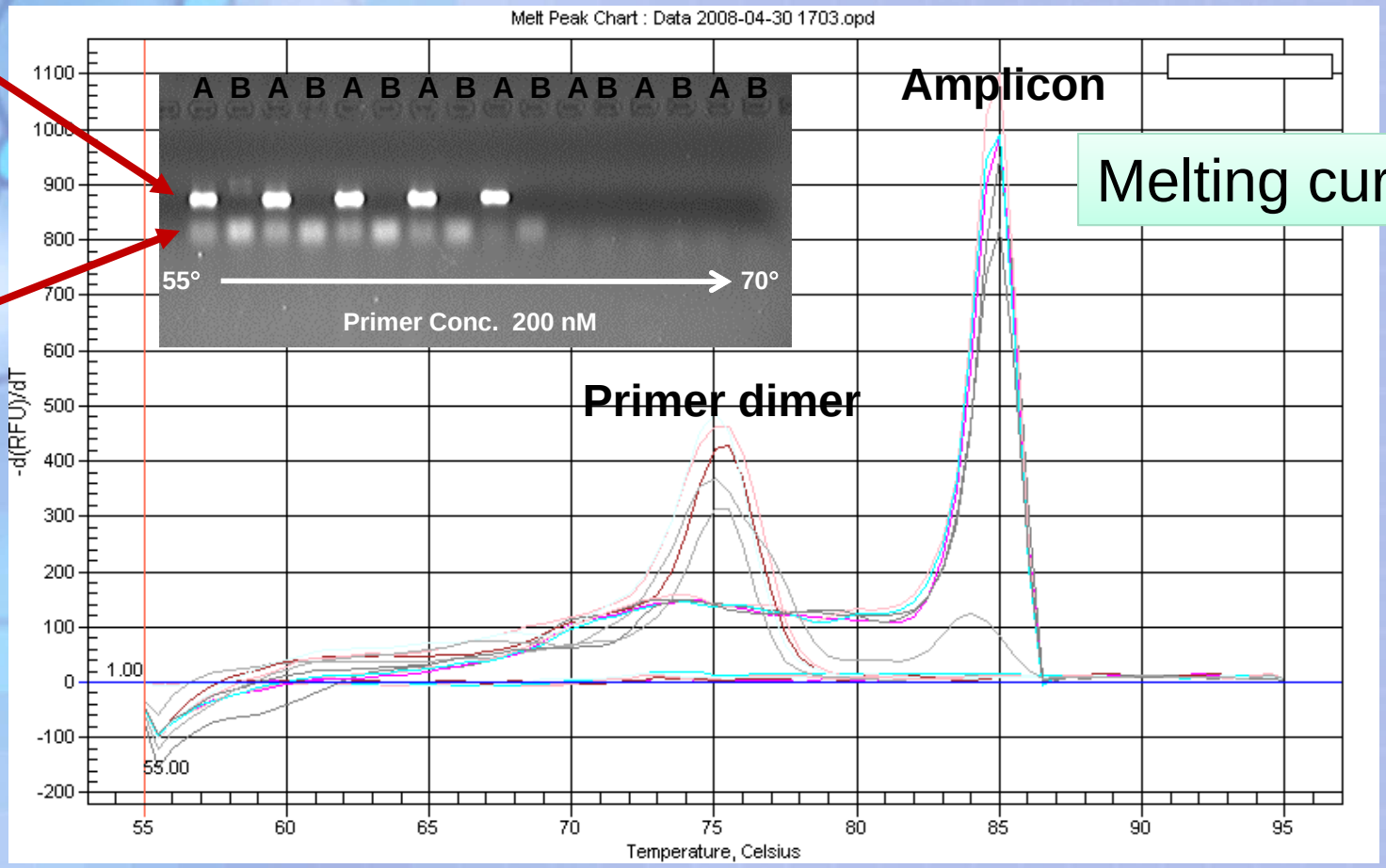
- Secondary structures
- Pipette calibration
- Dilution errors

Primer Dimers Raise the Apparent Efficiency

Optimization of RT-qPCR: Annealing Temperature

Specific Gene

Primer dimers

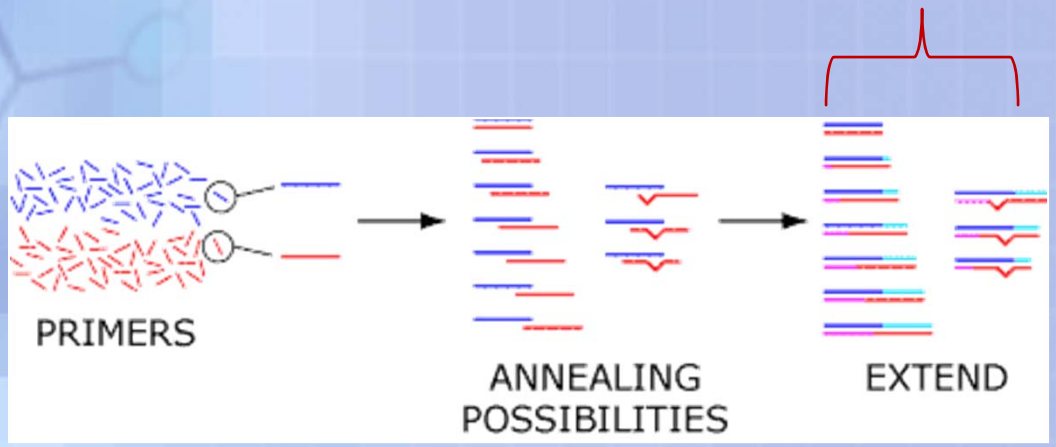


Melting curve

A = Template + primers B = Primers only (No template)

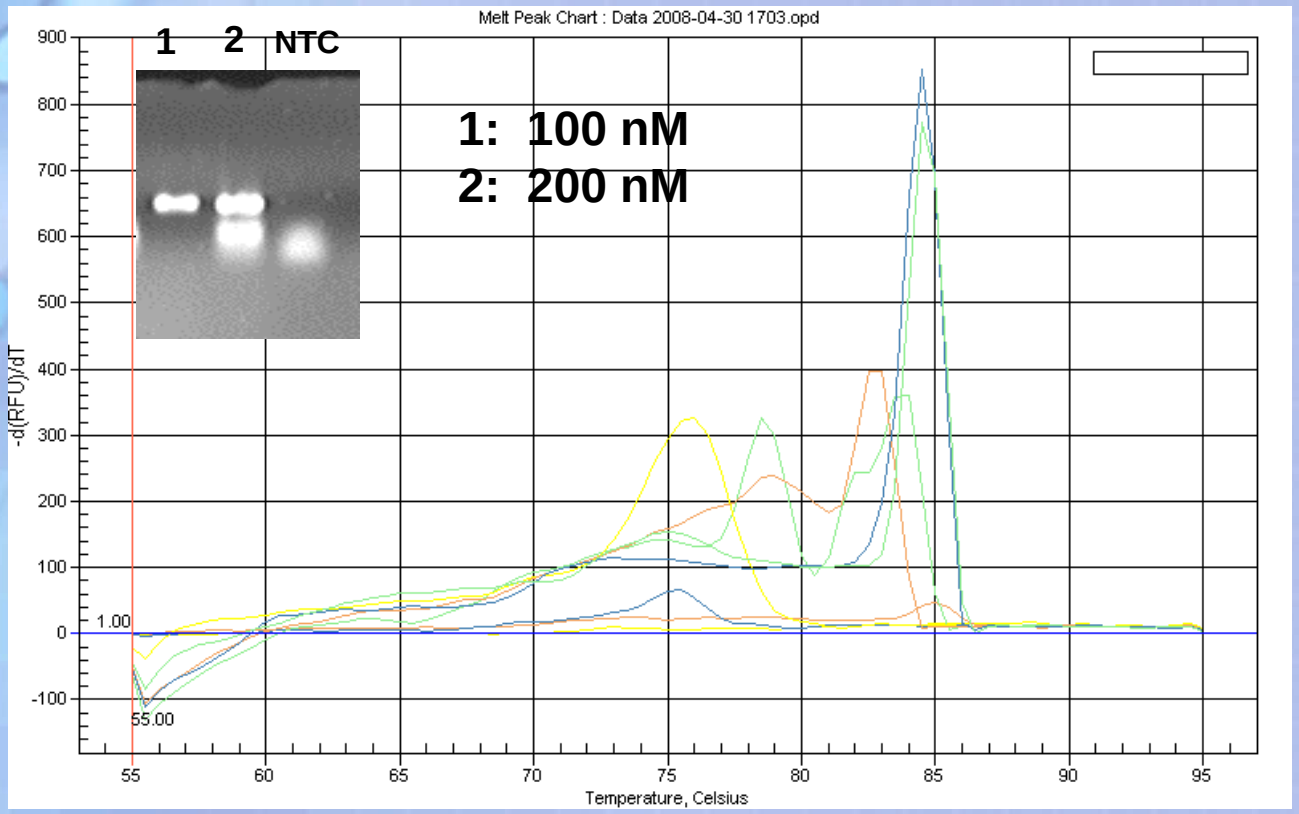
What Are Primer Dimers?

These double strand products are detected by SYBR Green



Primer Concentration Affects qPCR Efficiency

Optimization of RT-qPCR: Primer Concentration



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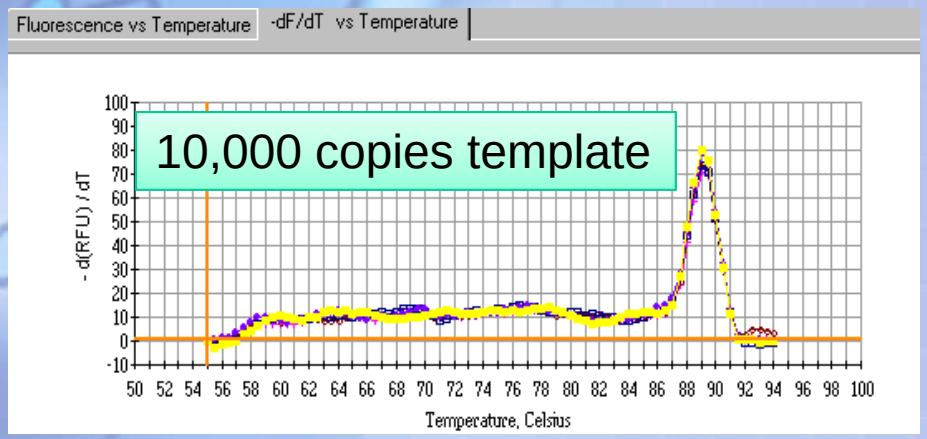
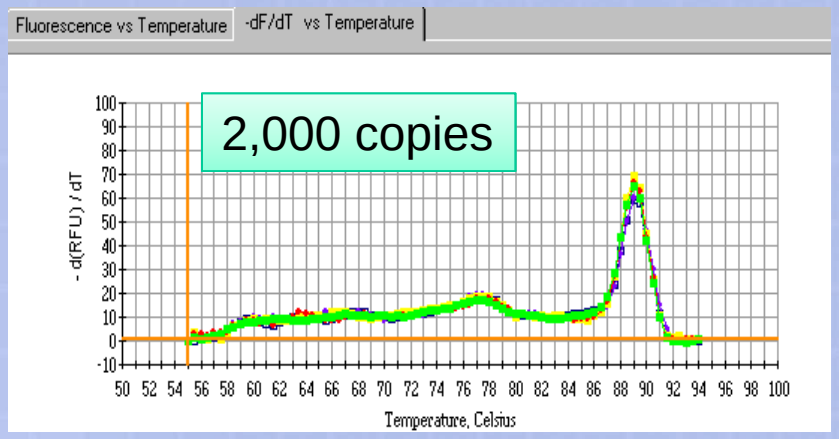
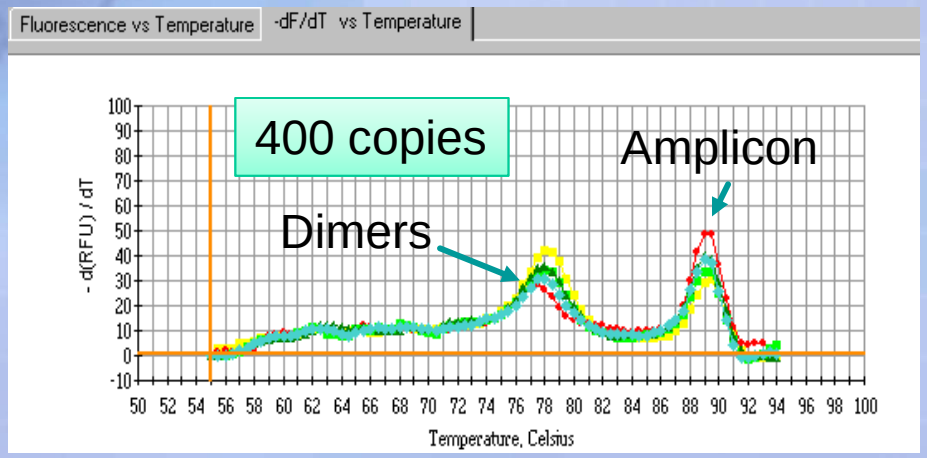
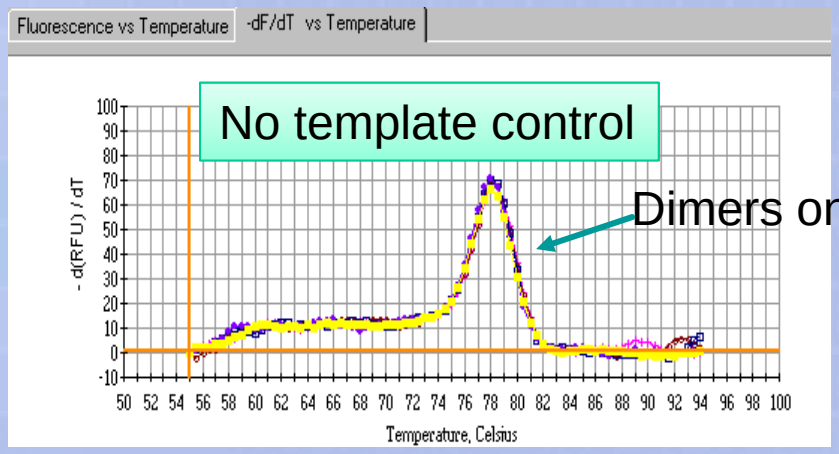
Primer Concentration

Primer-dimers can be influenced by template concentration









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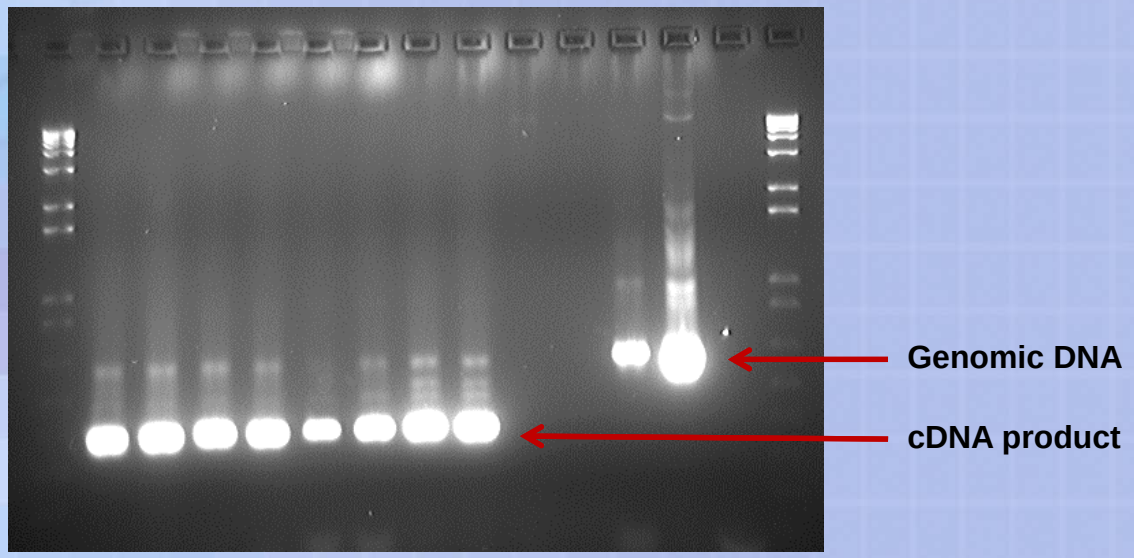
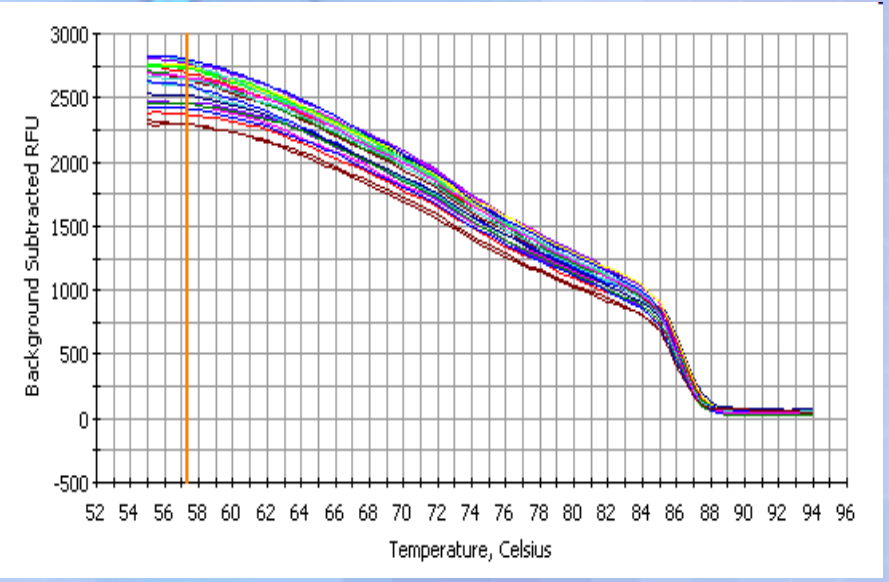
Genomic DNA Contamination Can Affect PCR Efficiency



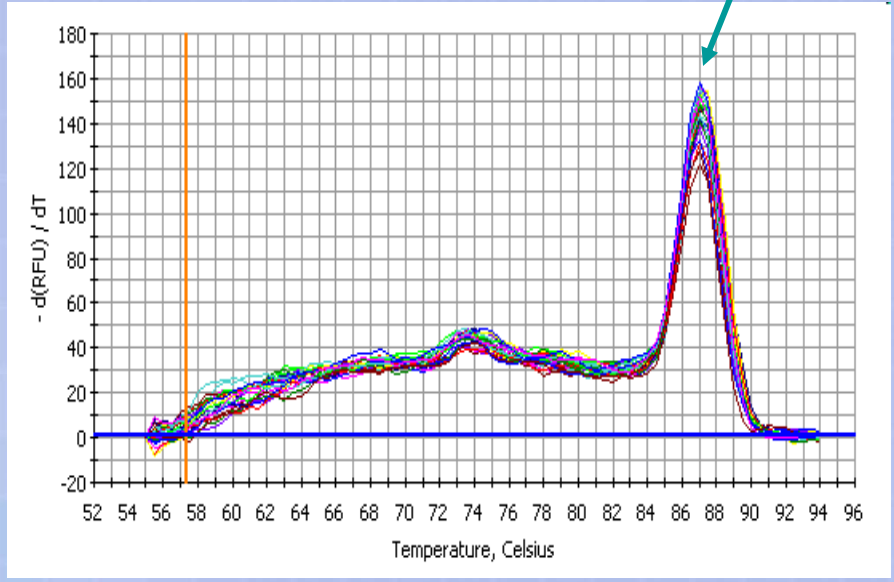
- DNAse treatment will reduce contamination
- Also design primers across intron/exon boundaries

Melt Curve Analysis

Analysis at the end of the qPCR run to determine the melting temperature (T_m) of PCR products



T_m of amplicon



Plot rate of change of fluorescence vs. temperature

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Why Do Melt Curve?

- Identification of non-specific products
- Mutation detection/allelic discrimination

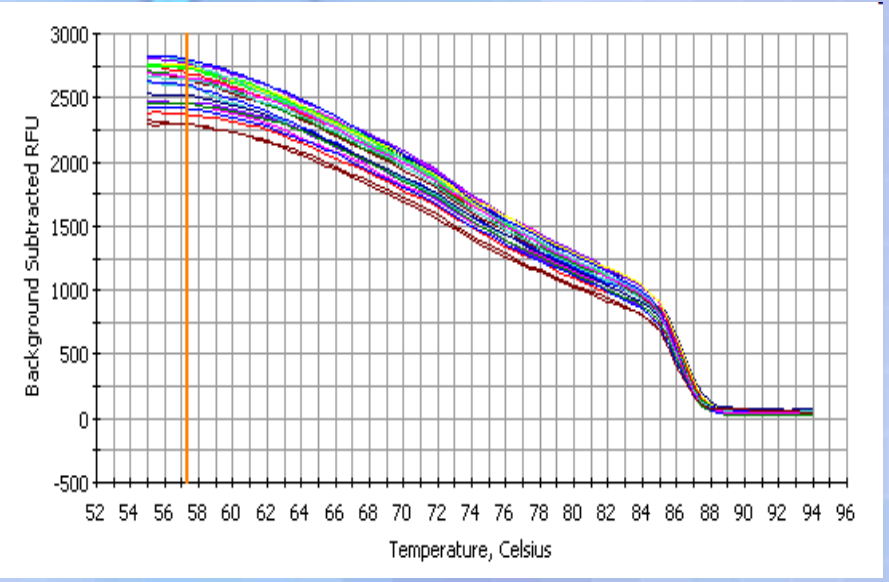
Optimizing Real time qPCR

Features for Reaction Optimization

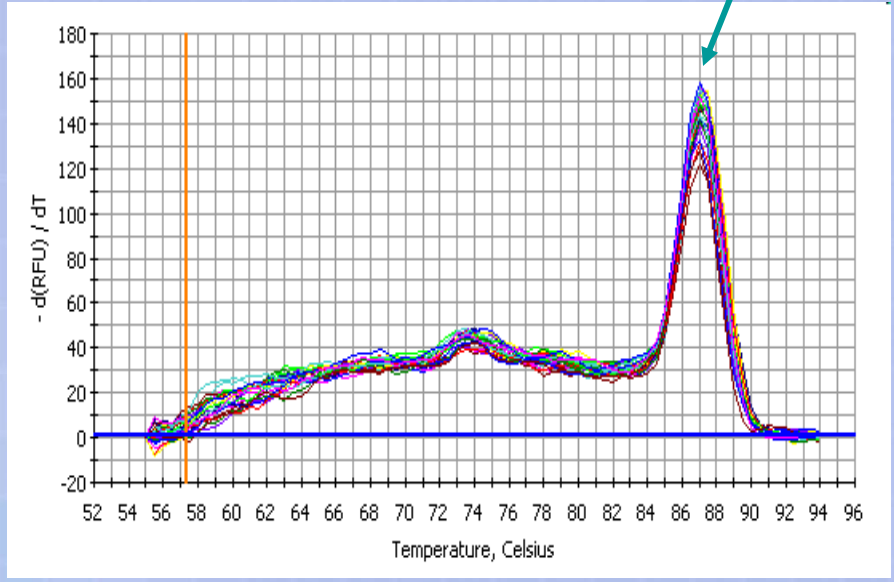
- Continuous Data Collection (iQ5/MYiQ)
- Thermal Gradient
- Melt Curve

Melt Curve Analysis

Analysis at the end of the qPCR run to determine the melting temperature (T_m) of PCR products



T_m of amplicon



Plot rate of change of fluorescence vs. temperature

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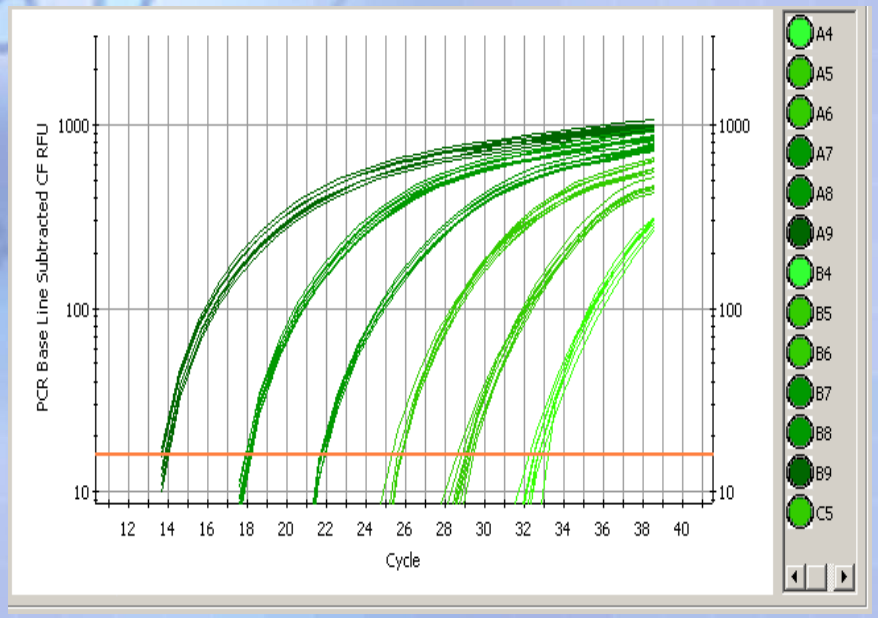
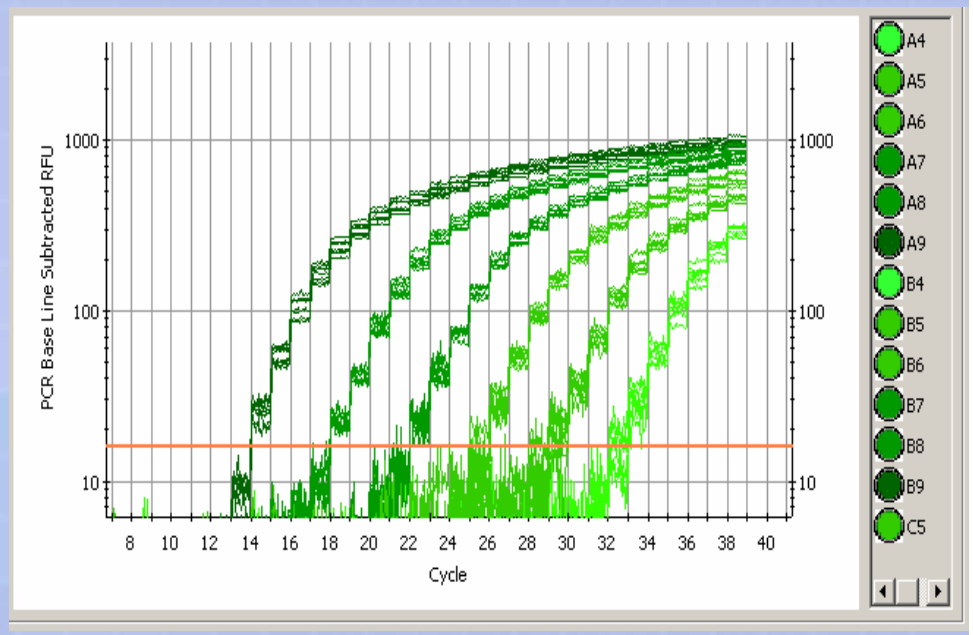
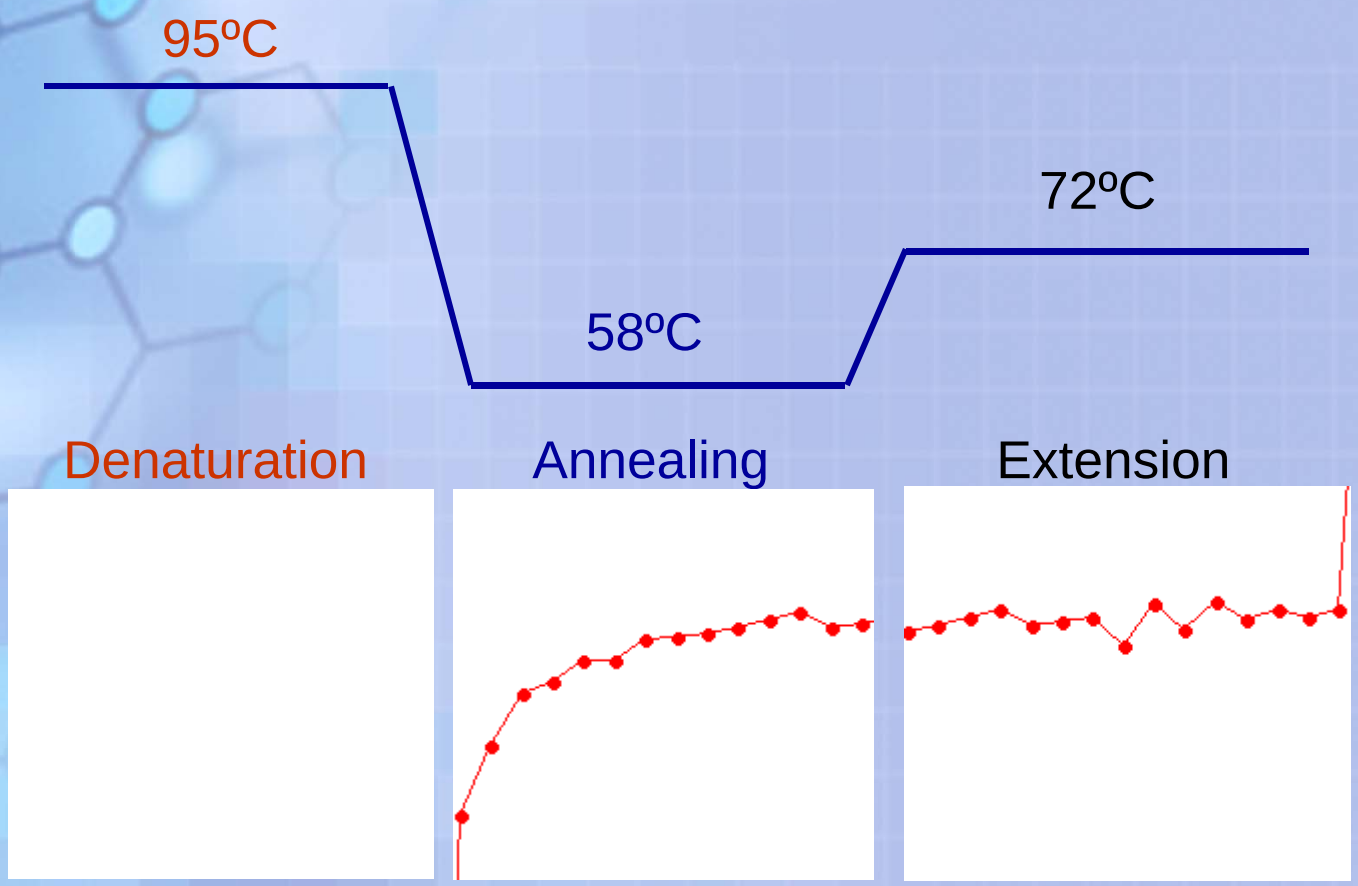
Continuous Data Collection

- iCycler iQ features continuous data collection that is designed to focus analytical scrutiny on subsets of the data gathered
  - Optimize annealing/extension time
  - Chemistry kinetics





Optimizing PCR Reactions: 2-Step PCR vs 3-Step



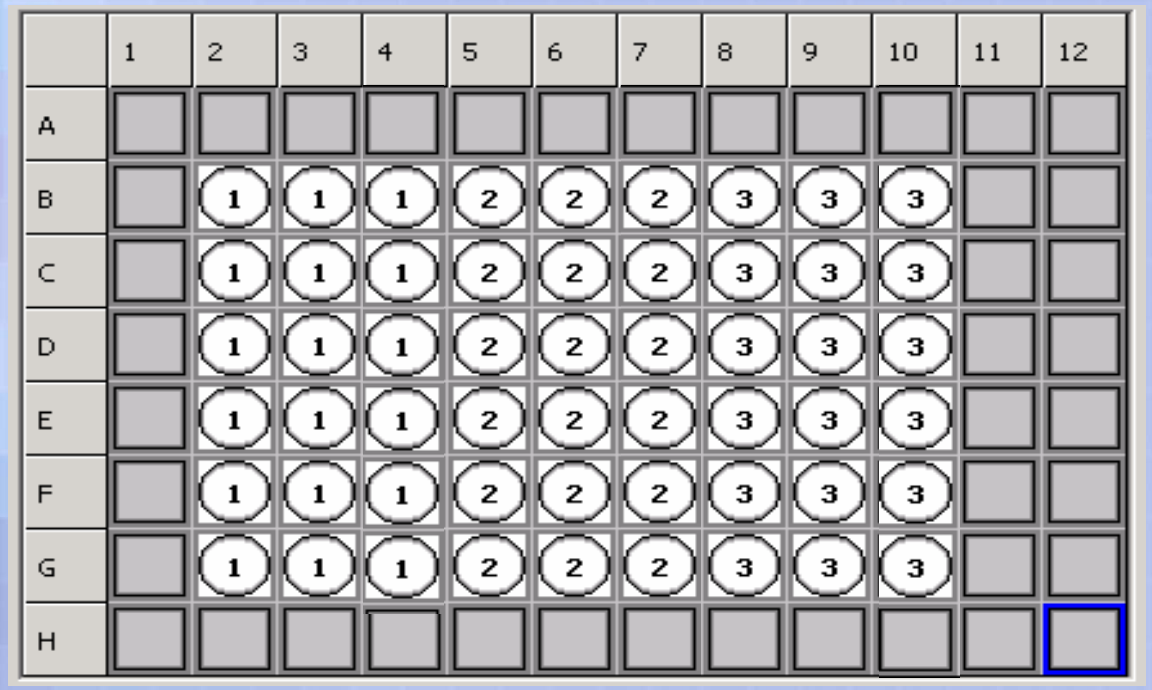
In some cases, can eliminate extension step since all amplification has already occurred during annealing

Thermal Gradient Optimization

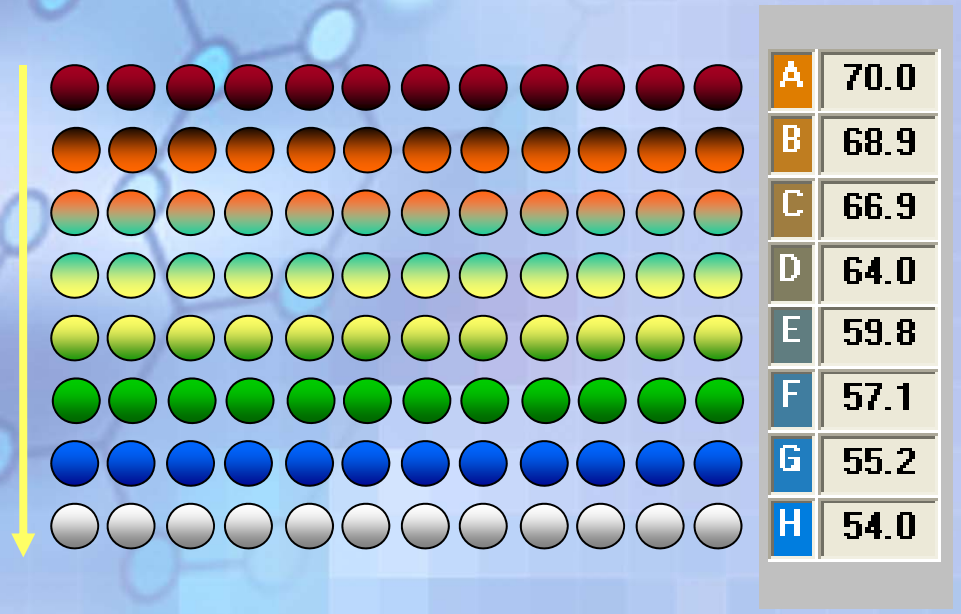
Look for specific product formation without primer dimers

Dilution series of primer [ ]

Temperature Gradient (annealing temperature)



iQ5/MYQ/CFX Thermal Gradient



- Used for annealing/extension temperature optimization for PCR reaction specificity and efficiency
- Up to 24ºC gradient range programmable across block

How do I get started?

Examples of Reagents and Plates

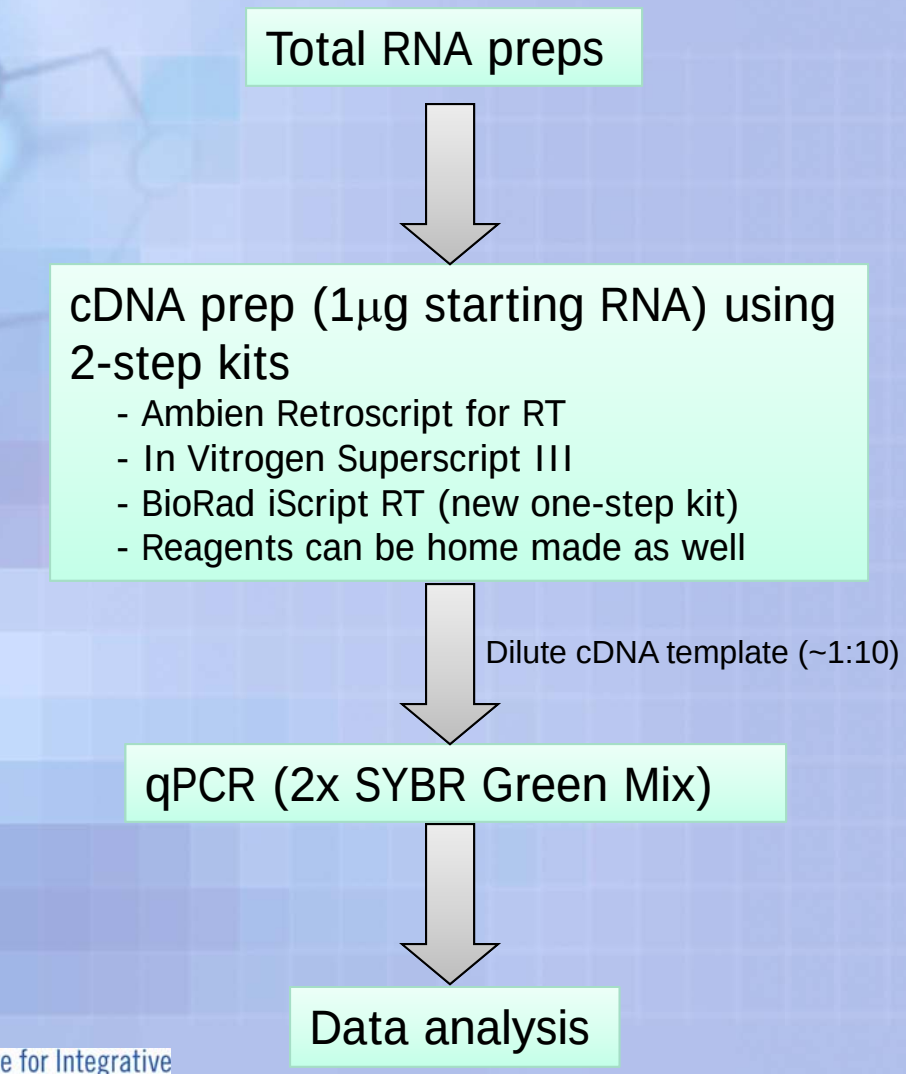
Disposables

- Low profile plates (CFX96) BioRad MLL9601 (Box of 25)
- Regular plates (iQ5) Biorad 2239441 (Box of 25)
- Microseal B Film Biorad MSB1001 (Pkg of 100)

Reagents

- iQ SYBR Green Supermix 500 x 50µl reaction (CFX and iQ5)
- iScript RT Supermix (1 step cDNA kit)
- SoAdvanced SYBRGreen Supermix 500 x 20µl reaction (CFX96 only; fast cycle reagent for <1 hr experiment)

Workflow


Total RNA preps
cDNA prep (1μg starting RNA) using 2-step kits
- Ambien Retroscript for RT
- In Vitrogen Superscript III
- BioRad iScript RT (new one-step kit)
- Reagents can be home made as well
Dilute cDNA template (~1:10)
qPCR (2x SYBR Green Mix)
Data analysis

Reaction Set-Up

qPCR reaction (per reaction)

Master mix	SYBRGreen SuperMix (Biorad)	12.5ul of 2x
	Primer forward (10uM)	0.25ul (100nM) (can use 100 to 500nM)
	Primer reverse (10uM)	0.25ul
	Water	10ul
		23ul
	Template	2ul (typically 1:10 dilution cDNA)
	Total volume	25ul

- Set-up can be at room temperature if using a hot-start Taq polymerase
- Can use smaller reaction volumes (15 to 20ul cost less)

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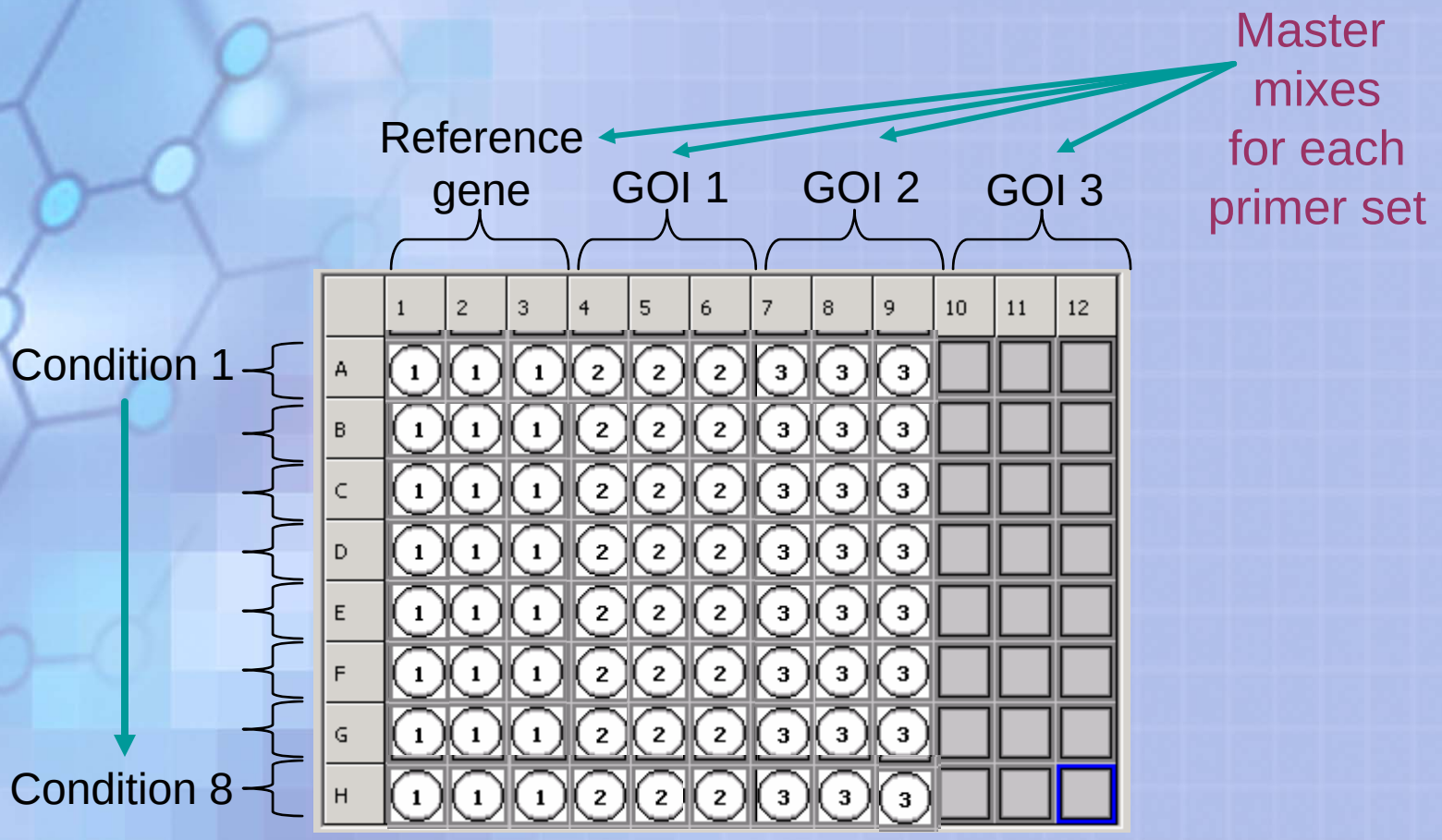
Plate Set-Up



Set-up can be at room temperature if using hot-start Taq

qPCR Optimization

A. Optimize conditions:

1) Annealing temperature
2) Primer concentration
3) Annealing/extension time
4) Sample prep protocol

B. Test efficiency of primers:

1) Primer-dimer
2) Secondary structure

C. Set up SYBR Green I experiments:

1) Standards and unknowns

SYBR Green I

Primer Design

There is nothing special about qPCR primers, but there are some considerations:

- Optimal Amplicon length 80-300 bp (can be longer)
- When designing primers, check for
  - predicted secondary structure
  - predicted primer-dimer formation
  - can also check for predicted hairpins in the amplicon
- Free tools at
  - IDT website (Google IDT)
    http://www.idtdna.com/SCITOOLS/scitools.aspx
  - mfold website
    http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi
  - Premier Biosoft
    http://www.premierbiosoft.com/index.html
- When designing multiple primers to be run on the same plate try to design primers with similar T_m

Template DNA

Genomic DNA

- Cut with restriction enzyme that does not cut within amplified region
- Boil DNA stock for 10 min and then onto ice

Plasmid DNA

- If it doesn't work, linearize plasmid with restriction enzyme that does not cut within amplified region

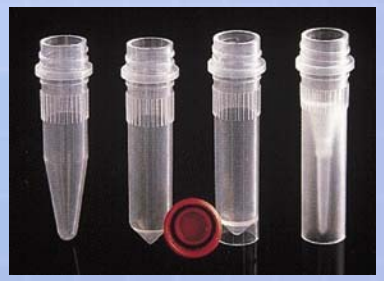
cDNA

- Treat total RNA with RNase-free DNase prior to reverse transcription
- Try to design primers at exon boundaries to avoid genomic DNA amplification

Getting Consistent Results

Do not underestimate the importance of using:

- Dedicated set of pipettors for real-time PCR
- Screwcap tubes
- Aerosol-barrier filter tips
- PCR-grade water
- Pipet carefully!



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Obtaining Consistent Results

- Wear gloves
- Create master mixes to average out error
- Mix very well by vortexing (at least 5 secs)
- No-template control to check for contamination
- Prepare reactions in replicate (ideally triplicate)
- Seal the optical sealer tape
- Inspect block for salt or dirt buildup
- Centrifuge to eliminate bubbles (bottom of well)

Data Analysis

- Relative Quantification
- Absolute Quantification
- Allelic Discrimination

Some References

Pfaffl method for relative gene quantification
Pfaffl (2001) Nucl acids Res 29(9): e45

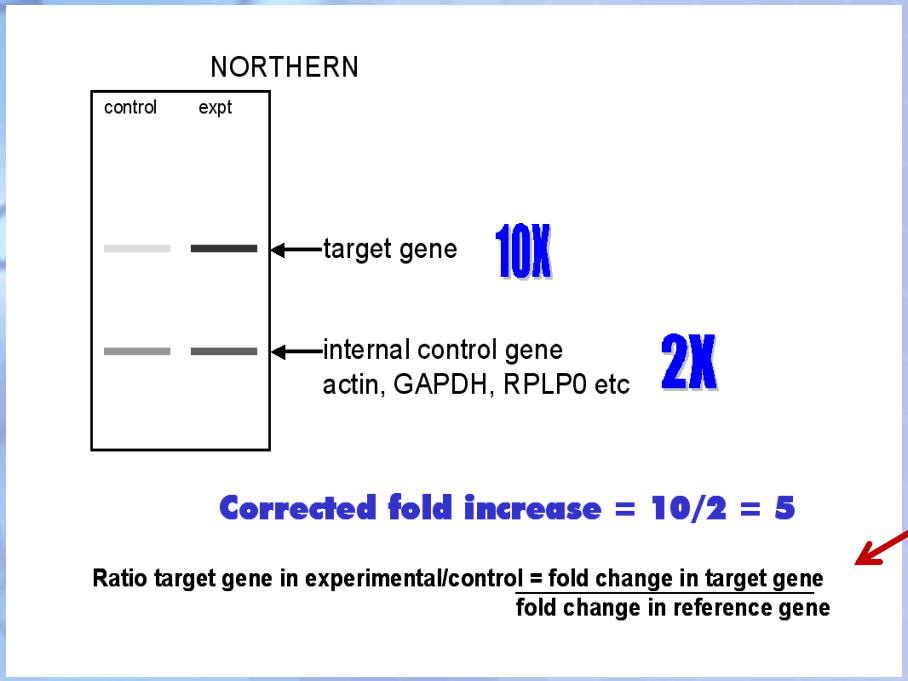
MIQE guidelines for publication of qPCR results
Bustin et al (2009) Clinical Chemistry 55(4):611
Taylor et al (2010) Methods 50:S1-S5

Reference gene selection and normalization
- Hellemans et al (2007) genome Biology8:R19 online at
http://genomebiology.com/2007/8/2/R19
- Vandesomple et al (2002) Genome Biology 3(7):research0034.1 online at
http://genomebiology.com/2002/3/7/research/0034

QPCR method and reviews
Bustin and Nolan (2004) J Biomolecular Techniques 15:155
Nolan et al (006) Nat Protocols 1(3):1559
D'Souza et al (2006) Pedriatrics 118:1664 (Why accurate results matter)

Principle of Relative Gene Expression

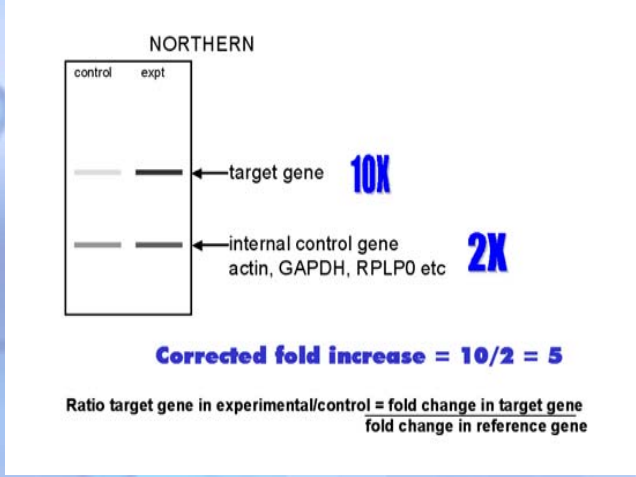
Example based on a Northern blot



Gene expression is normalized compared to a reference gene

Principle of Relative Gene Expression

Example based on a Northern blot



Normalized gene expression by qPCR (Pfaffl equation)

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

E = qPCR efficiency

Pfaffl (2001) Nucl acids Res 29(9): e45

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Calculating Relative Gene Expression

Pfaffl method: *(Pfaffl, 2001; Nucleic Acid Research)*

$$\text{Fold induction} = \frac{\text{Efficiency}_{\text{target}}^{\text{deltaCt}_{\text{target}}\ (\text{control-sample})}}{\text{Efficiency}_{\text{reference}}^{\text{deltaCt}_{\text{reference}}\ (\text{control-sample})}}$$

$$\text{Efficiency} = 10^{-1/\text{slope}}$$

Reference genes include Ubiquitin, actin, GAPDH, rDNA, and others

Calculating Relative Expression

Pfaffl method
(Efficiencies are normalized)

		Reference Primers (C_T)	GOI Primers (C_T)
	Tissue #1:	21	22
	Tissue #2:	20	24
(From Standard curve)	Efficiency:	90% = 1.9	100% = 2
	Delta C_T:	20-21 = -1	24-22 = 2

Fold induction in tissue #1 relative to tissue #2 $= \dfrac{2_{\text{target}}^{\text{deltaCt target}}\ (\mathbf{24\text{-}22 = 2})}{1.9_{\text{reference}}^{\text{deltaCt reference}}\ (\mathbf{20\text{-}21 = -1})} = \dfrac{4}{0.53} = 7.5$

All samples should be in triplicate with at least two biological replicates

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Calculating Relative Expression

Pfaffl method
(efficiencies are normalized)

	Reference Primers (C_T)	GOI Primers (C_T)
Mutant or treated:	21	22
Wild type or untreated:	20	24
(From Standard curve) Efficiency:	90% = 1.9	100% = 2
Delta C_T:	20-21 = -1	24-22 = 2

Fold induction in treated relative to untreated $= \dfrac{2_{\text{target}}^{\text{deltaCt target}}\ (\mathbf{24\text{-}22 = 2})}{1.9_{\text{reference}}^{\text{deltaCt reference}}\ (\mathbf{20\text{-}21 = -1})} = \dfrac{4}{0.53} = 7.5$

All samples should be in triplicate with at least two biological replicates

Displaying Results

Can export results to Excel within the iQ5 software

	Well	Fluor	Type	Identifier	Replicate #	Threshold Cycle (Ct)	Ct Mean	Ct Std. Dev
30 mins	A01	SYBR	Unkn	1A_DMSO30_Ub	1	21.65	22.19	0.487
	A02	SYBR	Unkn	1A_DMSO30_Ub	1	22.58		
	A03	SYBR	Unkn	1A_DMSO30_Ub	1	22.35		
	A04	SYBR	Unkn	1B_DMSO30_BL	2	30.56	30.42	0.147
	A05	SYBR	Unkn	1B_DMSO30_BL	2	30.27		
	A06	SYBR	Unkn	1B_DMSO30_BL	2	30.44		
	A07	SYBR	Unkn	2A_Pru30_Ub	3	21.64	21.83	0.175
	A08	SYBR	Unkn	2A_Pru30_Ub	3	21.86		
	A09	SYBR	Unkn	2A_Pru30_Ub	3	21.99		
	A10	SYBR	Unkn	2B_PrU30_BL	4	29.22	29.65	0.436
	A11	SYBR	Unkn	2B_PrU30_BL	4	30.09		
	A12	SYBR	Unkn	2B_PrU30_BL	4	29.63		
	B01	SYBR	Unkn	3A_BL30_Ub	5	21.5	21.34	0.14
	B02	SYBR	Unkn	3A_BL30_Ub	5	21.32		
	B03	SYBR	Unkn	3A_BL30_Ub	5	21.22		
	B04	SYBR	Unkn	3B_BL30_BL	6	27.67	27.32	0.324
	B05	SYBR	Unkn	3B_BL30_BL	6	27.25		
	B06	SYBR	Unkn	3B_BL30_BL	6	27.04		
	B07	SYBR	Unkn	4A_NAA30_Ub	7	21.35	21.53	0.172
	B08	SYBR	Unkn	4A_NAA30_Ub	7	21.57		
	B09	SYBR	Unkn	4A_NAA30_Ub	7	21.69		
	B10	SYBR	Unkn	4B_NAA30_BL	8	30.43	29.98	0.402
	B11	SYBR	Unkn	4B_NAA30_BL	8	29.68		
	B12	SYBR	Unkn	4B_NAA30_BL	8	29.82		
	C01	SYBR	Unkn	5A_BLPrU30_Ub	9	21.62	21.39	0.197
	C02	SYBR	Unkn	5A_BLPrU30_Ub	9	21.29		
	C03	SYBR	Unkn	5A_BLPrU30_Ub	9	21.26		
	C04	SYBR	Unkn	5B_BLPrU30_BL	10	28.15	28.48	0.309
	C05	SYBR	Unkn	5B_BLPrU30_BL	10	28.76		
	C06	SYBR	Unkn	5B_BLPrU30_BL	10	28.52		
	C07	SYBR	Unkn	6A_NAAPrU30_Ub	11	21	21.19	0.19
	C08	SYBR	Unkn	6A_NAAPrU30_Ub	11	21.18		
	C09	SYBR	Unkn	6A_NAAPrU30_Ub	11	21.38		
	C10	SYBR	Unkn	6B_NAAPrU30_BL	12	29.77	29.74	0.242
	C11	SYBR	Unkn	6B_NAAPrU30_BL	12	29.48		
	C12	SYBR	Unkn	6B_NAAPrU30_BL	12	29.96		

BioRad iQ5

Displaying Results

All software permits export of results to Excel

	Well	Fluor	Type	Identifier	Replicate #	Threshold Cycle (Ct)	Ct Mean	Ct Std. Dev
30 mins	A01	SYBR	Unkn	1A_DMSO30_Ub	1	21.65	22.19	0.487
	A02	SYBR	Unkn	1A_DMSO30_Ub	1	22.58		
	A03	SYBR	Unkn	1A_DMSO30_Ub	1	22.35		
	A04	SYBR	Unkn	1B_DMSO30_BL	2	30.56	30.42	0.147
	A05	SYBR	Unkn	1B_DMSO30_BL	2	30.27		
	A06	SYBR	Unkn	1B_DMSO30_BL	2	30.44		
	A07	SYBR	Unkn	2A_Pru30_Ub	3	21.64	21.83	0.175
	A08	SYBR	Unkn	2A_Pru30_Ub	3	21.86		
	A09	SYBR	Unkn	2A_Pru30_Ub	3	21.99		
	A10	SYBR	Unkn	2B_PrU30_BL	4	29.22	29.65	0.436
	A11	SYBR	Unkn	2B_PrU30_BL	4	30.09		
	A12	SYBR	Unkn	2B_PrU30_BL	4	29.63		
	B01	SYBR	Unkn	3A_BL30_Ub	5	21.5	21.34	0.14
	B02	SYBR	Unkn	3A_BL30_Ub	5	21.32		
	B03	SYBR	Unkn	3A_BL30_Ub	5	21.22		
	B04	SYBR	Unkn	3B_BL30_BL	6	27.67	27.32	0.324
	B05	SYBR	Unkn	3B_BL30_BL	6	27.25		
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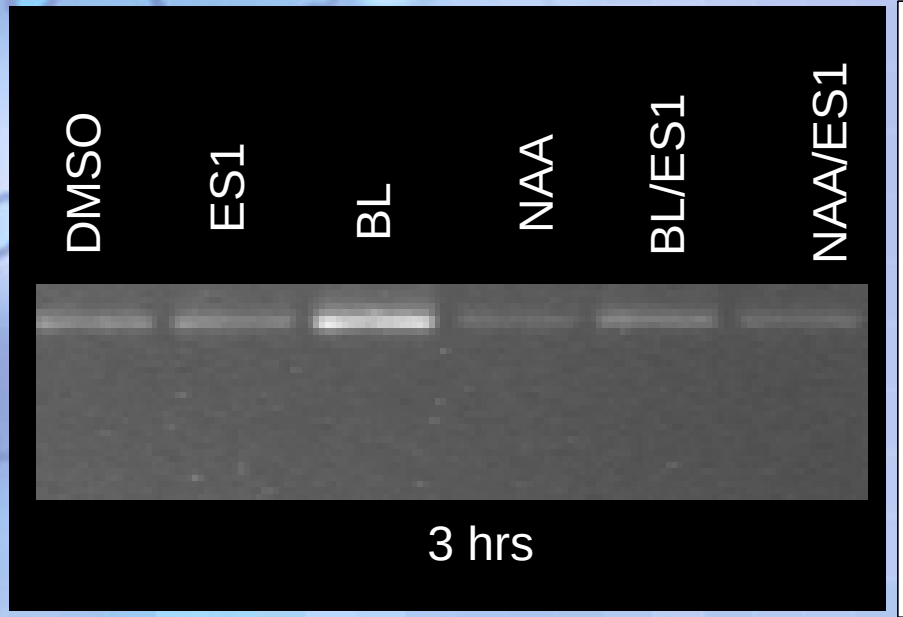
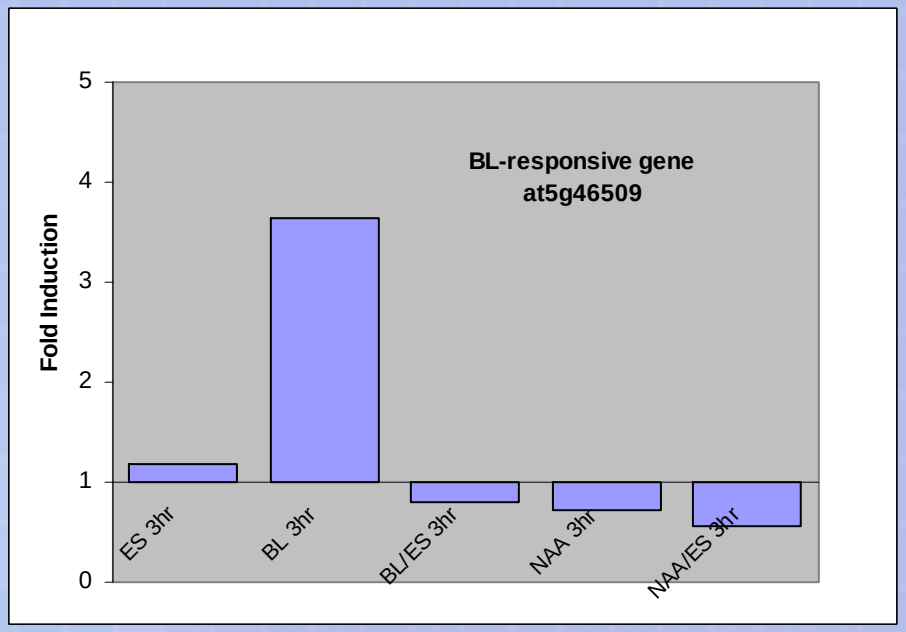
	B07	SYBR	Unkn	4A_NAA30_Ub	7	21.35	21.53	0.172
	B08	SYBR	Unkn	4A_NAA30_Ub	7	21.57		
	B09	SYBR	Unkn	4A_NAA30_Ub	7	21.69		
	B10	SYBR	Unkn	4B_NAA30_BL	8	30.43	29.98	0.402
	B11	SYBR	Unkn	4B_NAA30_BL	8	29.68		
	B12	SYBR	Unkn	4B_NAA30_BL	8	29.82		
	C01	SYBR	Unkn	5A_BLPrU30_Ub	9	21.62	21.39	0.197
	C02	SYBR	Unkn	5A_BLPrU30_Ub	9	21.29		
	C03	SYBR	Unkn	5A_BLPrU30_Ub	9	21.26		
	C04	SYBR	Unkn	5B_BLPrU30_BL	10	28.15	28.48	0.309
	C05	SYBR	Unkn	5B_BLPrU30_BL	10	28.76		
	C06	SYBR	Unkn	5B_BLPrU30_BL	10	28.52		
	C07	SYBR	Unkn	6A_NAAPrU30_Ub	11	21	21.19	0.19
	C08	SYBR	Unkn	6A_NAAPrU30_Ub	11	21.18		
	C09	SYBR	Unkn	6A_NAAPrU30_Ub	11	21.38		
	C10	SYBR	Unkn	6B_NAAPrU30_BL	12	29.77	29.74	0.242
	C11	SYBR	Unkn	6B_NAAPrU30_BL	12	29.48		
	C12	SYBR	Unkn	6B_NAAPrU30_BL	12	29.96		

Displaying Results

Semi-quantitative RT-PCR



Quantitative-PCR



- Comparison of chemical vs non-treated samples
- Histogram generated in Excel

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Reference Genes (Vandesompele Method)

- There are no true “House keeping” genes
- This method uses more than 1 reference gene (3 is recommended) and takes the geometric mean to normalize fold expression
- Using a single reference gene can lead to erroneous normalization up to 3.0-fold and 6.4-fold in 25% and 10% of the cases, respectively, with sporadic values above 20-fold
- geNorm site: http://medgen.ugent.be/~jvdesomp/genorm/
- geNorm is a popular algorithm to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel
- The CFX96 will provide Target Stability Values (M) to help assess reference genes

Vandesomple et al (2002) Genome Biology 3(7):research0034.1 online at http://genomebiology.com/2002/3/7/research/0034

Why Careful Results Matter

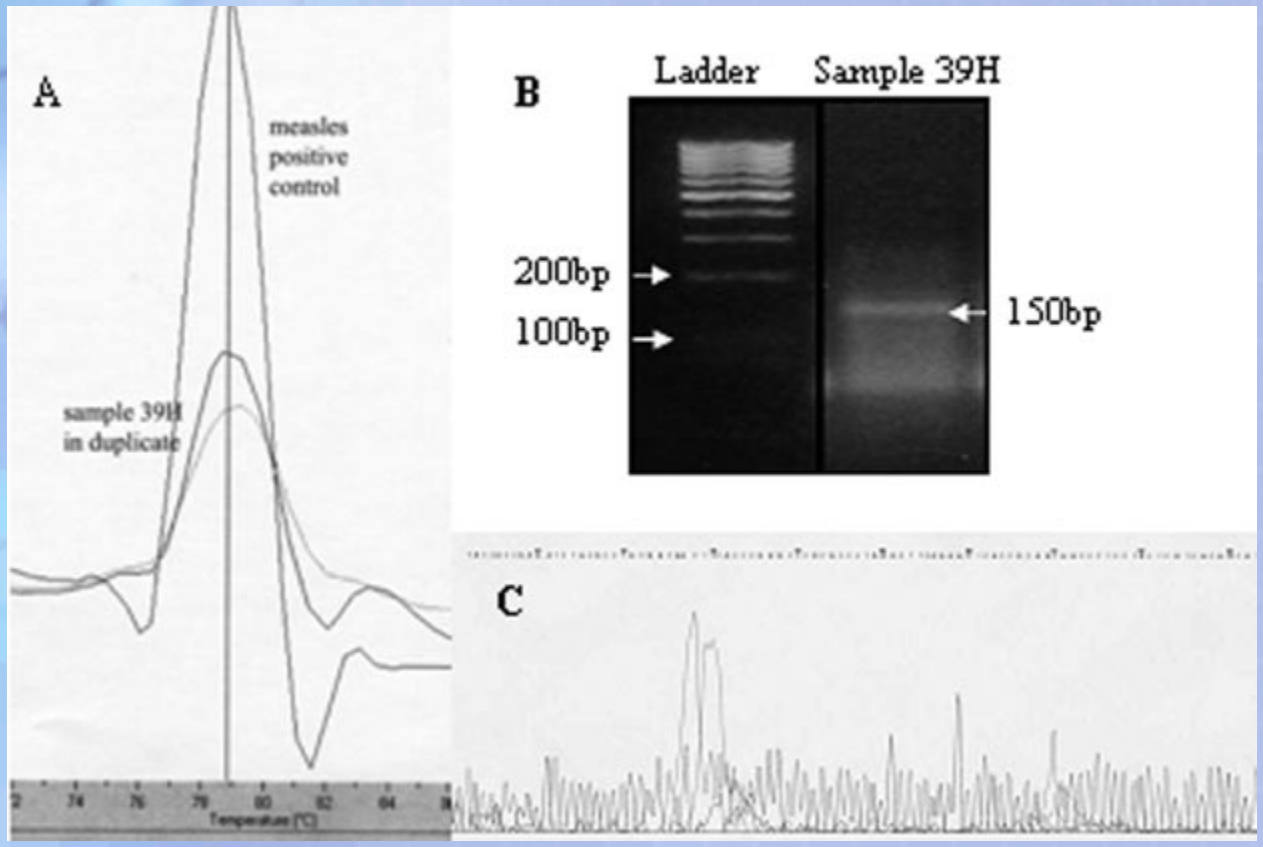
CONTROVERSY OVER MEASLES-MUMPS-RUBELLA (MMR) vaccine erupted in 1998 when it was suggested that the measles virus (MV) component of the vaccine was responsible for autistic enterocolitis, a new form of autism spectrum disorder (ASD) characterized by the presence of ileo-colonic lymphonodular hyperplasia, chronic inflammatory colonic disease, and loss of acquired cognitive skills after a period of normal development.

Wakefield AJ, Murch SH, Anthony A, et al. Ileal-lymphoidnodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children. *Lancet.* 1998;351:637–641

Fear has reduced MMR vaccinations in the US

Poor Technique with Serious Consequences

Non-specific products have correct melt temp for measles product, but not confirmed by sequence



D'Souza et al (2006) Pediatrics 118:1664

Reagents and Plates

Disposables

- Low profile plates (CFX96) BioRad MLL9601 (Box of 25) -$76
- Regular plates (iQ5) Biorad 2239441 (Box of 25) - $76
- Microseal B Film BIORAD MSB1001 (Pkg of 100) - $109.80

Reagents

- iQ SYBR Green Supermix 500 x 50μl reaction (CFX and iQ5) - $568
- iScript RT Supermix (1 step cDNA kit) - $387
- SsoAdvanced SYBR Supermix 1000 x 20μl reaction (CFX fast cycle reagent for <1 hr experiment) - $430

Data Analysis

FINIS