Introduction to Real-Time PCR and CFX System

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2021/02/2`
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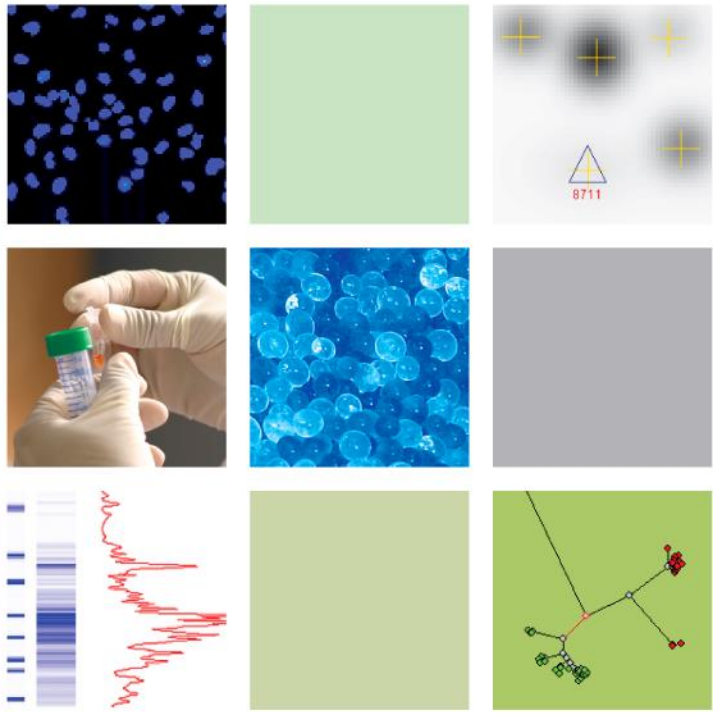
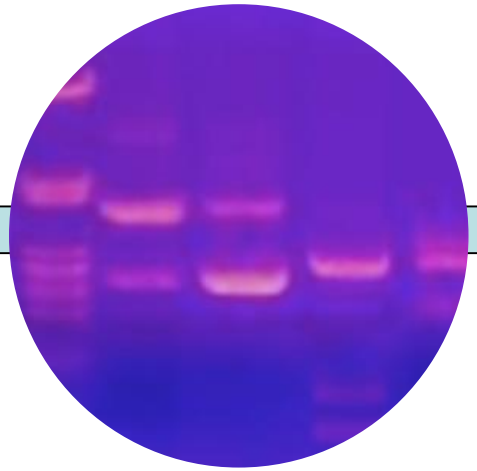


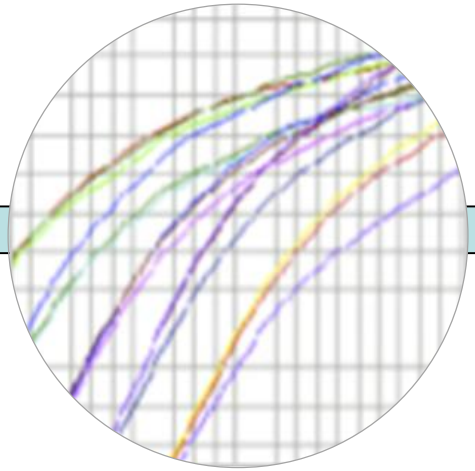
Generations of PCR

1st Generation



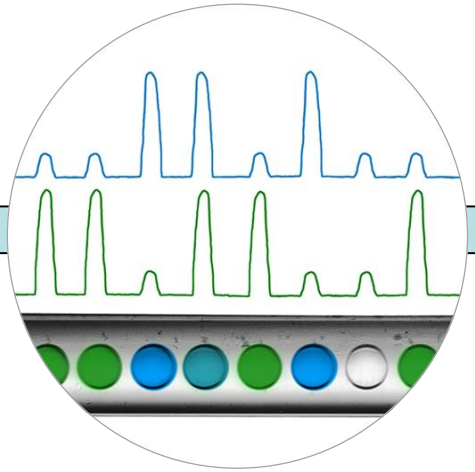
Gel Electrophoresis
(qualitative)

2nd Generation



Real-Time PCR
(indirect quantification)

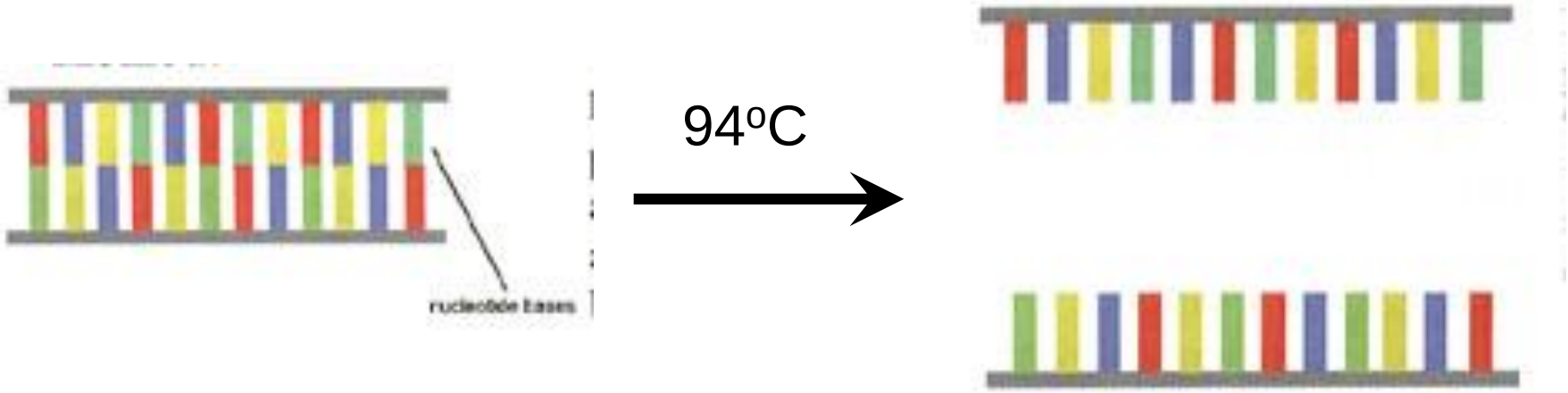
3rd Generation



Droplet Digital PCR
(absolute quantification)

Targeted DNA replication using thermostable DNA polymerase

DENATURE

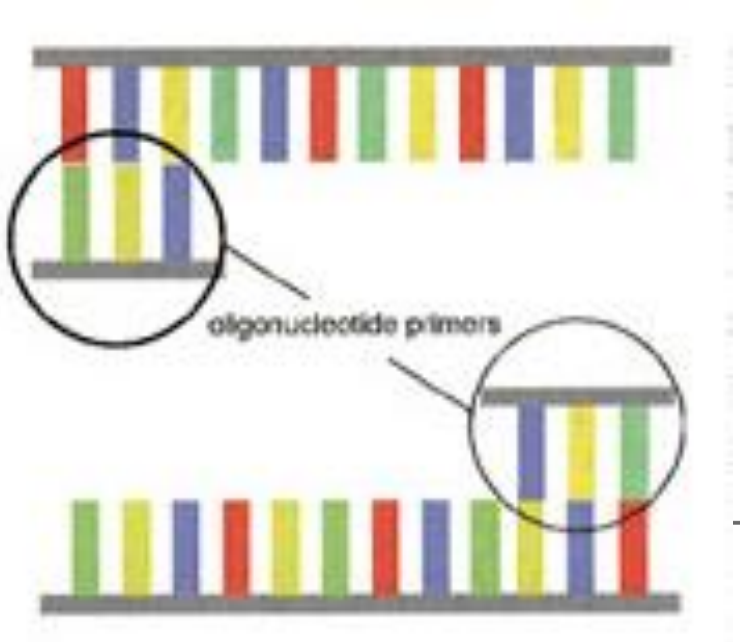


Polymerase Chain Reaction (PCR)

The use of two primers allows targeting of specific sequences

Primers are complementary to opposite strands of target region but not complementary to any other sequences

ANNEAL PRIMERS

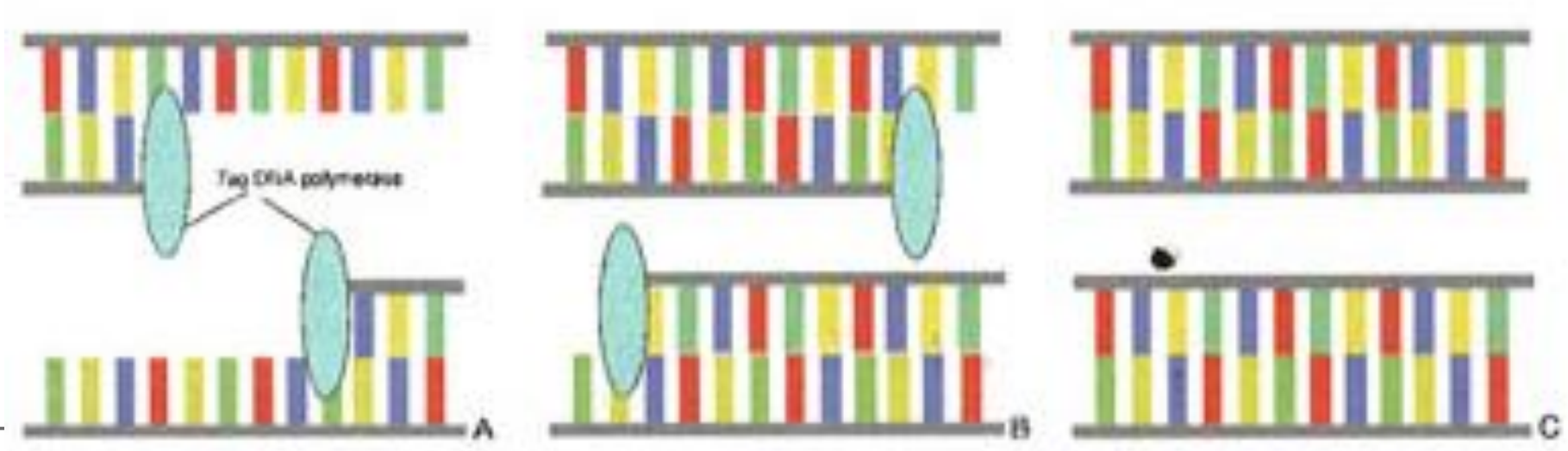


50 – 65°C

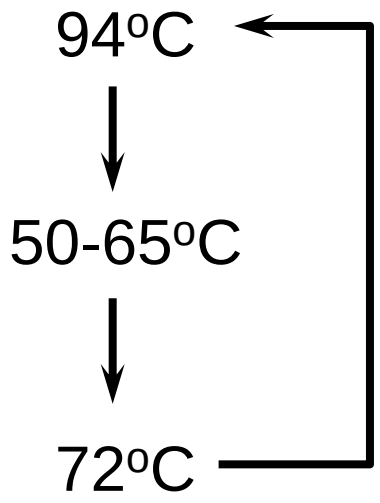
Taq polymerase synthesizes DNA complementary to template in 5` to 3` direction

EXTEND STRANDS

72°C



Polymerase Chain Reaction (PCR)



Each cycle of PCR doubles the number of progeny DNA duplexes (which can then act as template as well)

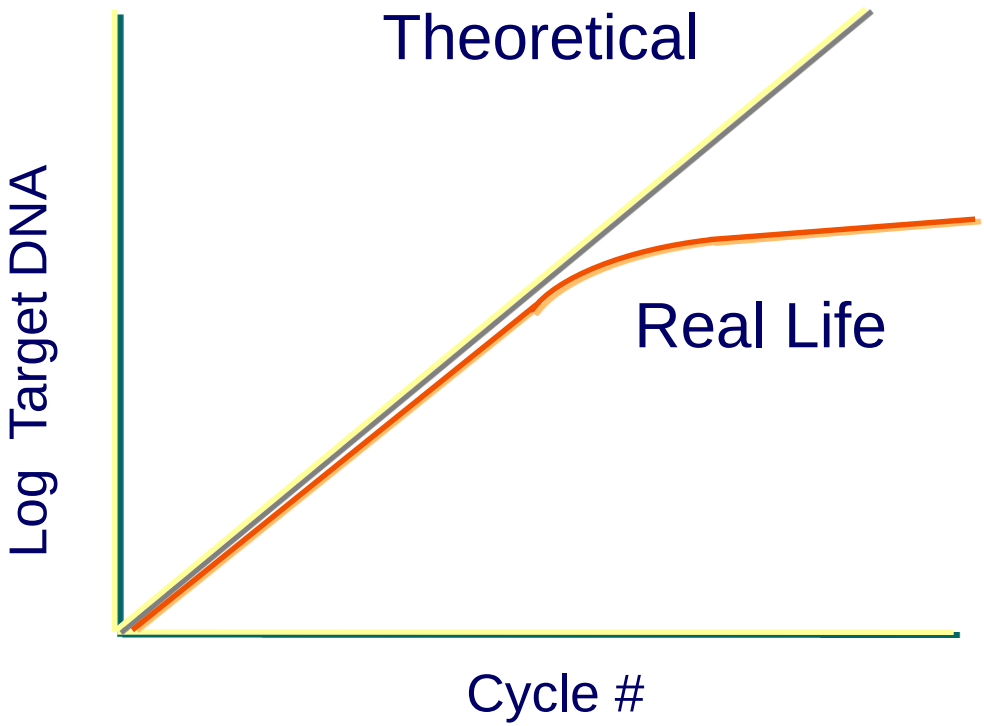
1 cycle = 2^1 copies of starting template

25 cycles = 2^{25} copies of starting template (~33554432)

Amplification is exponential, but the exponential increase is limited:

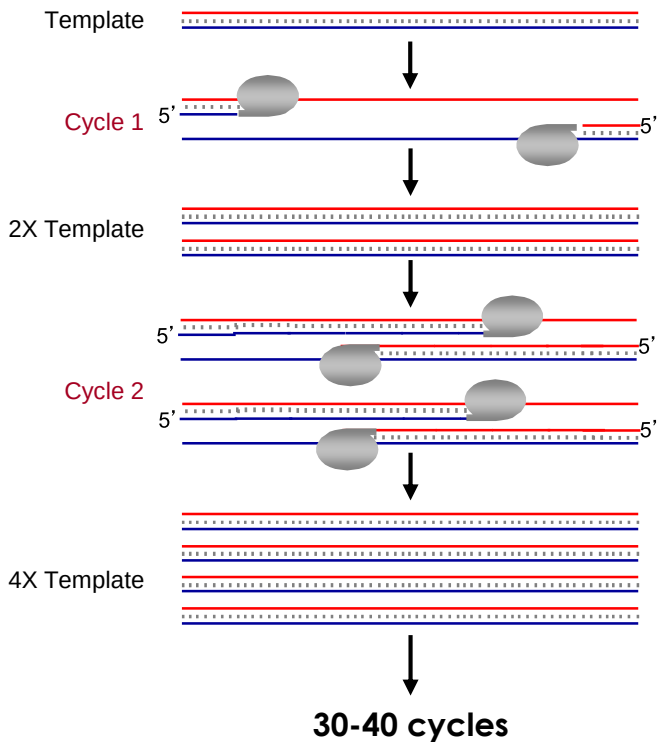
- **A linear increase follows exponential**
- **Eventually plateaus**

Real-Time PCR allows us to 'see' the exponential phase so we can calculate how much we started with.

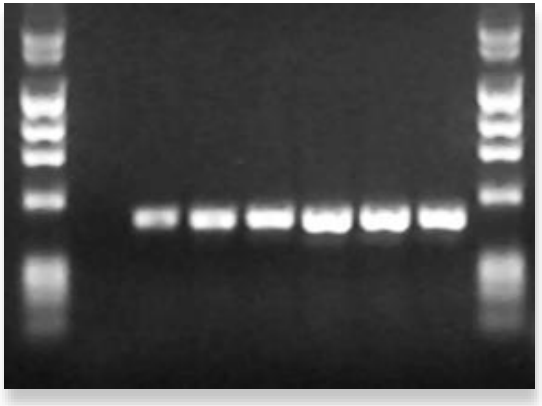


PCR – 1st Generation

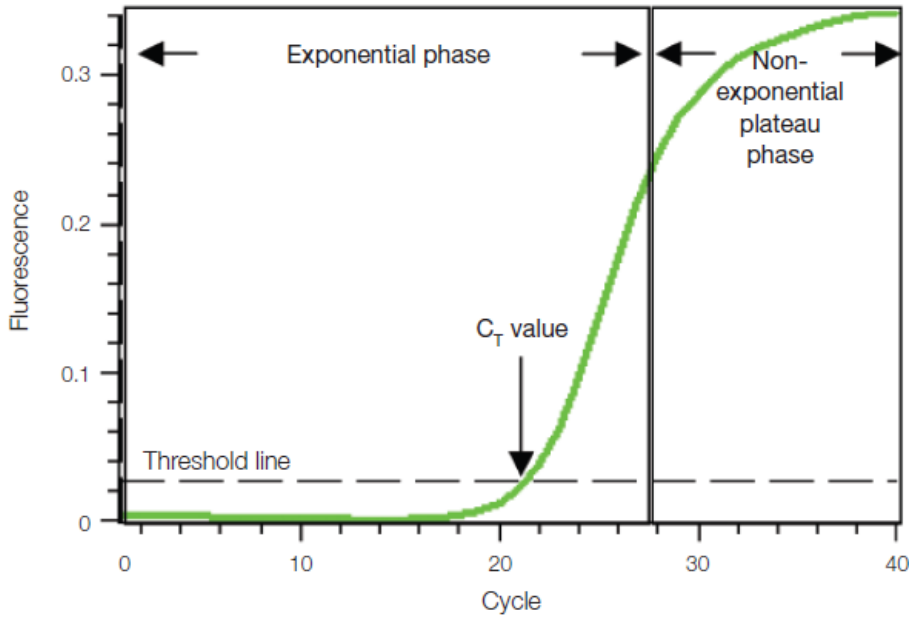
- Amplify target DNA with end point analysis to distinguish products
  - –No relationship between end point and starting target copies

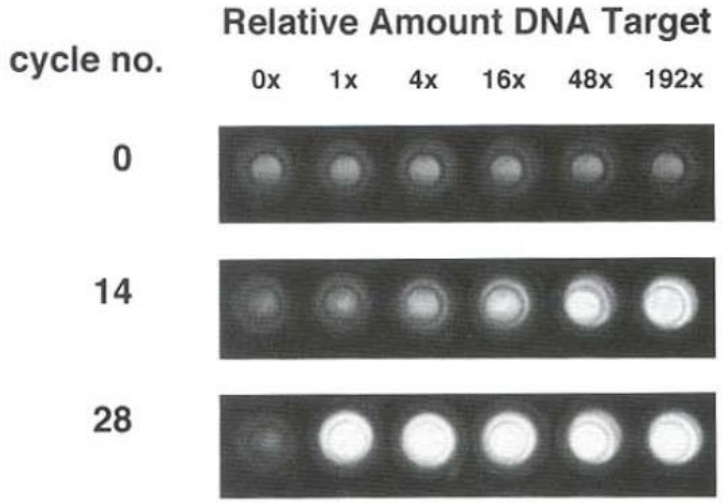


End point analysis



qPCR Principle

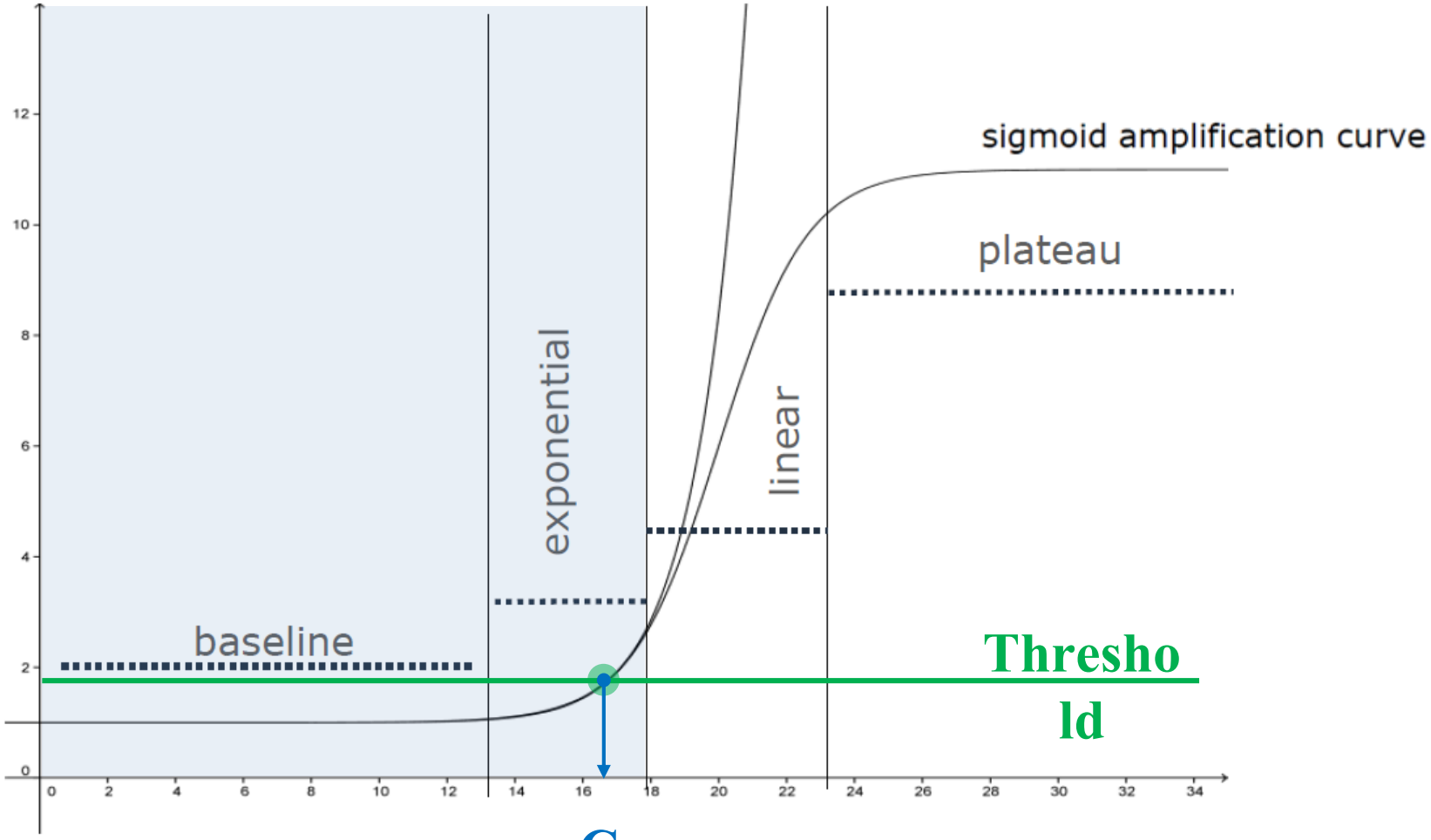




- Record target-specific fluorescence during the PCR and then determination C*q* Value for sample
- An inverse, linear relationship exists between the logarithm of the initial target quantity and the Cq
- Signal threshold determines Cq value (formerly Ct)
- Logarithmic base 2 scale

Determining Threshold and Cq

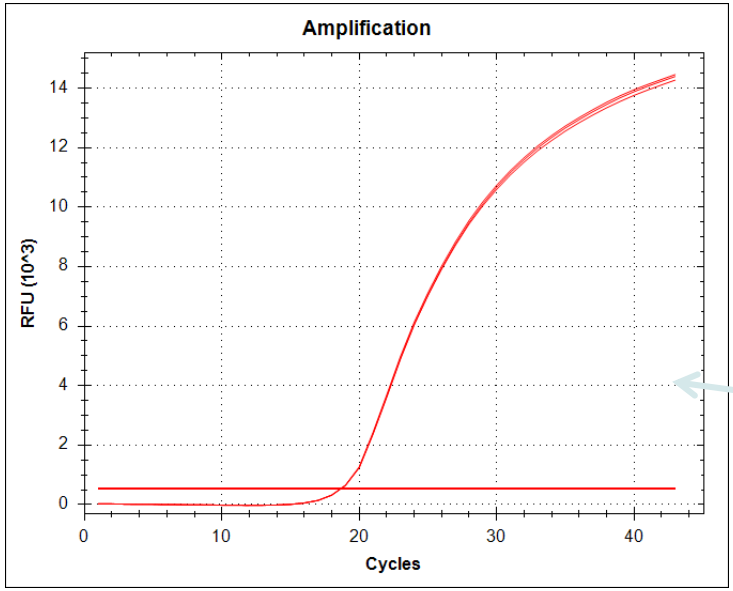
$$X_n = X_0(1+E)^n$$

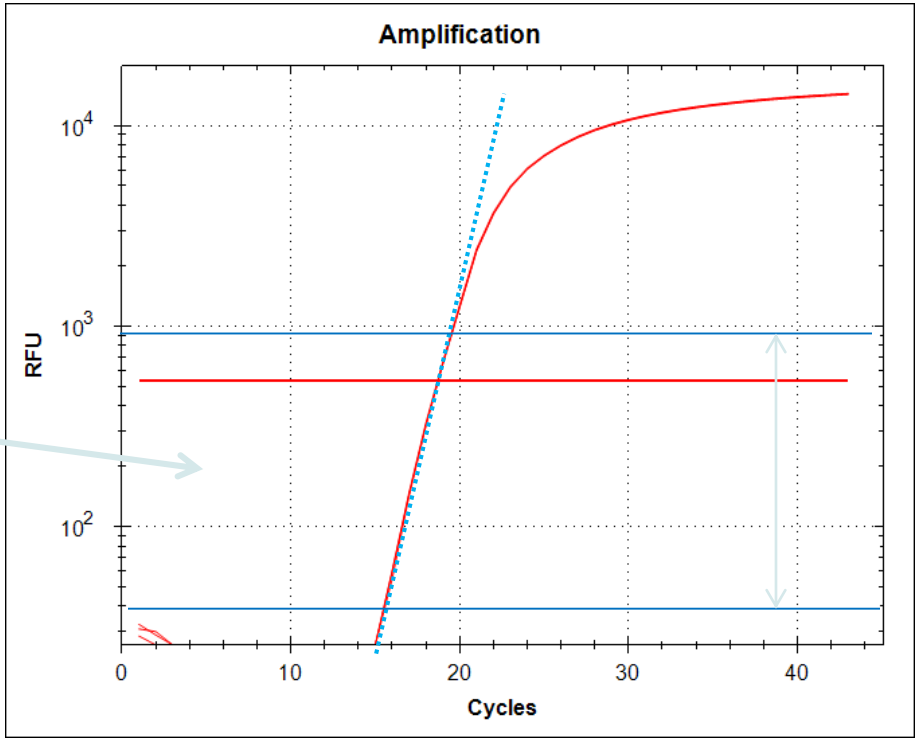


Cq

Determining Threshold and Cq

- Normal view versus Log view





C_T value v.s. concentration

- 1 cycle = 2 fold difference
- 3.32 cycles ≅ 10 fold difference

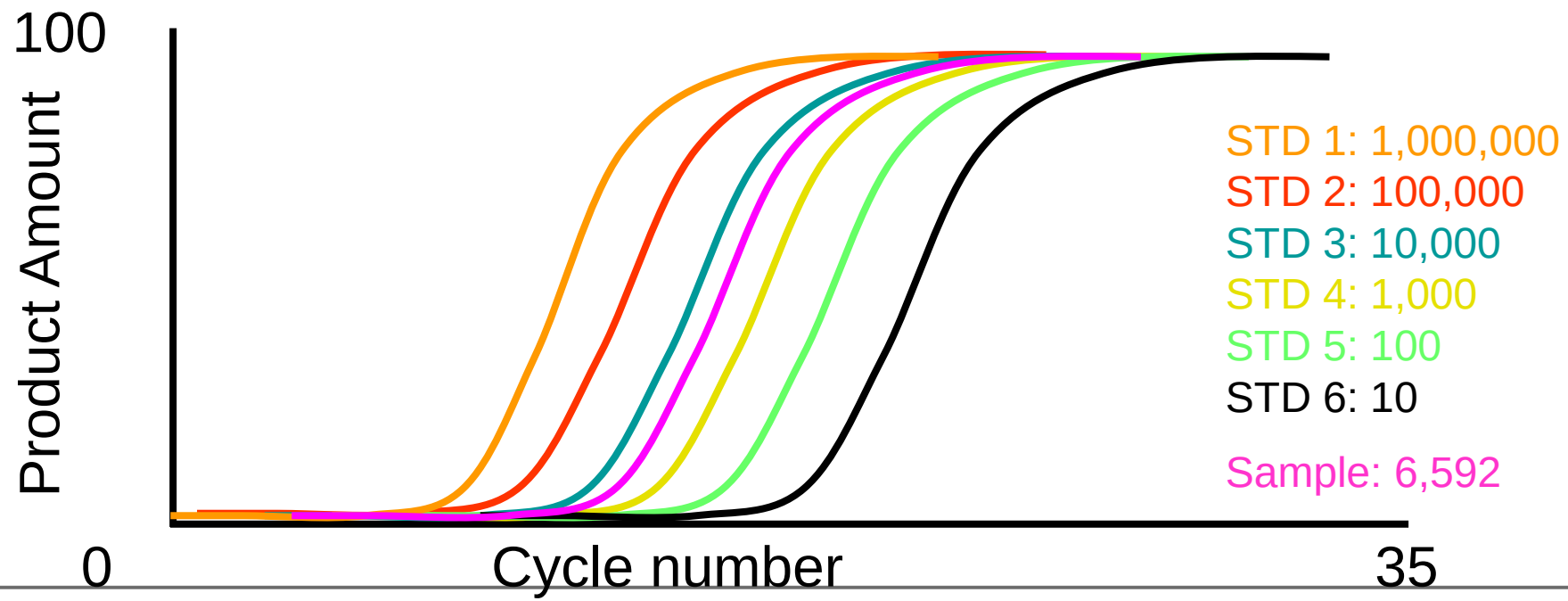
$Y = N_0\ 2^n$ → $Y = N_0\ (1+E)^n$

- Assumes 100% efficiency

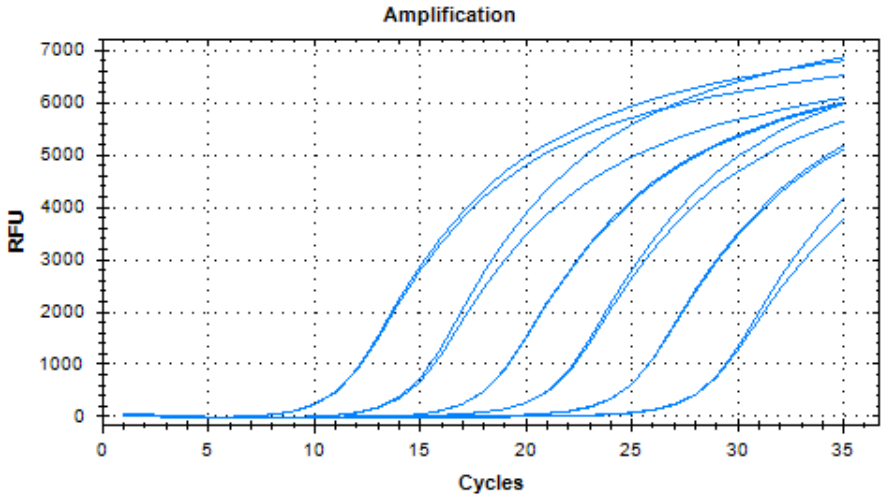
Quantitative PCR (qPCR)

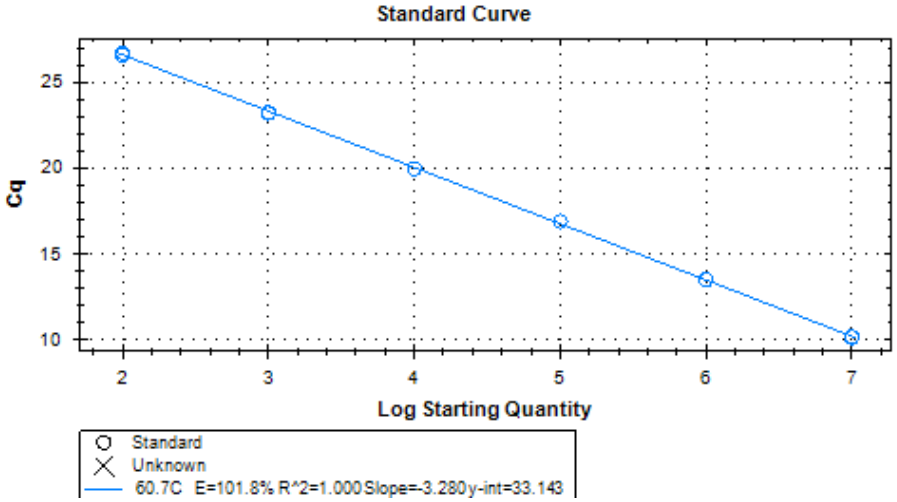
Create a **standard curve** with 10-fold serial dilutions of PCR product – assign arbitrary values

Compare values from standards with values for unknown sample



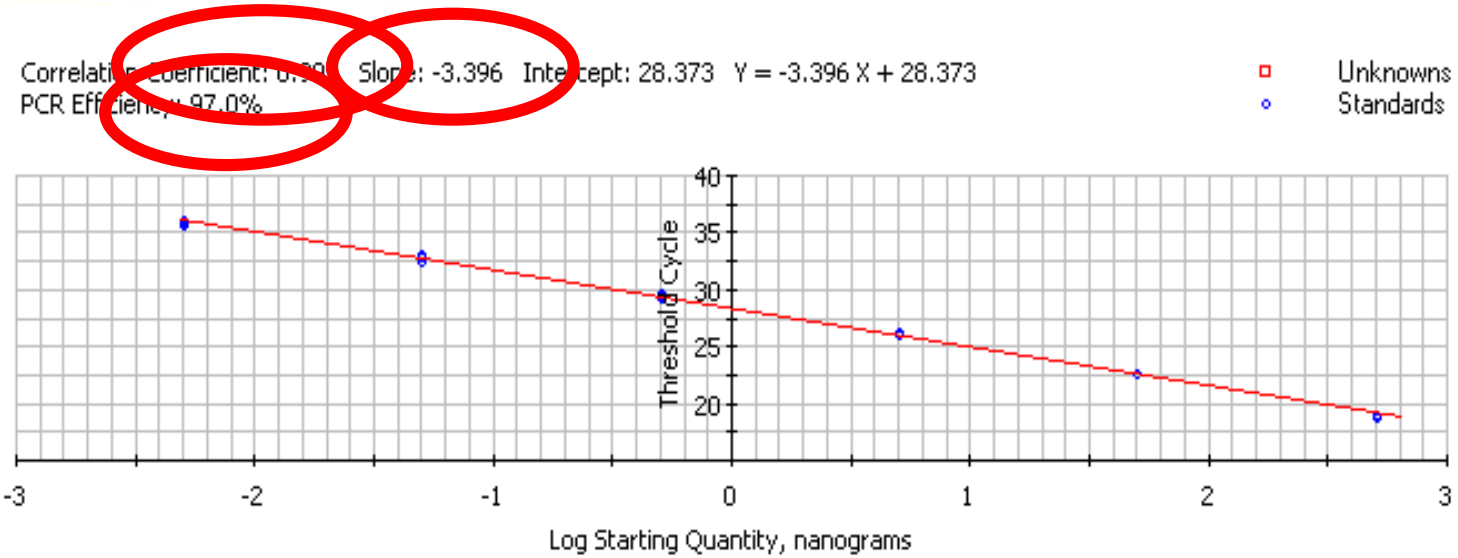
Standard Curves





A standard curve allows you to determine the efficiency of your assay

C_T is linear with the log of starting copy number (standard curve)



The slope of the standard curve can be directly correlated to the efficiency of the reactions:

r = is a measure of how well the actual data fit to the standard curve.

= (explained variation/total variation)

Aim for R value (Correlation Coefficient) of ≥0.98

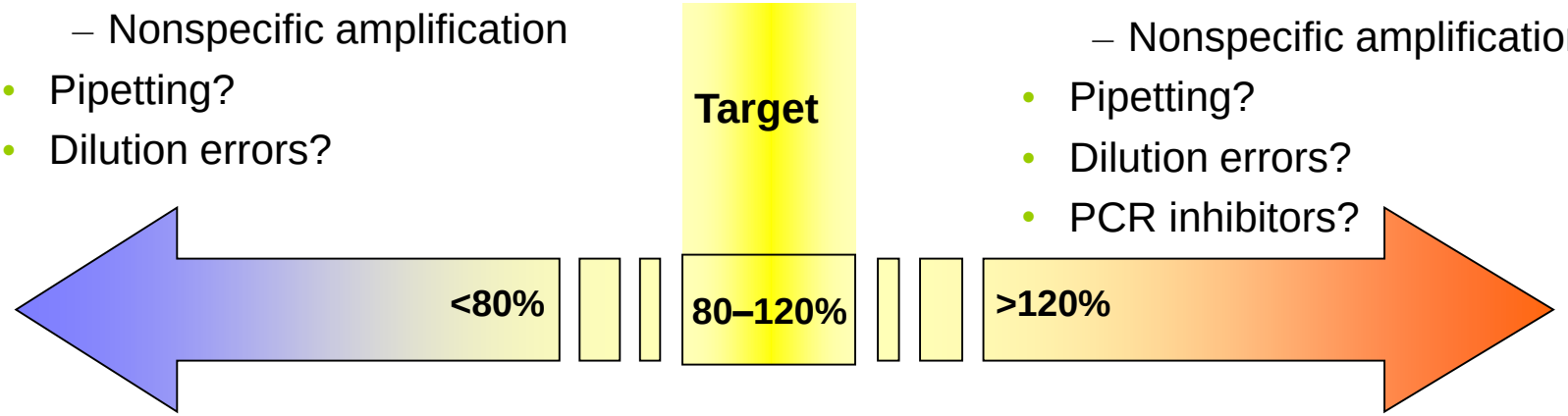
Efficiency (E) = $[10^{(-1/\text{slope})}] - 1$

when slope = -3.32, Efficiency = 100%

Efficiency Requirements

- Assay design
  - Secondary structures?
  - Primer-dimers?
  - Nonspecific amplification
- Pipetting?
- Dilution errors?

- Assay design
  - Secondary structures?
  - Primer-dimers?
  - Nonspecific amplification
- Pipetting?
- Dilution errors?
- PCR inhibitors?



E	Slope
0.5	-5.679
0.6	-4.899
0.7	-4.339
0.8	-3.917
0.9	-3.587
1	-3.322
1.1	-3.103
1.2	-2.920
1.3	-2.765

Slope = - [1/log (1+E)]

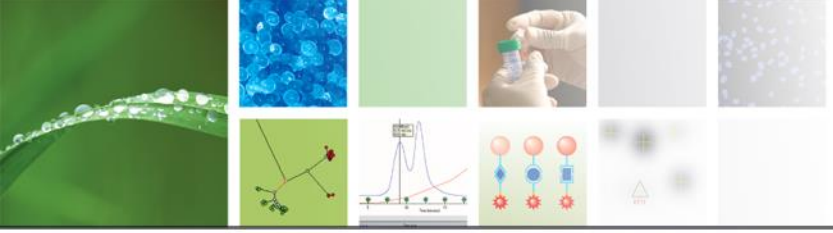
log (1+E) = - (1/slope)

$E = 10^{[-1/slope]} - 1$

Aim for Efficiency Values:

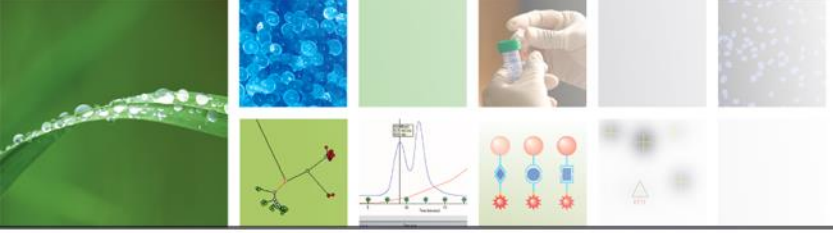
Good = 90 – 110%

Fantastic = 95 – 105%



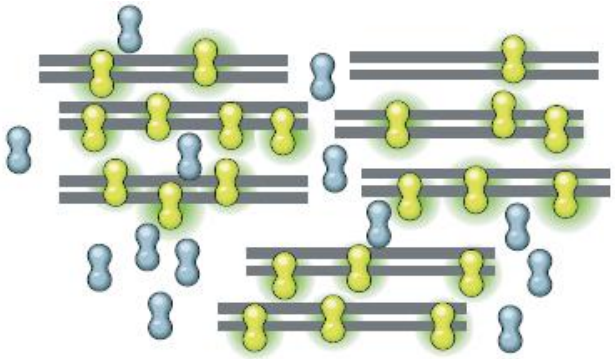
Points to Remember

- Threshold Cycle values (C_T) have a direct relationship to the amount of starting template
- Check space between C_T values follows correct relationship (100% efficiency) 2^n = fold dilution
- Efficiency of reactions between 90-110%
- R value should be ≥*0.98*

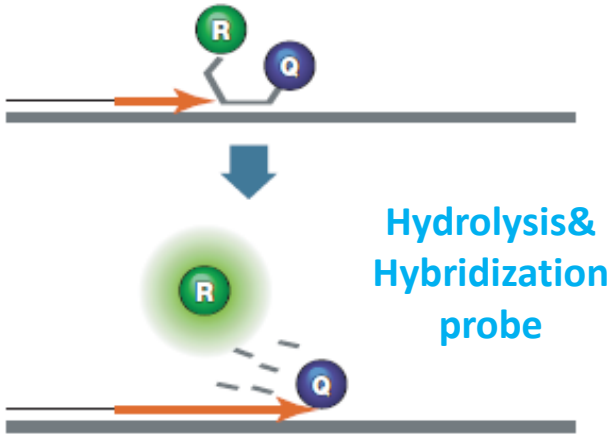


What are the most common detection strategies used for Real-Time PCR?

- The fluorescent molecules can be used
  - Non-specific DNA binding dyes
    - SYBR® Green I
  - Specific Hybridization Probes/Primers
    - TaqMan™

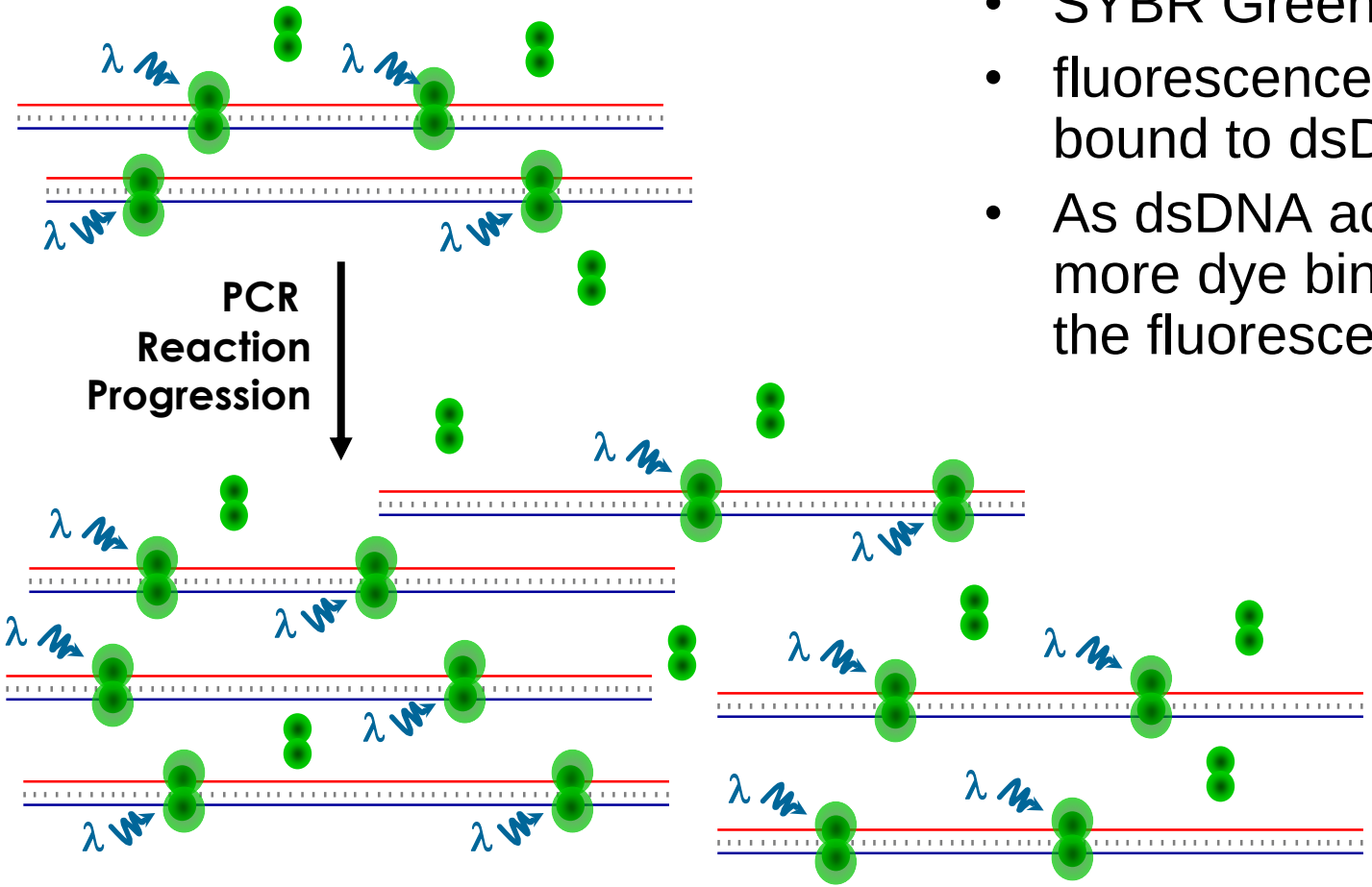


Nucleic Acid
Intercalating dye

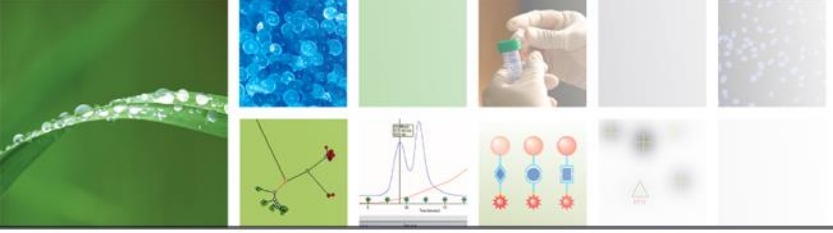


Hydrolysis&
Hybridization
probe

Real-Time Chemistry: SYBR Green



- SYBR Green I binds dsDNA
- fluorescence increases when bound to dsDNA
- As dsDNA accumulates, more dye binds the DNA and the fluorescence increase



Intercalation dyes: SYBR and EvaGreen

Advantages

- Intercalation dyes-based assays are more economical than probes-based ones
- Allow melt curve analysis to test assay specificity

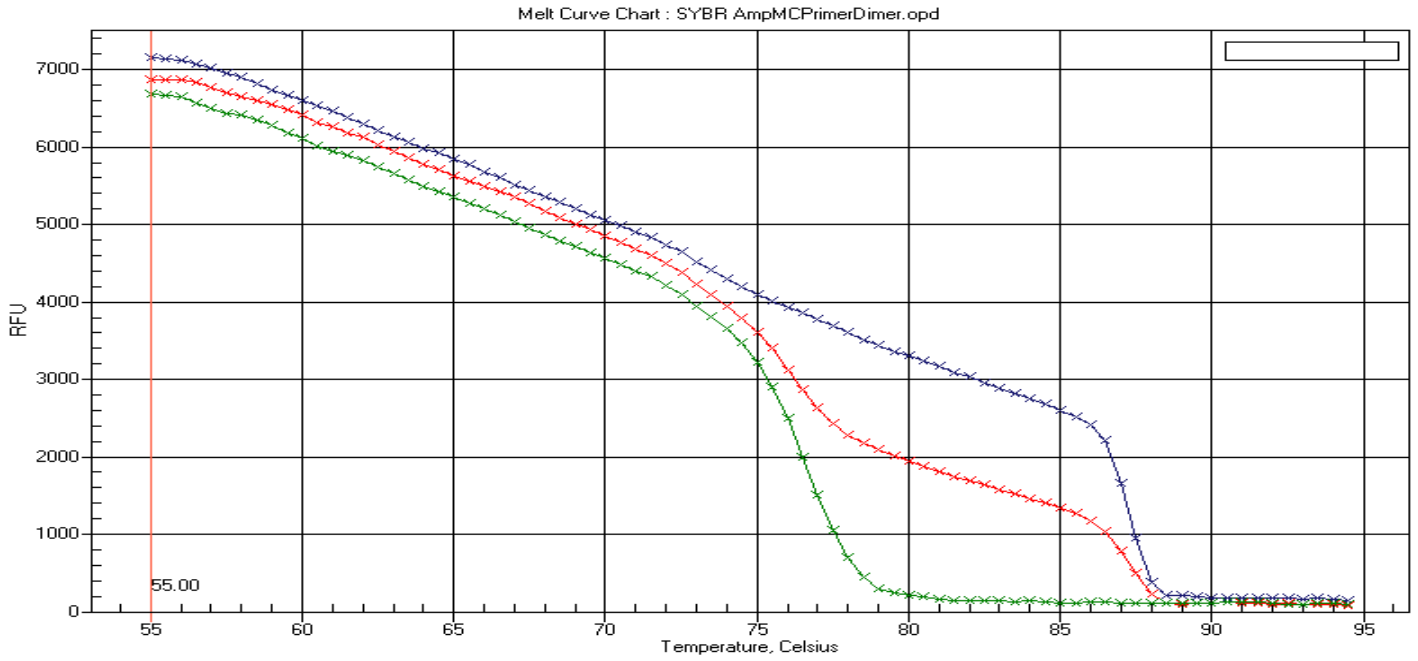
Disadvantages

- You can not multiplex with dyes
- Dyes do not discriminate and will bind any dsDNA in your sample
  - This includes primer dimers
  - Problems if assay is not specific

Melt Curve Analysis

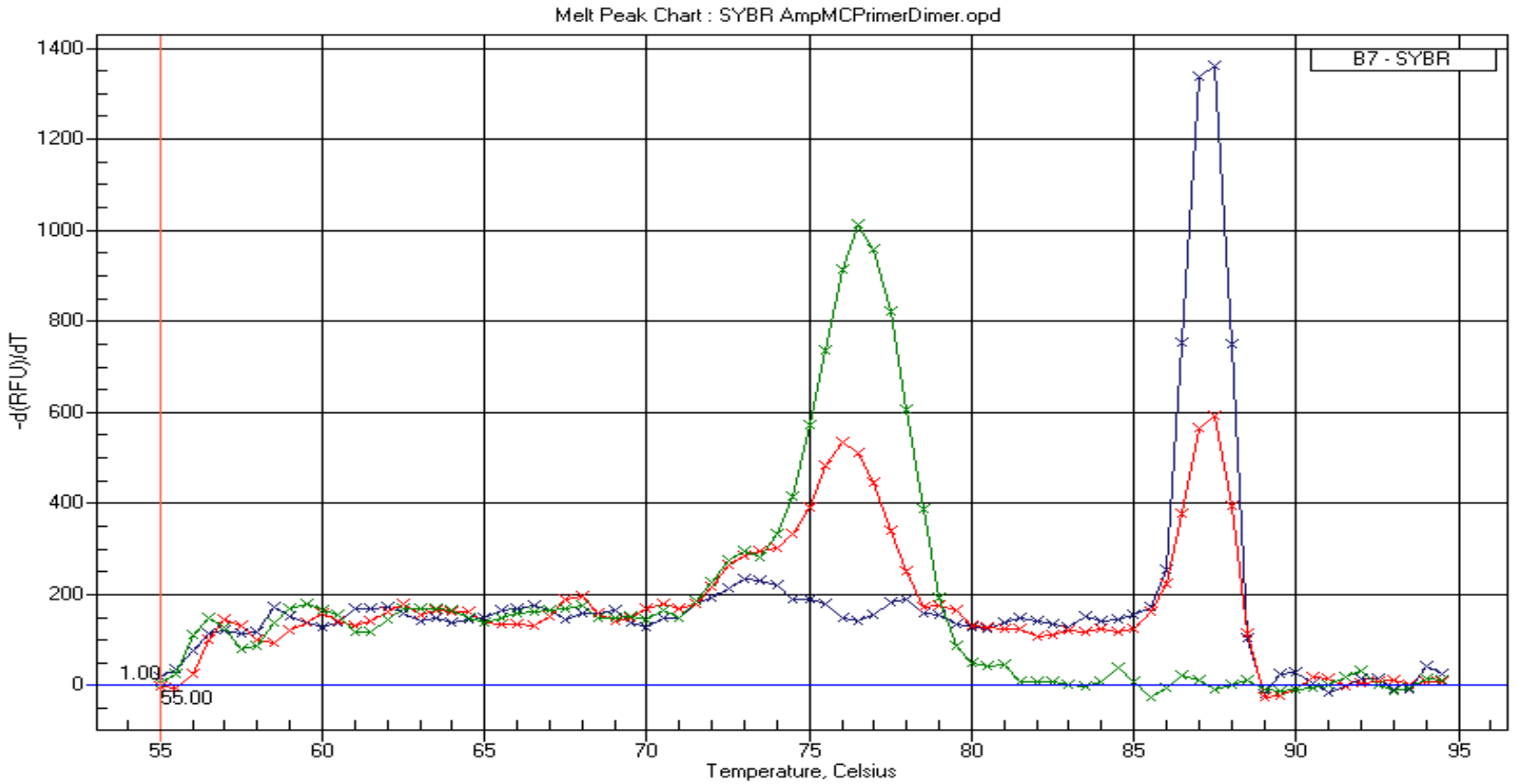
Fluorescence decrease as the temperature increase:

1. DNA strands start to separate
2. SYBR green looses its binding to the DNA
3. Fluorescence rapidly decreases

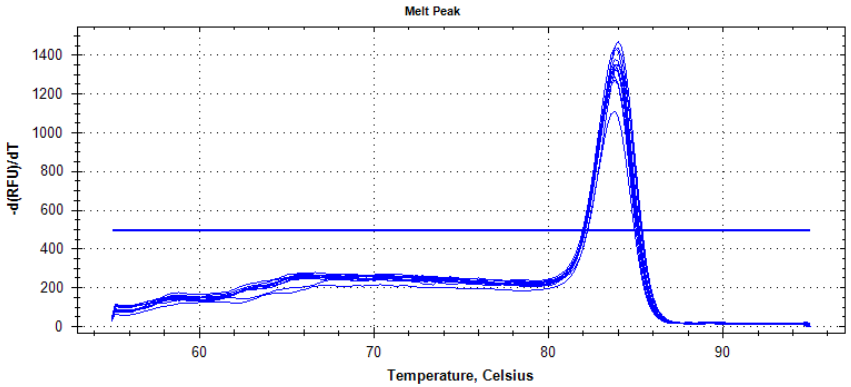


Melt Curve Analysis

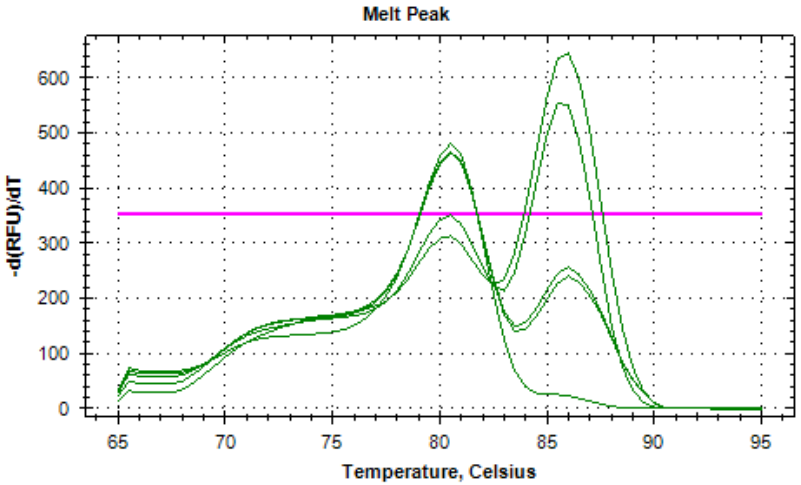
-The melting temperature of the amplicon can easily be detected.
-Contaminating DNA, primer dimer or false priming is seen as an additional peak.



Melt Curve Analysis



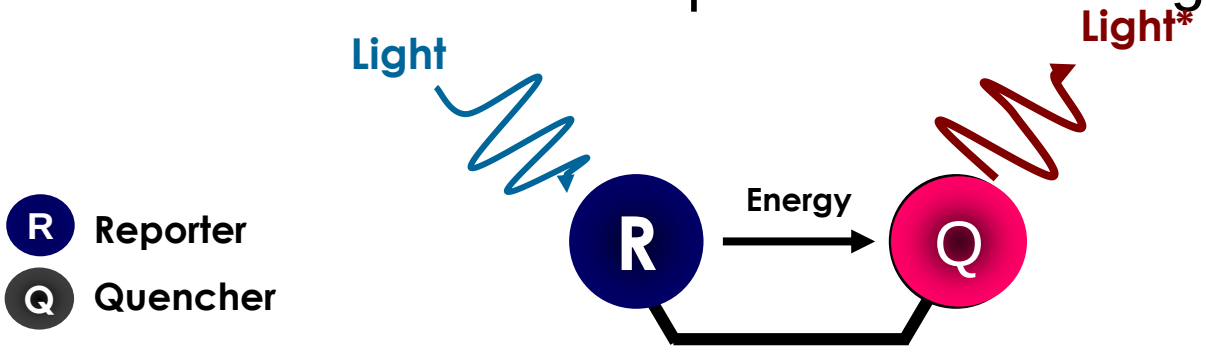
Multiple wells, same assay, single peaks at high melting temperatures (+80ºC). This means my assay is specific!



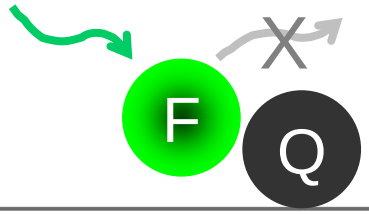
Multiple wells, same assay, multiple peaks! This means off-target amplification and/or primer dimers!

Real-Time Chemistry: Probes (TaqMan)

- Target specific hybridization probe
- 5’ reporter and 3’ quencher
- Utilizes FRET (Fluorescence Resonance Energy Transfer) quenching
- Relies on a validated primer set with high specificity



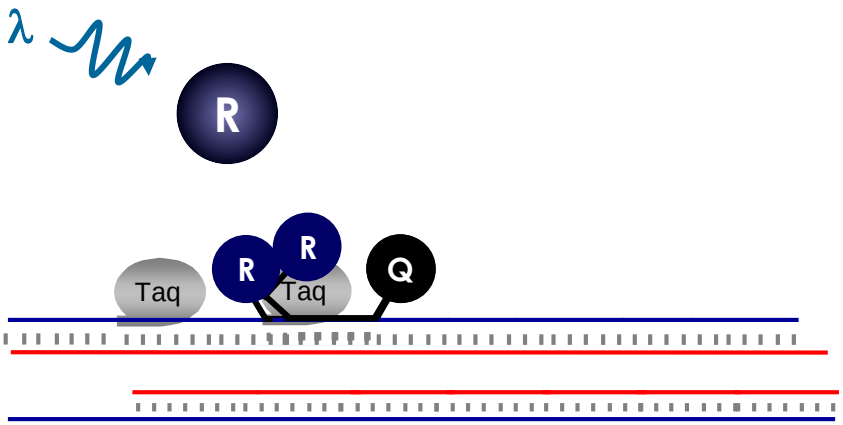
* heat for BHQs

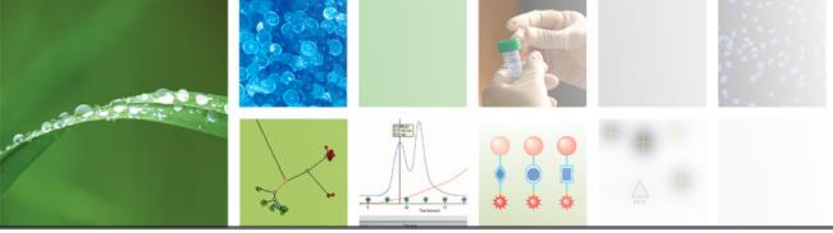


Dark Quenchers :Ground state or static quenching
Energy is dissipated as heat
Molecular interactions inhibit fluorescence

Mechanism of probe chemistry

1. During PCR, probe hybridizes to target sequence
2. Probe is partially displaced during extension
3. Probe cleaved by 5'- 3' nuclease activity of polymerase, releasing reporter from quencher
4. Illuminated reporter exhibits unquenched fluorescence





Hybridization probes

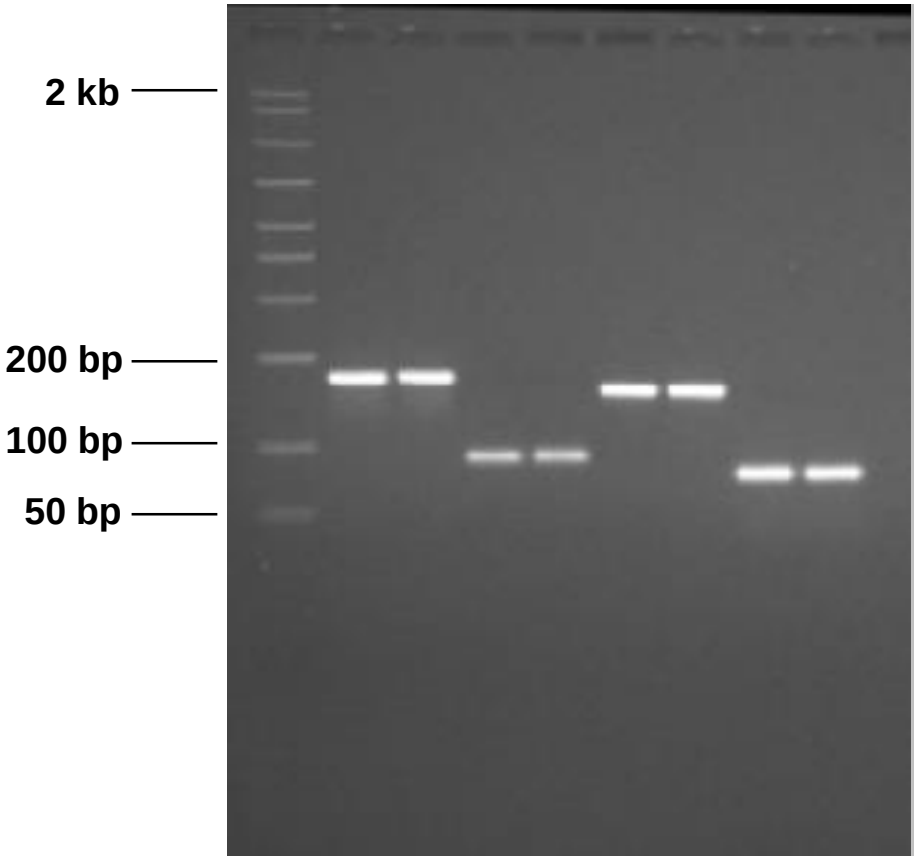
Advantages

- Allows multiplexing by using different reporter dye
- Removes concerns about primer dimers and off-target amplifications (More specific)
- SNP genotyping application

Disadvantages

- More expensive than dye-based assays
- To test assay specificity you'll need to run products on gels

Gel confirmation



SYBR vs. Probes: How do I choose?

- Is your sample limited or rare?
- Is time-to-results a very significant constraint?
- Are you differentiating similar sequences?
- Do you have specificity concerns?
- Do you need multiple data points out of the same sample?

Sso Advanced Universal SYBR® Green Supermix
1725270

Sso Advanced Universal Probes Supermix
1725280

Real-time PCR Sample Preparation

SYBR Green Chemistry

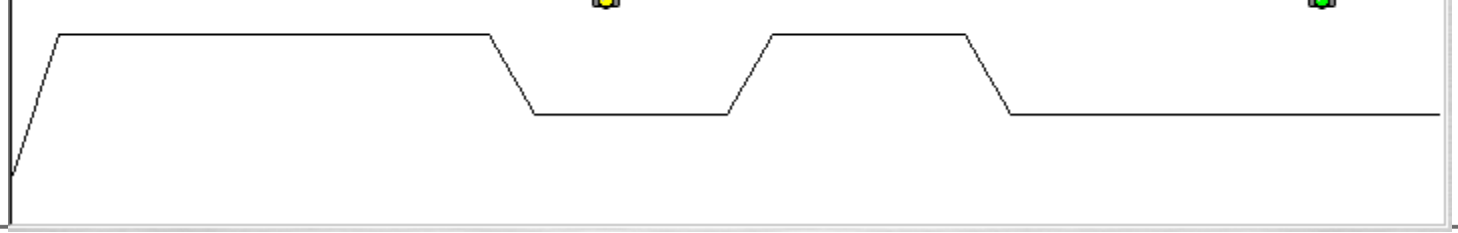
Component	Volume per reaction	Final concentration
iQ SYBR Green Supermix	25 µl	1X
Primer 1	x µl	100 nM–500 nM
Primer 2	x µl	100 nM–500 nM
Sterile water	x µl	
DNA template	x µl	
Total Volume	50 µl	

Probe Chemistry

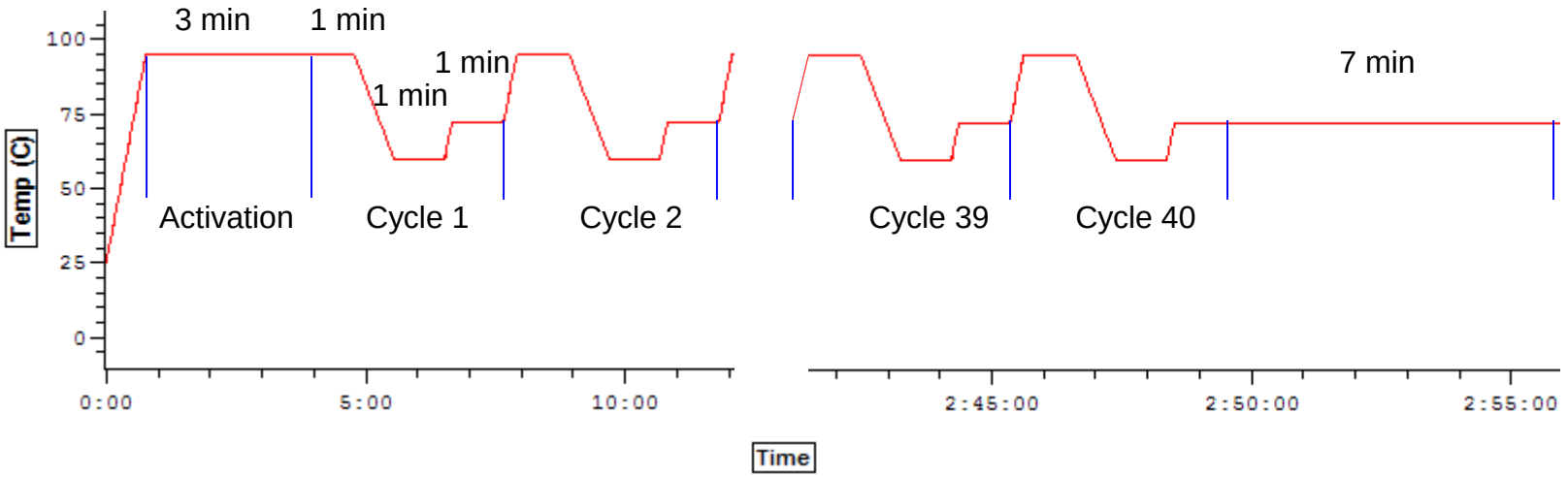
Component	Volume per reaction	Final concentration
iQ Supermix	25 µl	1X
Primer 1	x µl	100 nM–500 nM
Primer 2	x µl	100 nM–500 nM
Probe	x µl	100 nM–500 nM
Sterile water	x µl	
DNA template	x µl	
Total Volume	50 µl	

Hot Start PCR Melting Curve

Cycle 1	Cycle 2 40X		Cycle 3	Cycle 4	Cycle 5 81X
Step 1	Step 1	Step 2	Step 1	Step 1	Step 1
95.0	95.0	55.0	95.0	55.0	55.0
3:00	0:10	0:30	1:00	1:00	0:10



3 Step PCR



3 min Polymerase Activation
40 min Denaturing
40 min Annealing
40 min Extension
7 min Final Extension

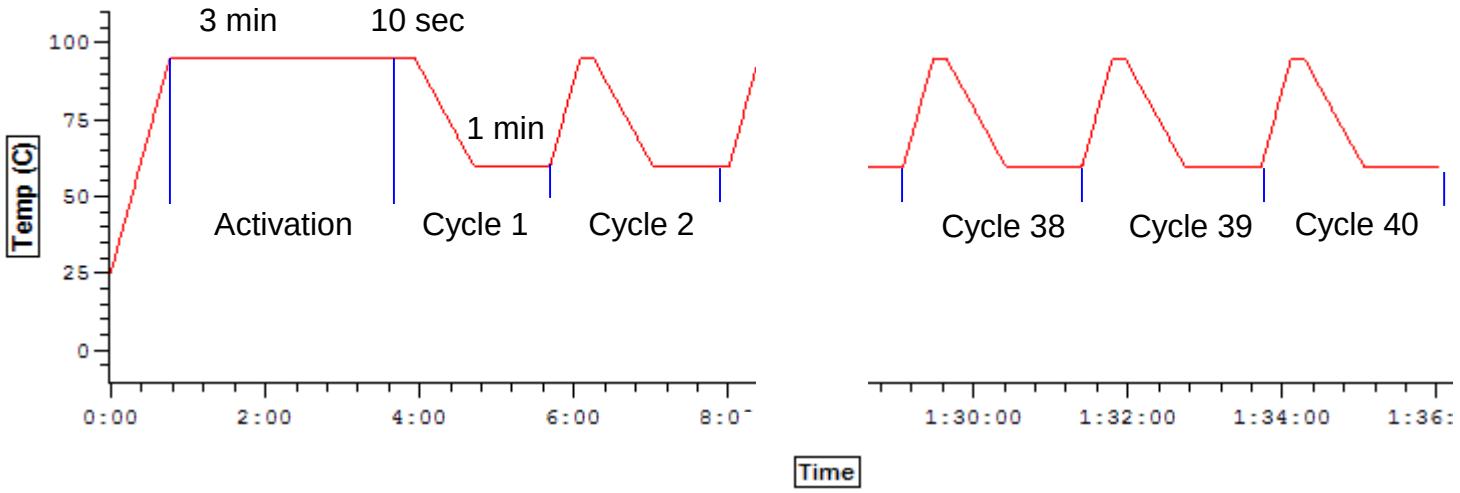
130 min total in incubations

Initial Ramp to 95°
40 X Ramp down to 60°
40 X Ramp up to 72°
39 X Ramp up to 95°

46 min total in ramping

Total time 2 hours 56 minutes

2 Step PCR



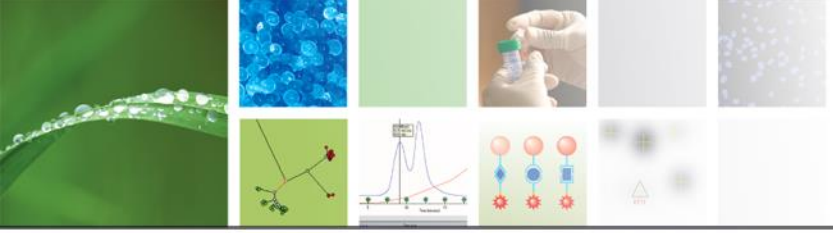
3 min Polymerase Activation
7 min Denaturing
40 min Annealing

50 min total in incubations

Initial Ramp to 95°
40 X Ramp down to 60°
39 X Ramp up to 95°

46 min total in ramping

Total time 1 hour 36 minutes



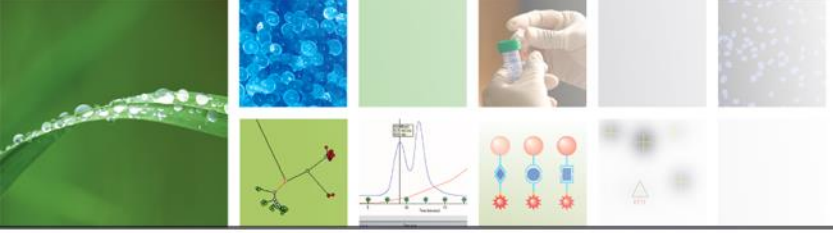
General QPCR Working Process -- Data analysis

- ***Relative Quantity (ΔCT)***
  - –Not normalized
  - –Normalization accomplished via equal loading of samples
  - –Post analysis normalization
- ***Normalized Expression (ΔΔCT)***
  - –Accounts for loading differences
  - –Usually normalize to reference gene
  - –Relative quantity of Target is normalized by the relative quantity of the reference genes

Relative Quantity (ΔCT)

	Target
Tissue #1:	22
Tissue #2:	24
Delta Ct:	24-22 = 2

Fold induction = $2^2 = 4$



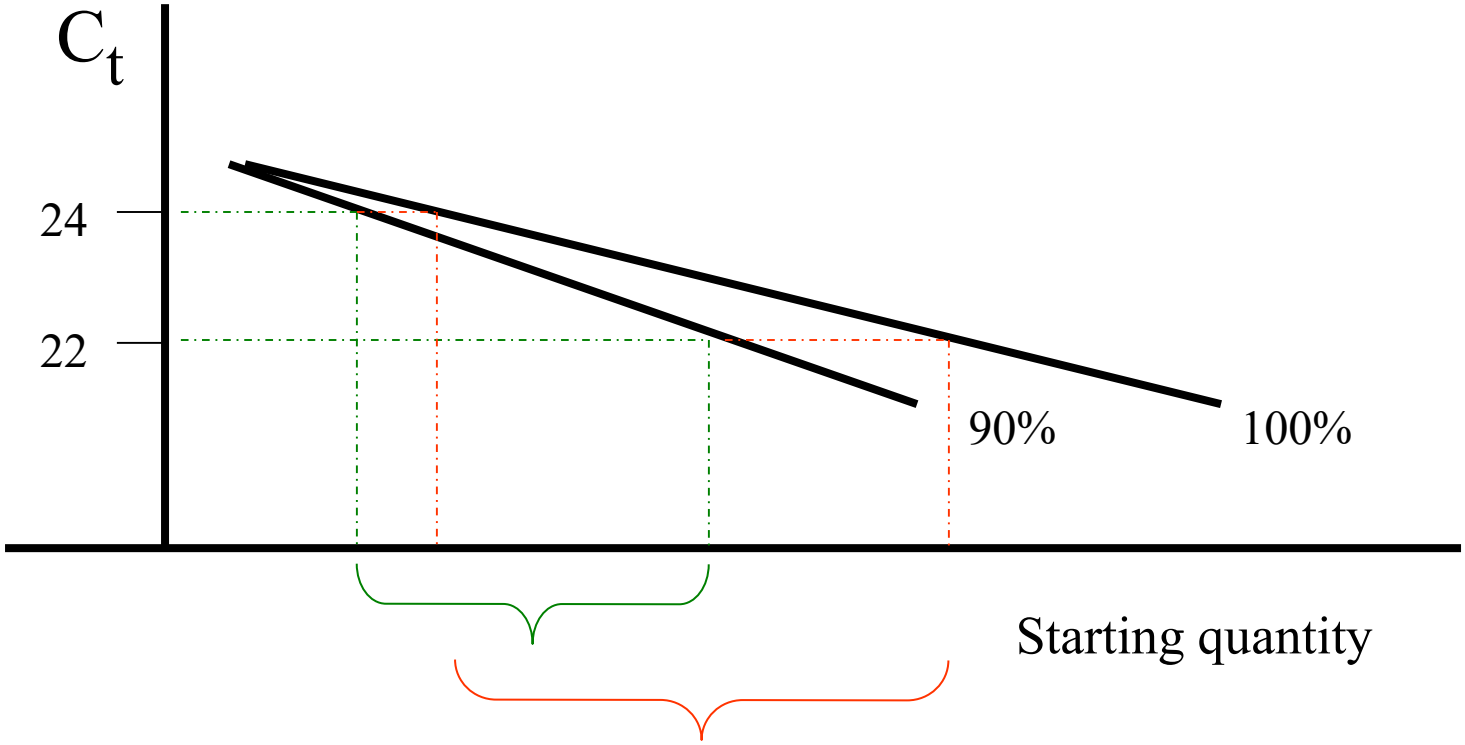
Normalized Expression (ΔΔCT)

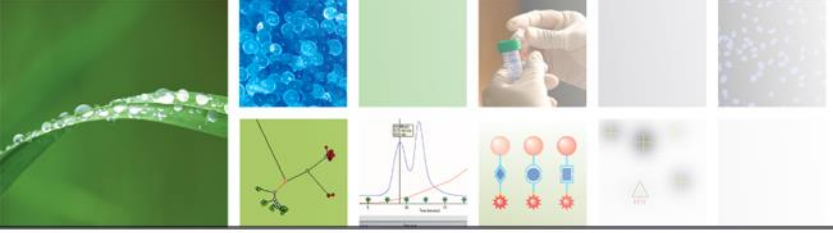
		Reference	Target
Tissue #1 (Control):		21	22
Tissue #2 (Test) :		20	24
1st Delta	Delta Ct #1:		22-21 = 1
	Delta Ct #2:		24-20 = 4
2nd Delta	Delta Ct:		1-4 = -3

Fold induction = $2^{-(-3)} = 8$

Relative Quantification

- Problem with the ΔΔCT
- Slopes are not parallel

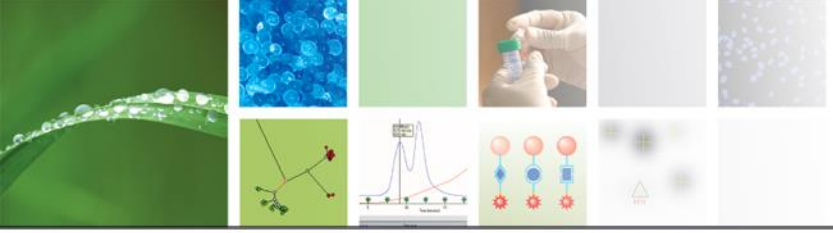




Pfaffl modification

	Primer set #1 Reference	Primer set #2 Target
Tissue #1 (Control):	21	22
Tissue #2 (Test) :	20	24
(From Standard curve) Efficiency:	90% = 1.9	100% = 2
Delta Ct:	20-21 = -1	24-22 = 2

$$\text{Fold induction} = \frac{2_{\text{target}}^{\text{deltaCt }_{\text{target}}\,(\mathbf{24\text{-}22 = 2})}}{1.9_{\text{reference}}^{\text{deltaCt }_{\text{reference}}\,(\mathbf{20\text{-}21 = -1})}} = \frac{4}{0.56} = 7.1$$

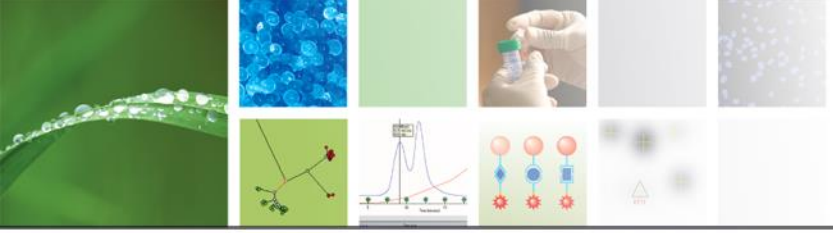


- ΔCt method: (*no reference gene*)
  - Fold induction : 4
- ΔΔCt method: (*reference gene*)
  - Fold induction : 8
- Pfaffl modification: (*reference gene and efficiency*)
  - Fold induction : 7.1

Remember...

Real-Time PCR is not ‘cookbook chemistry’ - a real-time instrument will not optimize your experiments for you

However, once you do **optimize your reactions**, you will get **reproducible, accurate results**



Assay and Primer Design

- Primer design considerations
  - –Assay type (SYBR vs Probe/TaqMan)
  - –Exonic vs intron spanning
  - –Selection of primary genome viewer (NCBI, Ensembl, UCSC)
  - –SNP locations
  - –Selection of best fragments for optimal transcript coverage and maximum intron size
  - –Secondary structures (UNAfold)
  - –Specificity (BiSearch)

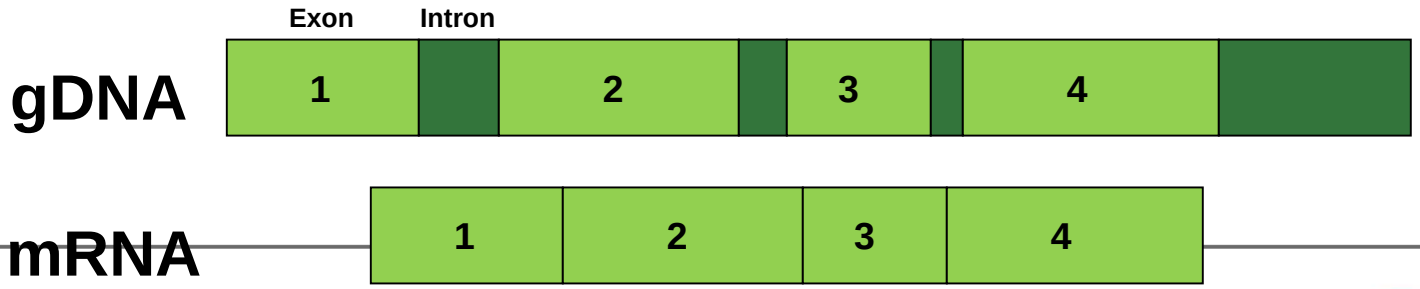
Choosing between intron/exon coverage

Exonic designs

- Assay completely located within a single exon
- Amplifes both cDNA & gDNA
- gDNA can be used as positive control
- Can be applied to all genes

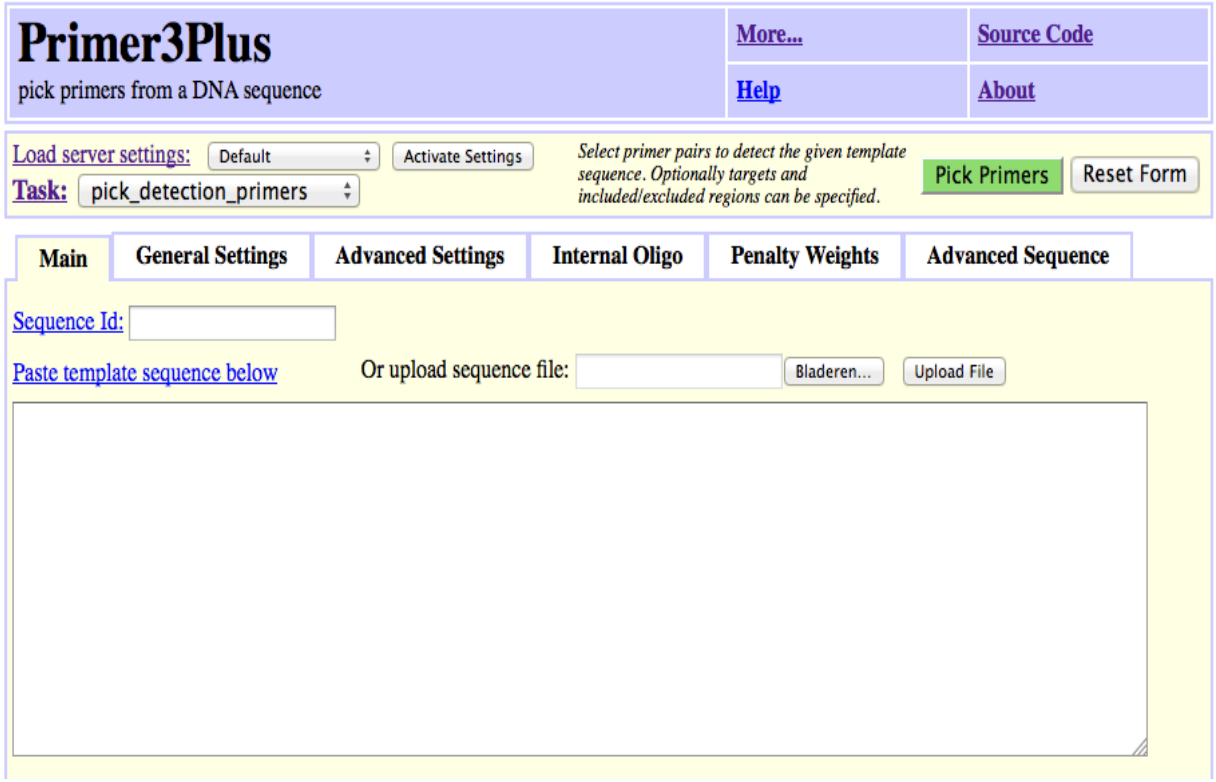
Intron-spanning designs

- At least one intron between forward & reverse primer
- Limits co-amplification of contaminating gDNA
- Not possible for single exon genes or genes with small introns (~ 9% of the genome)
- May not help for genes with processed pseudogenes (~15% of genome)
- Decreased design space



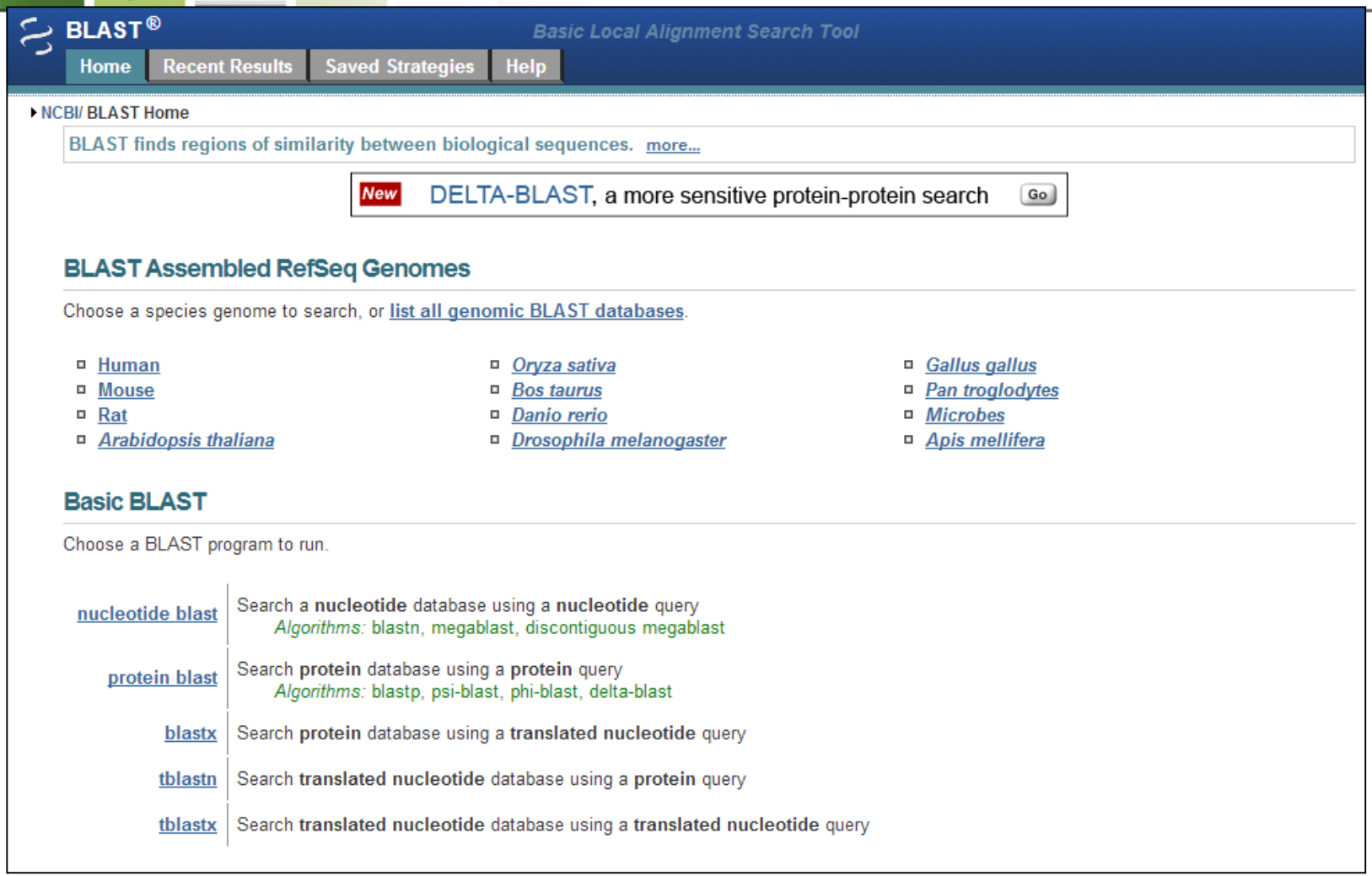
Primer3Plus Primer Design

- http://www.primer3plus.com

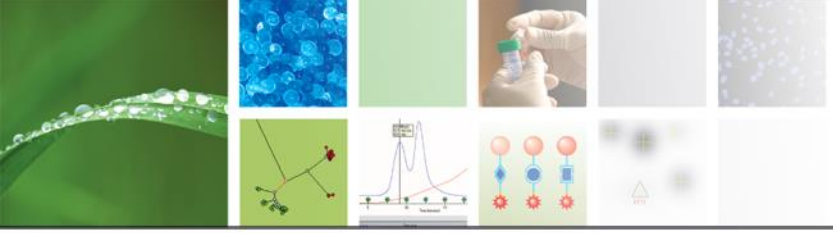


- PCR settings
  - mix_salt_divalent
  - mix_salt_monovalent
  - mix_dntp_conc
  - mix_dna_conc
  - mix_pcr_temperature
- amplicon properties
  - amplicon_size
- Tm prediction
  - SantaLucia 1998
- primer properties
  - primer_size
  - primer_tm
  - primer_max_tm_diff
  - primer_gc
  - primer_max_3prime_gc
  - primer_max_3_poly
  - primer_max_self_end
  - primer_max_self_any
  - primer_pair_max_compl_end

Specificity Analysis



http://blast.ncbi.nlm.nih.gov/Blast.cgi



Assays – What are your choices?

- Design new assays
  - –Take advantage of the latest information and design tools and do the proper wet-lab validation in order to meet MIQE guidelines

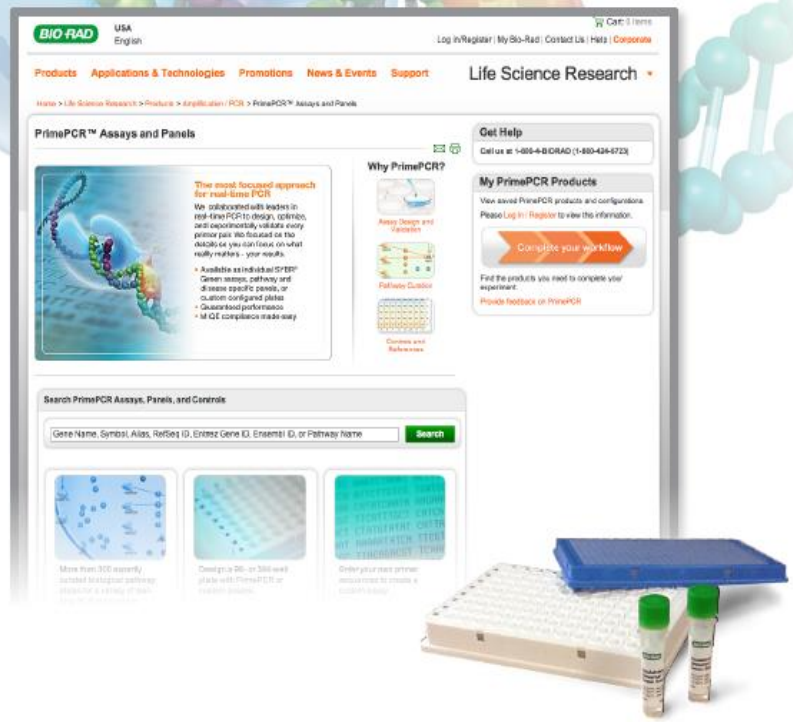
- Use existing custom designed assays
  - –Possible unknown design criteria
  - –Have they been validated to MIQE standards?

- Use pre-designed assays from a vendor
  - –Unknown design criteria (for most)
  - –In-silico validation vs. wet-lab validation
    - **Not all assays are created equally!**
  - Bio-Rad's PrimePCR assays/plate arrays
  - **www.Bio-Rad.com/PrimePCR**

PrimePCR Assays

Available Species

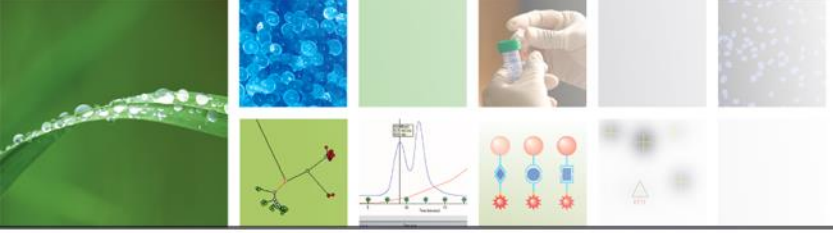
Human	Rabbit
Mouse	Rhesus Monkey
Rat	Yeast
Chicken	Zebrafish
Arabidopsis	Cow
Pig	Dog
	Rice



Also: Human lncRNA assays!

www.bio-rad.com/PrimePCR

PrimePCR Pathway & Collection Panels



Diseases

- Cancer (133)
- Cardiac hypertrophy (7)
- Cardiovascular diseases (15)
- Congenital, hereditary, and neonatal diseases and abnormalities (73)
- Cystic fibrosis (37)
- Digestive system diseases (13)
- Endocrine system diseases (20)
- Hemic and lymphatic diseases (51)
- Immune system diseases (53)
- Mental disorders (11)
- Musculoskeletal diseases (6)
- Nervous system diseases (16)
- Nutritional and metabolic diseases (46)
- Respiratory tract diseases (31)
- Skin and connective tissue diseases (19)

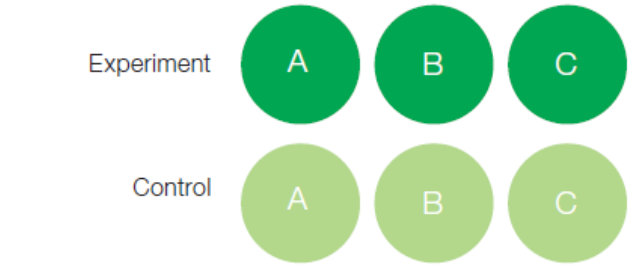
96-well	384-well
ABI 7300 - 96	ABI 7900 - 384
ABI 7500 - 96 Fast	ABI QuantStudio - 384
ABI 7500 - 96 Std	ABI ViiA 7 - 384
ABI 7900 - 96 Fast	Bio-Rad CFX384
ABI 7900 - 96 Std	Bio-Rad CFX384 Touch
ABI QuantStudio - 96 Fast	Roche LC480 - 384
ABI QuantStudio - 96 Std	
ABI StepOnePlus - 96	
ABI ViiA 7 - 96 Fast	
ABI ViiA 7 - 96 Std	
Bio-Rad CFX Connect - 96	
Bio-Rad CFX96	
Bio-Rad CFX96 Touch	
Bio-Rad Chromo4 - 96	
Bio-Rad iQ - 96	
Bio-Rad iQ5 - 96	
Bio-Rad MyiQ - 96	
Bio-Rad MyiQ2 - 96	
Bio-Rad Opticon - 96	
Bio-Rad Opticon 2 - 96	
Eppendorf Realplex - 96	
Roche LC480 - 96	
Stratagene Mx - 96	

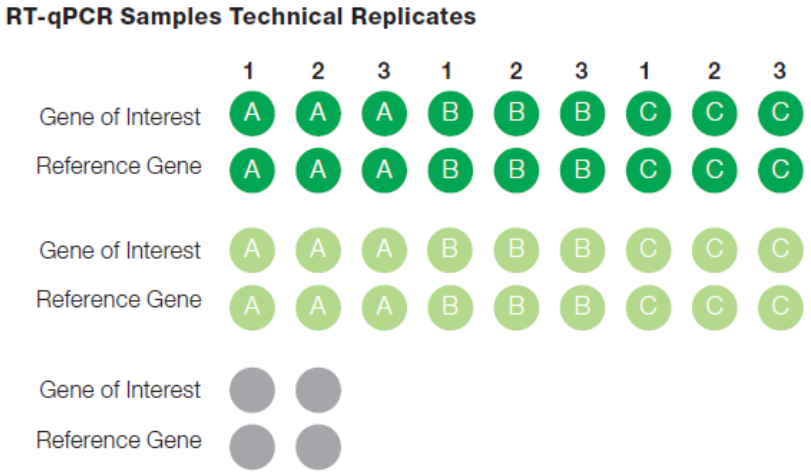
abolism

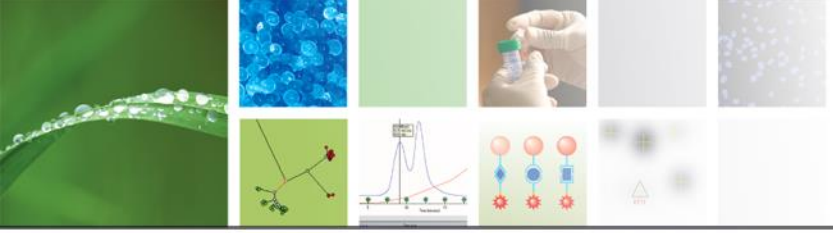
- no acid metabolism (4)
- bohydrate metabolism (4)
- l metabolism (5)
- leotide metabolism (3)
- ulation of lipid metabolism (3)
- bid metabolism (5)
- nin and cofactor
- abolism (4)
- biotic metabolism (6)

Additional tips: Experimental Design

- Technical vs. Biological replication
  - Reviewers typically like technical triplicates
  - Smaller fold changes require a higher *n* of samples
- Multiple reference genes can stabilize basal gene expression
  - Critical if you measure small fold changes





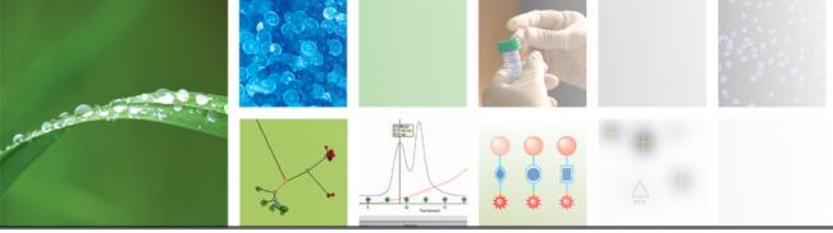


Additional tips: Experimental Design

- Volumes matter!
  - St Dev goes up as volume of reaction goes down
  - Avoid pipetting small volumes (1-2 ul)
  - Better to add more of a diluted template

Example well:
- 10 ul of 2x Supermix (dNTPs/Taq/Mg++/Buffer)
- 1 ul of 20x diluted F+R primer mix
- 3 ul of water
- 5 ul template

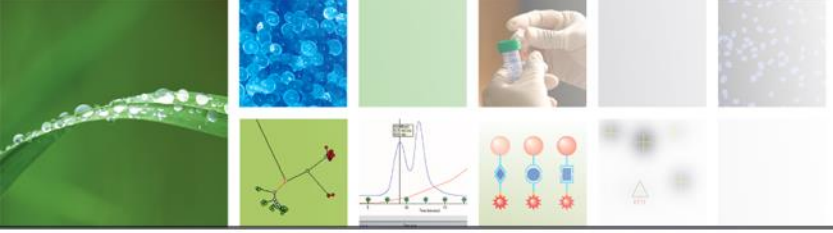
- Errors increase with each pipetting step
- Make up a master mix of everything minus template
  - Usually total samples +10% - always need overage
  - Minimize error, lower total pipetting steps



Additional tips: Essential Controls

- Non-template control (NTC)
  - Add water in lieu of template
  - Any signal before cycle 38 should be checked
  - Either due to primer dimers or contamination
  - Best to have one NTC well per assay (primer set)

- No reverse transcription (NRT)
  - Checks for DNA carry over in your RNA purification
  - Add equivalent amount of non-converted RNA as you did cDNA
  - ΔCq to cDNA should be above ~4 cycles
  - More important on a per sample/prep basis



Additional tips: Essential Controls

- Negative control (Neg)
  - Add template from another species/system/knock-out that does not contain your sequence of interest
  - Differs from NTC – checks for *specificity*
  - Essential to catch off target effects early

- Positive control (Pos)
  - Add your template of interest
  - Look for a signal <Cq=30
  - Used primarily in troubleshooting inhibitory samples

Experiment design

Sample maximization method

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 Actb	Unk-1 Actb	Unk-1 Actb	Unk-9 Actb	Unk-9 Actb	Unk-9 Actb	Unk-17 Actb	Unk-17 Actb	Unk-17 Actb	Unk-25 Actb	Unk-25 Actb	Unk-25 Actb
B	Unk-2 Actb	Unk-2 Actb	Unk-2 Actb	Unk-10 Actb	Unk-10 Actb	Unk-10 Actb	Unk-18 Actb	Unk-18 Actb	Unk-18 Actb	Unk-26 Actb	Unk-26 Actb	Unk-26 Actb
C	Unk-3 Actb	Unk-3 Actb	Unk-3 Actb	Unk-11 Actb	Unk-11 Actb	Unk-11 Actb	Unk-19 Actb	Unk-19 Actb	Unk-19 Actb	Unk-27 Actb	Unk-27 Actb	Unk-27 Actb
D	Unk-4 Actb	Unk-4 Actb	Unk-4 Actb	Unk-12 Actb	Unk-12 Actb	Unk-12 Actb	Unk-20 Actb	Unk-20 Actb	Unk-20 Actb	Unk-28 Actb	Unk-28 Actb	Unk-28 Actb
E	Unk-5 Actb	Unk-5 Actb	Unk-5 Actb	Unk-13 Actb	Unk-13 Actb	Unk-13 Actb	Unk-21 Actb	Unk-21 Actb	Unk-21 Actb	Unk-29 Actb	Unk-29 Actb	Unk-29 Actb
F	Unk-6 Actb	Unk-6 Actb	Unk-6 Actb	Unk-14 Actb	Unk-14 Actb	Unk-14 Actb	Unk-22 Actb	Unk-22 Actb	Unk-22 Actb	Unk-30 Actb	Unk-30 Actb	Unk-30 Actb
G	Unk-7 Actb	Unk-7 Actb	Unk-7 Actb	Unk-15 Actb	Unk-15 Actb	Unk-15 Actb	Unk-23 Actb	Unk-23 Actb	Unk-23 Actb	NTC Actb	NTC Actb	NTC Actb
H	Unk-8 Actb	Unk-8 Actb	Unk-8 Actb	Unk-16 Actb	Unk-16 Actb	Unk-16 Actb	[illegible]	[illegible]	Unk-24 Actb			

Actb

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 GAPDH	Unk-1 GAPDH	Unk-1 GAPDH	Unk-9 GAPDH	Unk-9 GAPDH	Unk-9 GAPDH	Unk-17 GAPDH	Unk-17 GAPDH	Unk-17 GAPDH	Unk-25 GAPDH	Unk-25 GAPDH	Unk-25 GAPDH
B	Unk-2 GAPDH	Unk-2 GAPDH	Unk-2 GAPDH	Unk-10 GAPDH	Unk-10 GAPDH	Unk-10 GAPDH	Unk-18 GAPDH	Unk-18 GAPDH	Unk-18 GAPDH	Unk-26 GAPDH	Unk-26 GAPDH	Unk-26 GAPDH
C	Unk-3 GAPDH	Unk-3 GAPDH	Unk-3 GAPDH	Unk-11 GAPDH	Unk-11 GAPDH	Unk-11 GAPDH	Unk-19 GAPDH	Unk-19 GAPDH	Unk-19 GAPDH	Unk-27 GAPDH	Unk-27 GAPDH	Unk-27 GAPDH
D	Unk-4 GAPDH	Unk-4 GAPDH	Unk-4 GAPDH	Unk-12 GAPDH	Unk-12 GAPDH	Unk-12 GAPDH	Unk-20 GAPDH	Unk-20 GAPDH	Unk-20 GAPDH	Unk-28 GAPDH	Unk-28 GAPDH	Unk-28 GAPDH
E	Unk-5 GAPDH	Unk-5 GAPDH	Unk-5 GAPDH	Unk-13 GAPDH	Unk-13 GAPDH	Unk-13 GAPDH	Unk-21 GAPDH	Unk-21 GAPDH	Unk-21 GAPDH	Unk-29 GAPDH	Unk-29 GAPDH	Unk-29 GAPDH
F	Unk-6 GAPDH	Unk-6 GAPDH	Unk-6 GAPDH	Unk-14 GAPDH	Unk-14 GAPDH	Unk-14 GAPDH	Unk-22 GAPDH	Unk-22 GAPDH	Unk-22 GAPDH	Unk-30 GAPDH	Unk-30 GAPDH	Unk-30 GAPDH
G	Unk-7 GAPDH	Unk-7 GAPDH	Unk-7 GAPDH	Unk-15 GAPDH	Unk-15 GAPDH	Unk-15 GAPDH	Unk-23 GAPDH	Unk-23 GAPDH	Unk-23 GAPDH	NTC GAPDH	NTC GAPDH	NTC GAPDH
H	Unk-8 GAPDH	[illegible]	[illegible]	[illegible]	[illegible]	Unk-16 GAPDH	Unk-24 GAPDH	Unk-24 GAPDH	Unk-24 GAPDH			

APDH

Good DATA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 P53	Unk-1 P53	Unk-1 P53	Unk-9 P53	Unk-9 P53	Unk-9 P53	Unk-17 P53	Unk-17 P53	Unk-17 P53	Unk-25 P53	Unk-25 P53	Unk-25 P53
B	Unk-2 P53	Unk-2 P53	Unk-2 P53	Unk-10 P53	Unk-10 P53	Unk-10 P53	Unk-18 P53	Unk-18 P53	Unk-18 P53	Unk-26 P53	Unk-26 P53	Unk-26 P53
C	Unk-3 P53	Unk-3 P53	Unk-3 P53	Unk-11 P53	Unk-11 P53	Unk-11 P53	Unk-19 P53	Unk-19 P53	Unk-19 P53	Unk-27 P53	Unk-27 P53	Unk-27 P53
D	Unk-4 P53	Unk-4 P53	Unk-4 P53	Unk-12 P53	Unk-12 P53	Unk-12 P53	Unk-20 P53	Unk-20 P53	Unk-20 P53	Unk-28 P53	Unk-28 P53	Unk-28 P53
E	Unk-5 P53	Unk-5 P53	Unk-5 P53	Unk-13 P53	Unk-13 P53	Unk-13 P53	Unk-21 P53	Unk-21 P53	Unk-21 P53	Unk-29 P53	Unk-29 P53	Unk-29 P53
F	Unk-6 P53	Unk-6 P53	Unk-6 P53	Unk-14 P53	Unk-14 P53	Unk-14 P53	Unk-22 P53	Unk-22 P53	Unk-22 P53	Unk-30 P53	Unk-30 P53	Unk-30 P53
G	Unk-7 P53	Unk-7 P53	Unk-7 P53	Unk-15 P53	Unk-15 P53	Unk-15 P53	Unk-23 P53	Unk-23 P53	Unk-23 P53	NTC P53	NTC P53	NTC P53
H	Unk-8 P53	Unk-8 P53	Unk-8 P53	Unk-16 P53	Unk-16 P53	Unk-16 P53	Unk-24 P53	Unk-24 P53	Unk-24 P53			

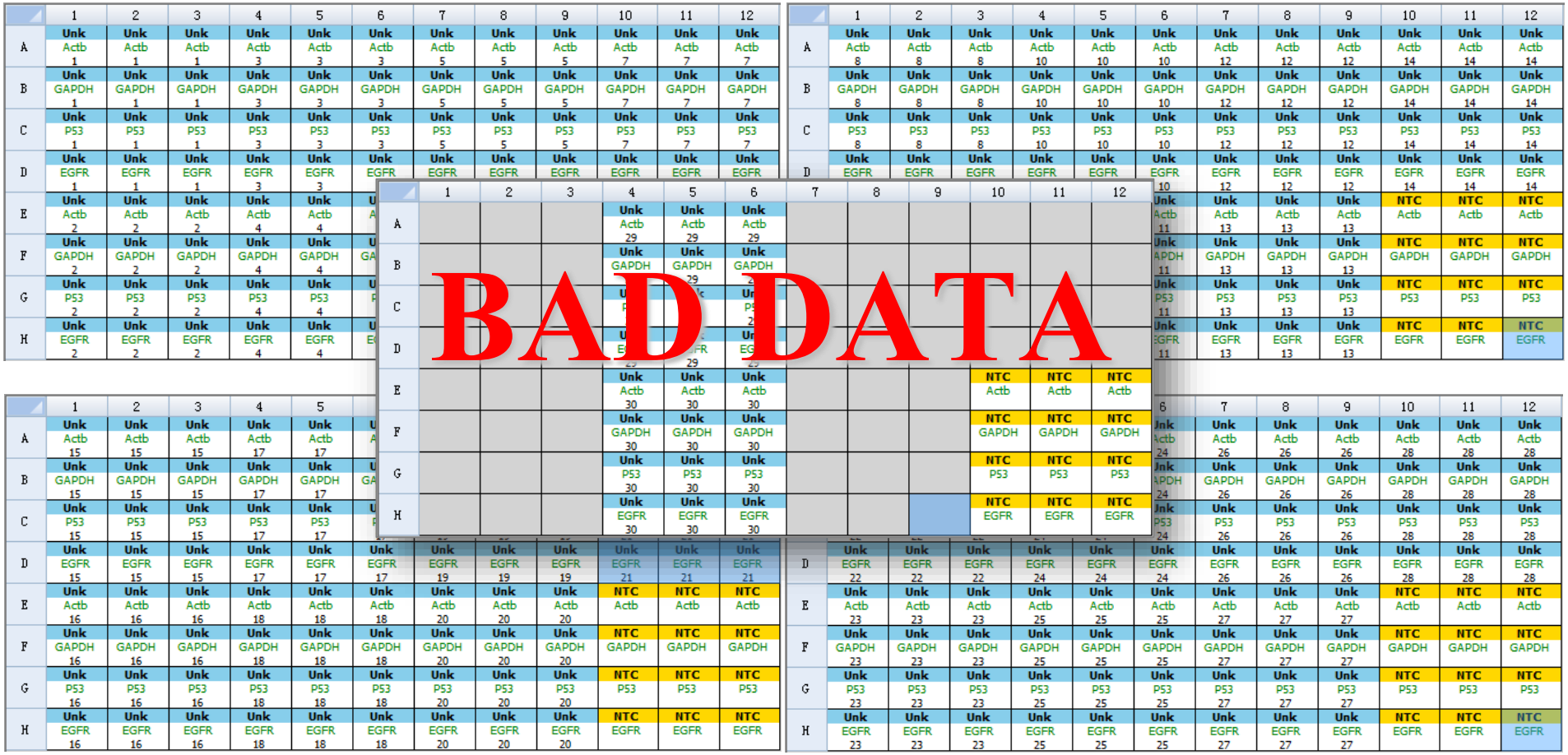
P53

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk-1 EGFR	Unk-1 EGFR	Unk-1 EGFR	Unk-9 EGFR	Unk-9 EGFR	Unk-9 EGFR	Unk-17 EGFR	Unk-17 EGFR	Unk-17 EGFR	Unk-25 EGFR	Unk-25 EGFR	Unk-25 EGFR
B	Unk-2 EGFR	Unk-2 EGFR	Unk-2 EGFR	Unk-10 EGFR	Unk-10 EGFR	Unk-10 EGFR	Unk-18 EGFR	Unk-18 EGFR	Unk-18 EGFR	Unk-26 EGFR	Unk-26 EGFR	Unk-26 EGFR
C	Unk-3 EGFR	Unk-3 EGFR	Unk-3 EGFR	Unk-11 EGFR	Unk-11 EGFR	Unk-11 EGFR	Unk-19 EGFR	Unk-19 EGFR	Unk-19 EGFR	Unk-27 EGFR	Unk-27 EGFR	Unk-27 EGFR
D	Unk-4 EGFR	Unk-4 EGFR	Unk-4 EGFR	Unk-12 EGFR	Unk-12 EGFR	Unk-12 EGFR	Unk-20 EGFR	Unk-20 EGFR	Unk-20 EGFR	Unk-28 EGFR	Unk-28 EGFR	Unk-28 EGFR
E	Unk-5 EGFR	Unk-5 EGFR	Unk-5 EGFR	Unk-13 EGFR	Unk-13 EGFR	Unk-13 EGFR	Unk-21 EGFR	Unk-21 EGFR	Unk-21 EGFR	Unk-29 EGFR	Unk-29 EGFR	Unk-29 EGFR
F	Unk-6 EGFR	Unk-6 EGFR	Unk-6 EGFR	Unk-14 EGFR	Unk-14 EGFR	Unk-14 EGFR	Unk-22 EGFR	Unk-22 EGFR	Unk-22 EGFR	Unk-30 EGFR	Unk-30 EGFR	Unk-30 EGFR
G	Unk-7 EGFR	Unk-7 EGFR	Unk-7 EGFR	Unk-15 EGFR	Unk-15 EGFR	Unk-15 EGFR	Unk-23 EGFR	Unk-23 EGFR	Unk-23 EGFR	NTC EGFR	NTC EGFR	NTC EGFR
H	Unk-8 EGFR	Unk-8 EGFR	Unk-8 EGFR	Unk-16 EGFR	Unk-16 EGFR	Unk-16 EGFR	Unk-24 EGFR	Unk-24 EGFR	Unk-24 EGFR			

EGFR

Experiment design

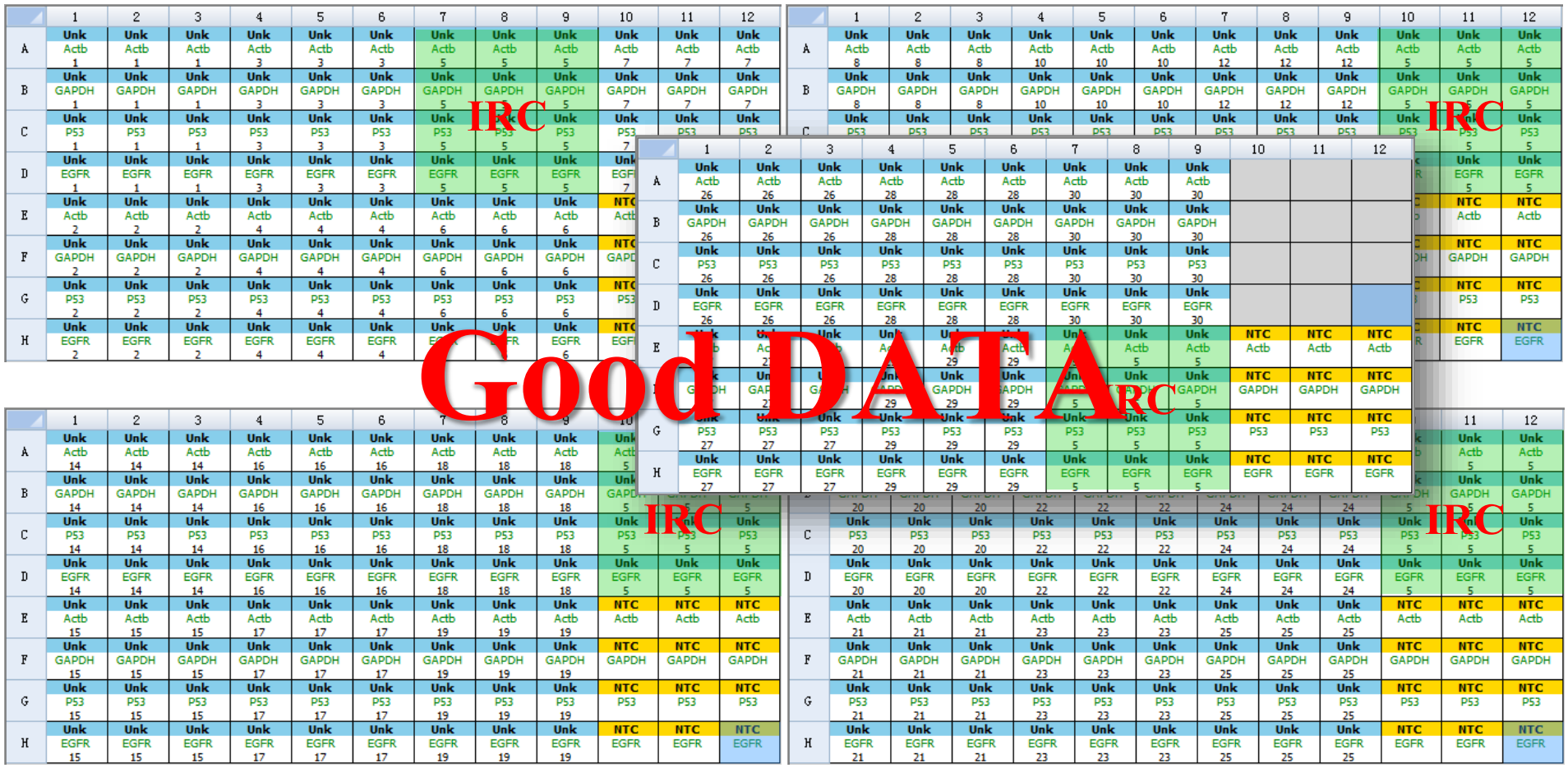
Someone did lay-out like this…



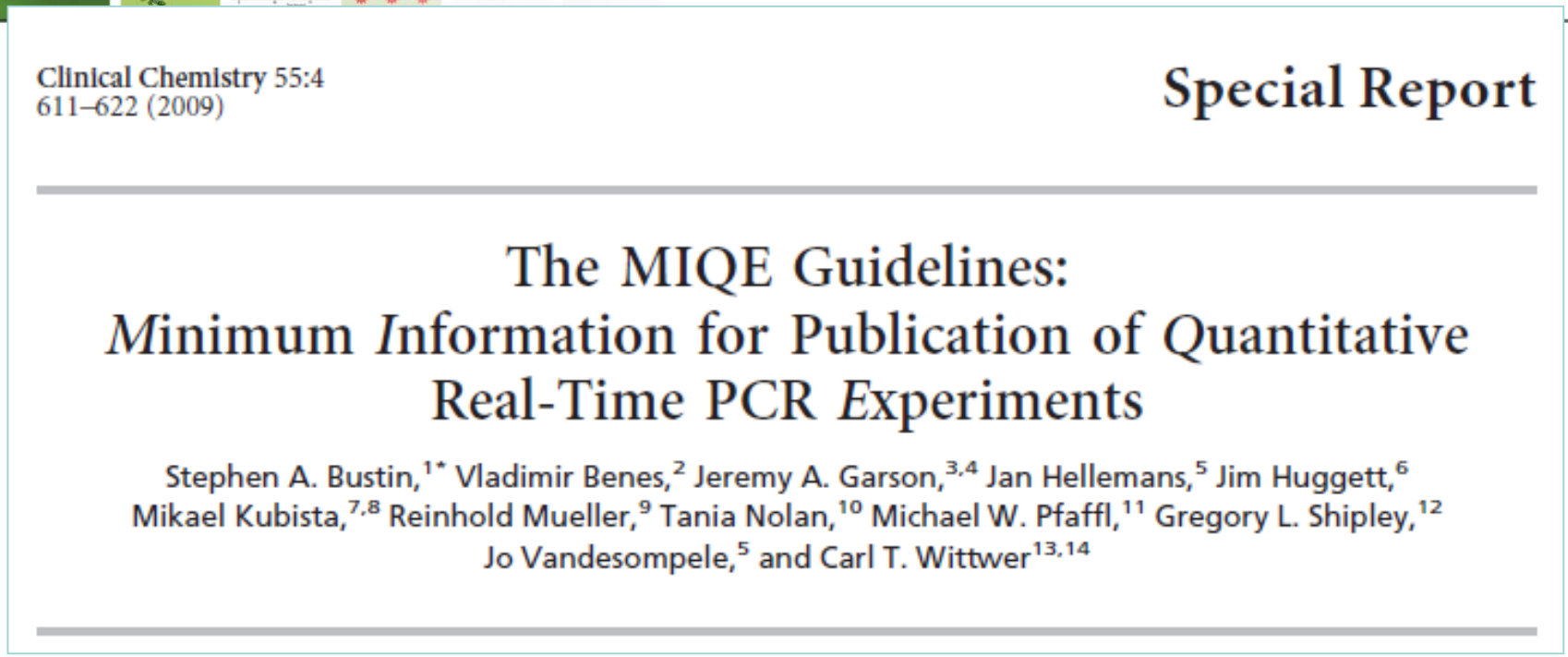
Without inter-run calibration!

Inter-run Calibrator

Gene maximization method



A new standard for qPCR



Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: *Minimum Information for Publication of Quantitative Real-Time PCR Experiments*

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

The MIQE guidelines… "target the **reliability** of results to help ensure the **integrity** of the scientific literature, promote **consistency** between laboratories, and increase experimental **transparency**."

>4000 Citations

What is MIQE: It's a Checklist

- qPCR community driven guidelines for essential and desired information in literature;
  - Experimental Design
  - Sample Information
  - Nucleic Acid Extraction
  - Reverse Transcription
  - qPCR Target Information
  - qPCR Oligonucleotides
  - qPCR Protocol
  - qPCR Validation
  - Data Analysis

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and *y* intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Inhibition testing (C_q dilutions, spike, or other)	E	r^2 of calibration curve	E
Reverse transcription		Linear dynamic range	E
Complete reaction conditions	E	C_q variation at LOD	E
Amount of RNA and reaction volume	E	CIs throughout range	D
Priming oligonucleotide (if using GSP) and concentration	E	Evidence for LOD	E
Reverse transcriptase and concentration	E	If multiplex, efficiency and LOD of each assay	E
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	D

[a] All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available. If primers are from RTPrimerDB, information on qPCR target, oligonucleotides, protocols, and validation is available from that source.
[b] FFPE, formalin-fixed, paraffin-embedded; RIN, RNA integrity number; RQI, RNA quality indicator; GSP, gene-specific priming; dNTP, deoxynucleoside triphosphate.
[c] Assessing the absence of DNA with a no–reverse transcription assay is essential when first extracting RNA. Once the sample has been validated as DNA free, inclusion of a no–reverse transcription control is desirable but no longer essential.
[d] Disclosure of the probe sequence is highly desirable and strongly encouraged; however, because not all vendors of commercial predesigned assays provide this information, it cannot be an essential requirement. Use of such assays is discouraged.

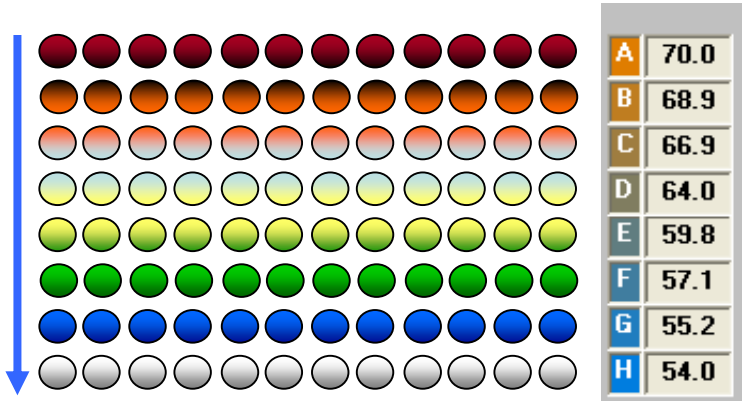
Life Science Group

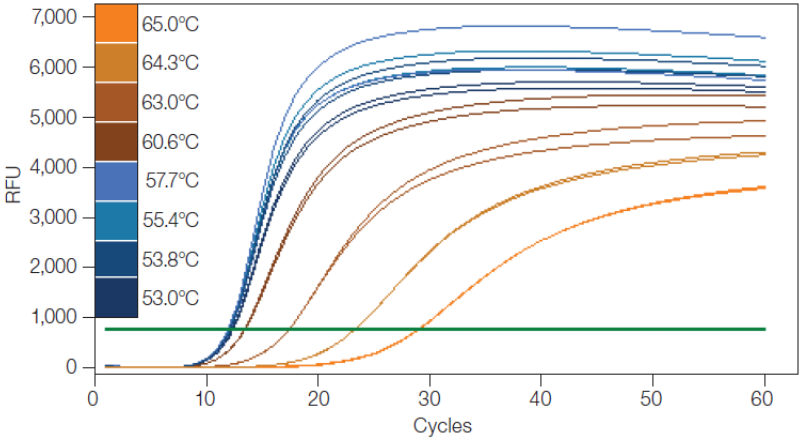
Clinical Chemistry 55:4 (2009) 613

BIO-RAD

Temperature Gradient

dynamic thermal gradient





Test 12 sets of primer

- MIQE guideline
  - –Evidence of optimization

Table 1. MIQE checklist for authors, reviewers, and editors.[a]

Item to check	Importance	Item to check	Importance
Experimental design		qPCR oligonucleotides	
Definition of experimental and control groups	E	Primer sequences	E
Number within each group	E	RTPrimerDB identification number	D
Assay carried out by the core or investigator's laboratory?	D	Probe sequences	D[d]
Acknowledgment of authors' contributions	D	Location and identity of any modifications	E
Sample		Manufacturer of oligonucleotides	D
Description	E	Purification method	D
Volume/mass of sample processed	D	qPCR protocol	
Microdissection or macrodissection	E	Complete reaction conditions	E
Processing procedure	E	Reaction volume and amount of cDNA/DNA	E
If frozen, how and how quickly?	E	Primer, (probe), Mg^{2+}, and dNTP concentrations	E
If fixed, with what and how quickly?	E	Polymerase identity and concentration	E
Sample storage conditions and duration (especially for FFPE[b] samples)	E	Buffer/kit identity and manufacturer	E
Nucleic acid extraction		Exact chemical composition of the buffer	D
Procedure and/or instrumentation	E	Additives (SYBR Green I, DMSO, and so forth)	E
Name of kit and details of any modifications	E	Manufacturer of plates/tubes and catalog number	D
Source of additional reagents used	D	Complete thermocycling parameters	E
Details of DNase or RNase treatment	E	Reaction setup (manual/robotic)	D
Contamination assessment (DNA or RNA)	E	Manufacturer of qPCR instrument	E
Nucleic acid quantification	E	qPCR validation	
Instrument and method	E	Evidence of optimization (from gradients)	D
Purity (A_{260}/A_{280})	D	Specificity (gel, sequence, melt, or digest)	E
Yield	D	For SYBR Green I, C_q of the NTC	E
RNA integrity: method/instrument	E	Calibration curves with slope and y intercept	E
RIN/RQI or C_q of 3′ and 5′ transcripts	E	PCR efficiency calculated from slope	E
Electrophoresis traces	D	CIs for PCR efficiency or SE	D
Temperature and time	E	Data analysis	
Manufacturer of reagents and catalogue numbers	D	qPCR analysis program (source, version)	E
C_qs with and without reverse transcription	D[c]	Method of C_q determination	E
Storage conditions of cDNA	D	Outlier identification and disposition	E
qPCR target information		Results for NTCs	E
Gene symbol	E	Justification of number and choice of reference genes	E
Sequence accession number	E	Description of normalization method	E
Location of amplicon	D	Number and concordance of biological replicates	D
Amplicon length	E	Number and stage (reverse transcription or qPCR) of technical replicates	E
In silico specificity screen (BLAST, and so on)	E	Repeatability (intraassay variation)	E
Pseudogenes, retropseudogenes, or other homologs?	D	Reproducibility (interassay variation, CV)	D
Sequence alignment	D	Power analysis	D
Secondary structure analysis of amplicon	D	Statistical methods for results significance	E
Location of each primer by exon or intron (if applicable)	E	Software (source, version)	E
What splice variants are targeted?	E	C_q or raw data submission with RDML	E

qPCR validation
Evidence of optimization (from gradients) D
Specificity (gel, sequence, melt, or digest) E

Bio-Rad's CFX series Real-time PCR Family

Price

CFX Connect

- Mid-price
- 96-well
- Fast block
- 2 targets

CFX96 Touch

- Premium
- 96-well
- Fast block
- 5 targets
- Stand-alone run

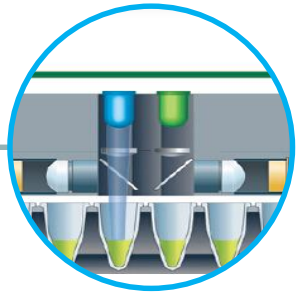
CFX384 Touch

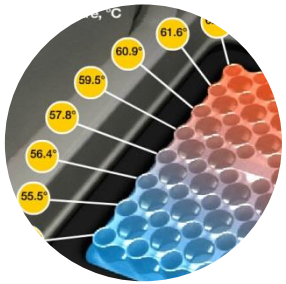
- Premium
- 384-well
- 4 targets
- Stand-alone
- High throughput
- Stand-alone run

Features & Flexibility

CFX Connect™

- 3 filtered LEDS
- 3 filtered photodiodes
- 2 targets multiplex



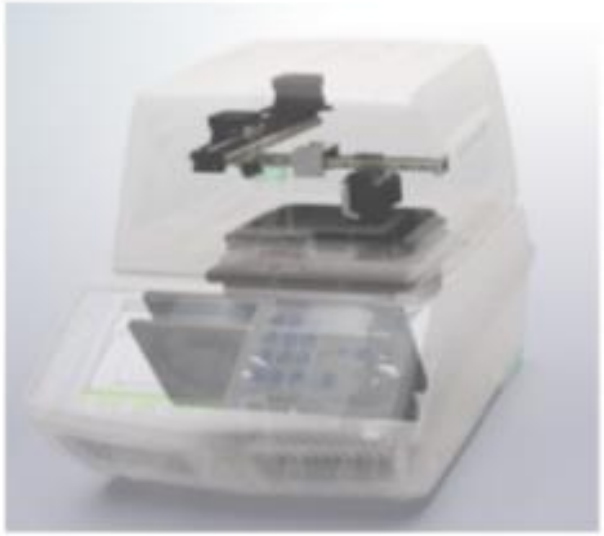


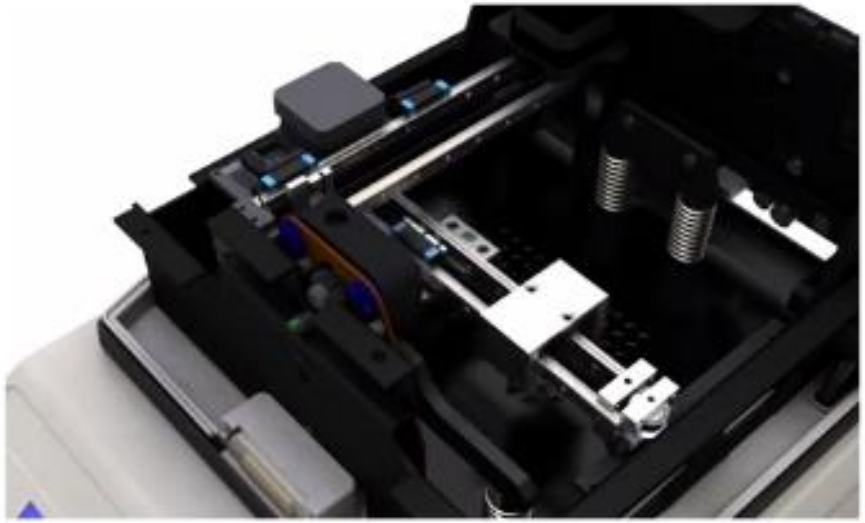
- Temperature Gradient
- High resolution melt

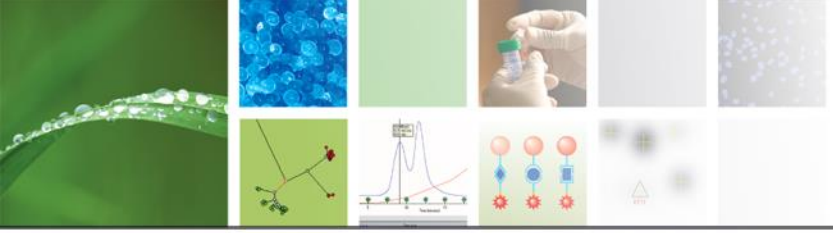
- 96 well 1-50µL
- Max ramp rate 5°C/s

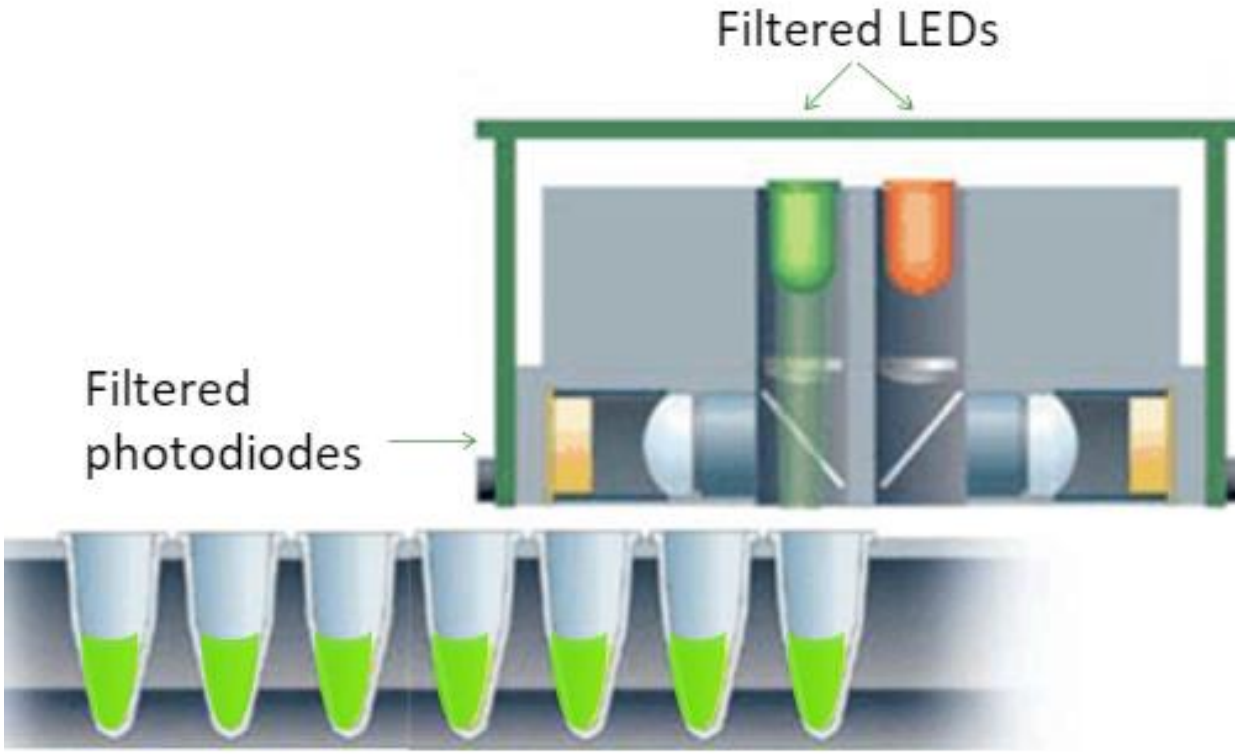
BIO-RAD

- Optics shuttle transverses the plate at the completion of each data acquisition step
- Optics shuttle centered above each well resulting in identical light path and intensity for each well
- No need to sacrifice one channel to data collection of a passive reference dye







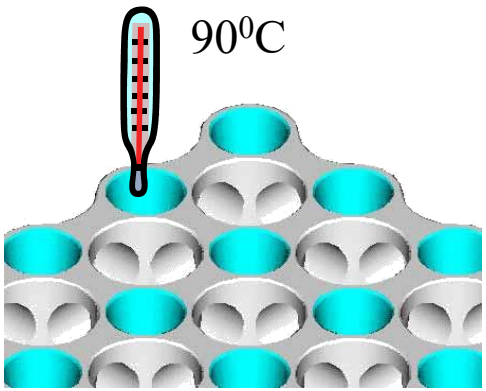


Strength of our optic module

- Accurate data collection
- Consistent light source
- Minimal maintainance
- Data collected from every well

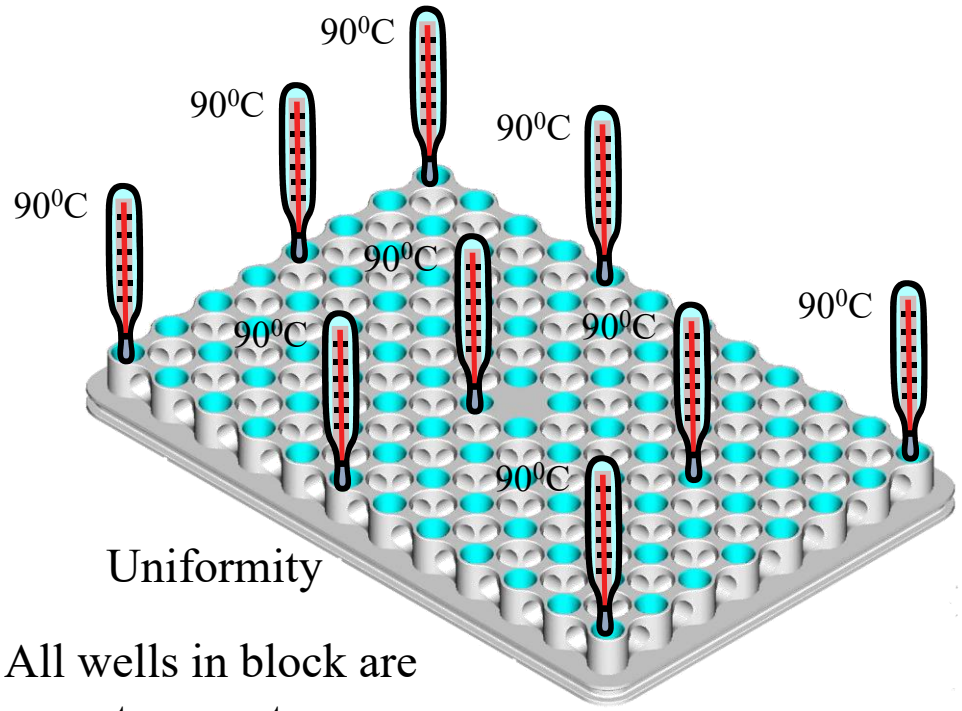
Patented Block Design

- Accuracy: +/- 0.2°C
- Uniformity: +/- 0.4°C within10 seconds



Accuracy

Programmed temperature
= Actual well temperature

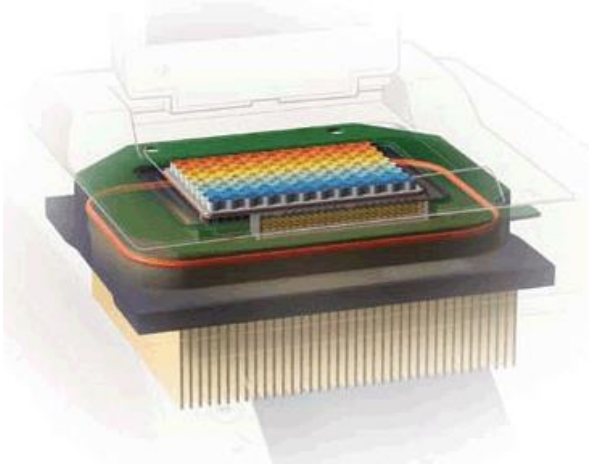


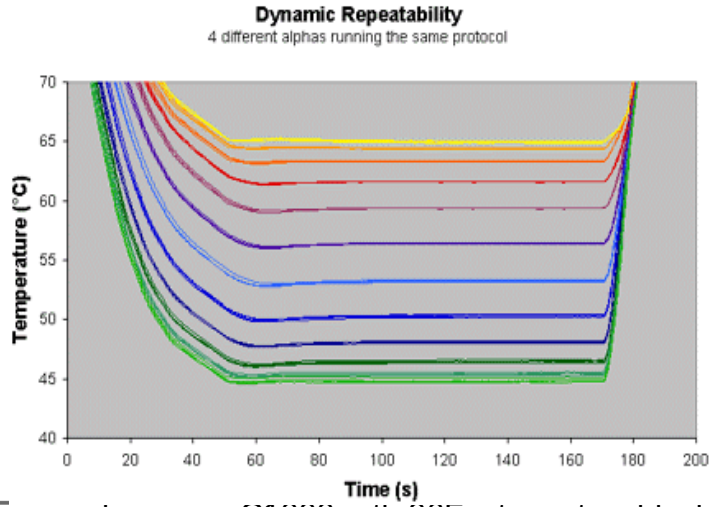
Uniformity

All wells in block are
same temperature

Performance
Thermal Cycler Gradient

- Annealing temp is critical for reaction specificity and efficiency
- Save time and reagents optimizing annealing temp in one run
  - Program up to a 24˚C gradient, back-to-front (back row is hotter)
  - 30-100˚C range
  - "Dynamic Ramping" – Wells hit set temperature point together, and then maintain the same dwell to eliminate time as a reaction variable

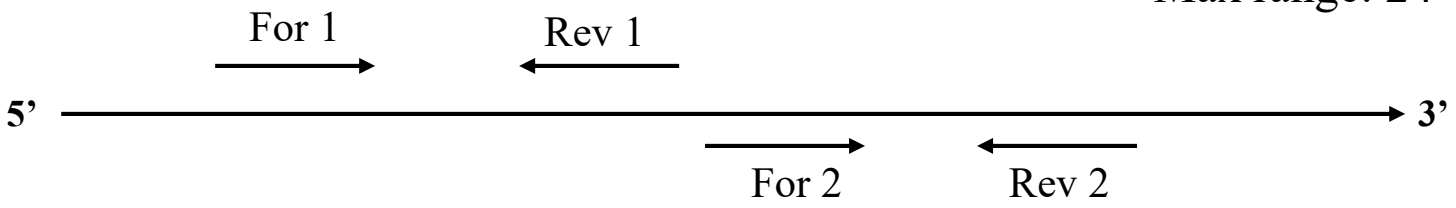




columns on C1000 with 96Fast reaction block

Assay optimization

Max range: 24°C



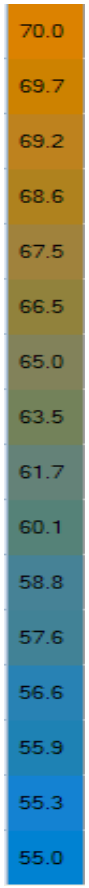
For 1 For 2
Rev 1 Rev 2

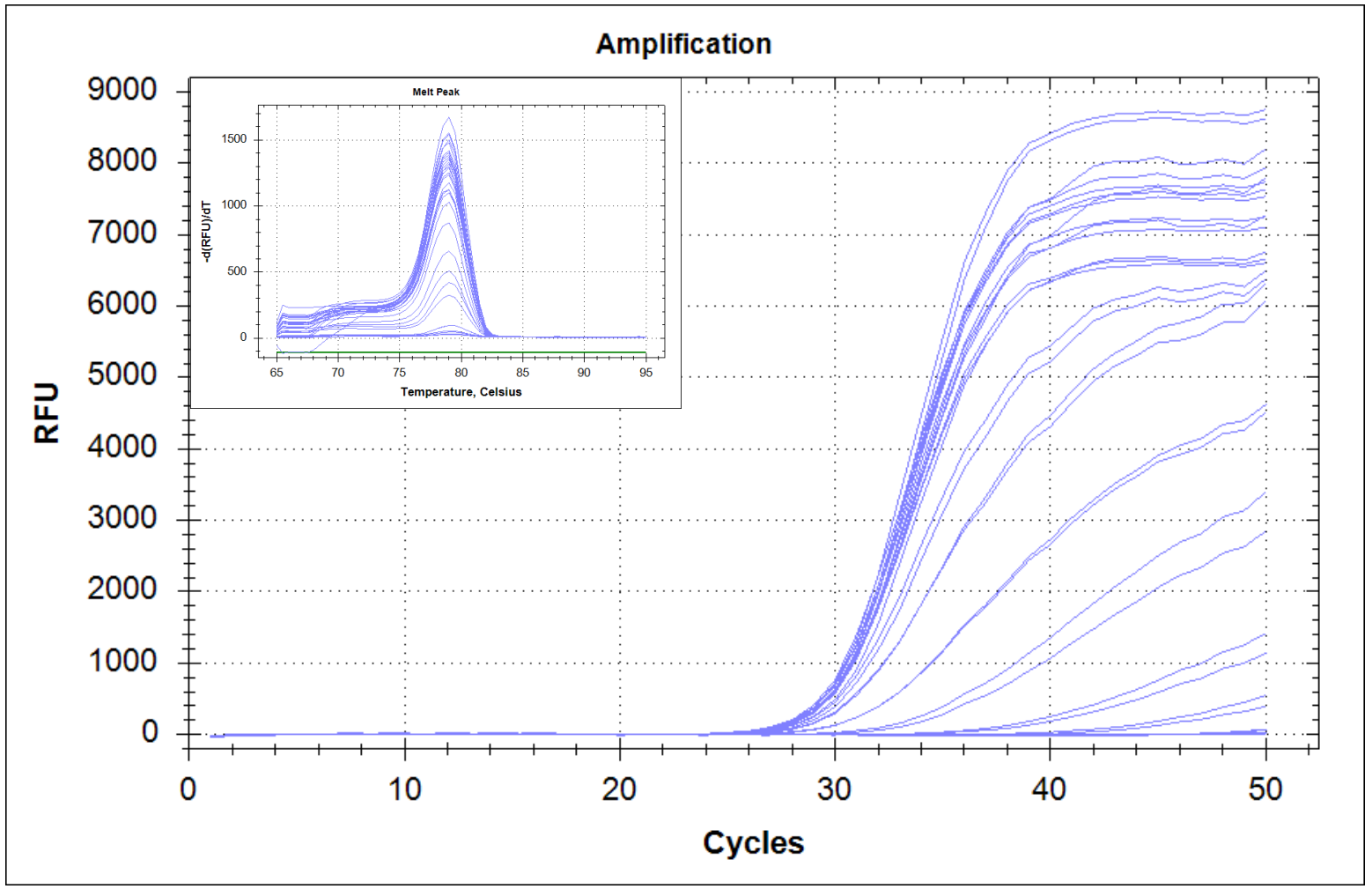
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	70.0	U	U		U	U																			
B	69.7	U	U		U	U																			
C	69.2	U	U		U	U																			
D	68.6	U	U		U	U																			
E	67.5	U	U		U	U																			
F	66.5	U	U		U	U																			
G	65.0	U	U		U	U																			
H	63.5	U	U		U	U																			
I	61.7	U	U		U	U																			
J	60.1	U	U		U	U																			
K	58.8	U	U		U	U																			
L	57.6	U	U		U	U																			
M	56.6	U	U		U	U																			
N	55.9	U	U		U	U																			
O	55.3	U	U		U	U																			
P	55.0	U	U		U	U																			

10° above design

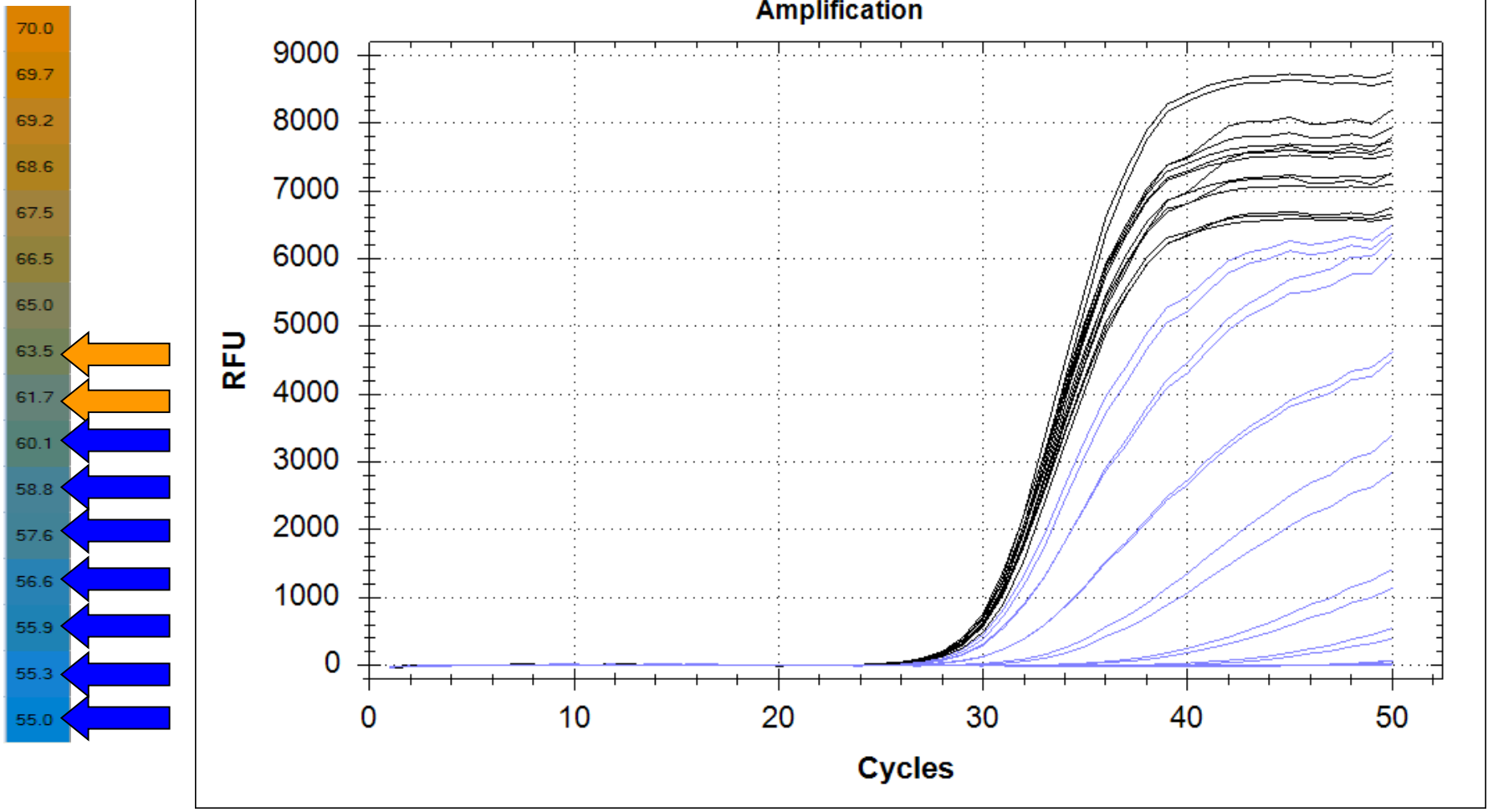
5° below design

Gradient analysis



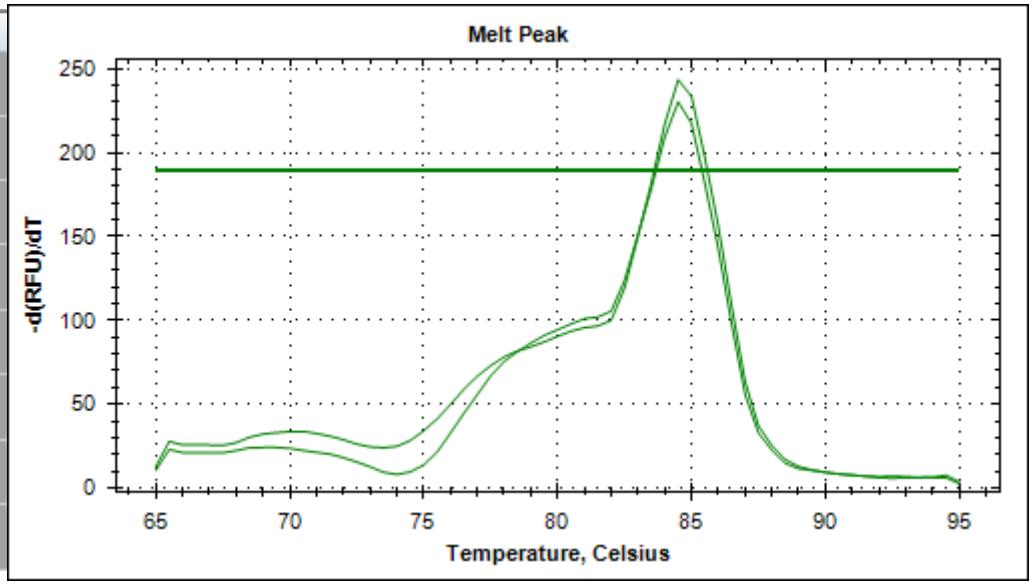


Optimal Annealing Range



Overcome non-specific amplification

		1	2	3	4
A	68.0			Unk	Unk
B	67.0			Unk	Unk
C	64.9			Unk	Unk
D	61.4			Unk	Unk
E	57.0			Unk	Unk
F	53.5			Unk	Unk
G	51.2			Unk	Unk
H	50.0			Unk	Unk



Overcome non-specific amplification

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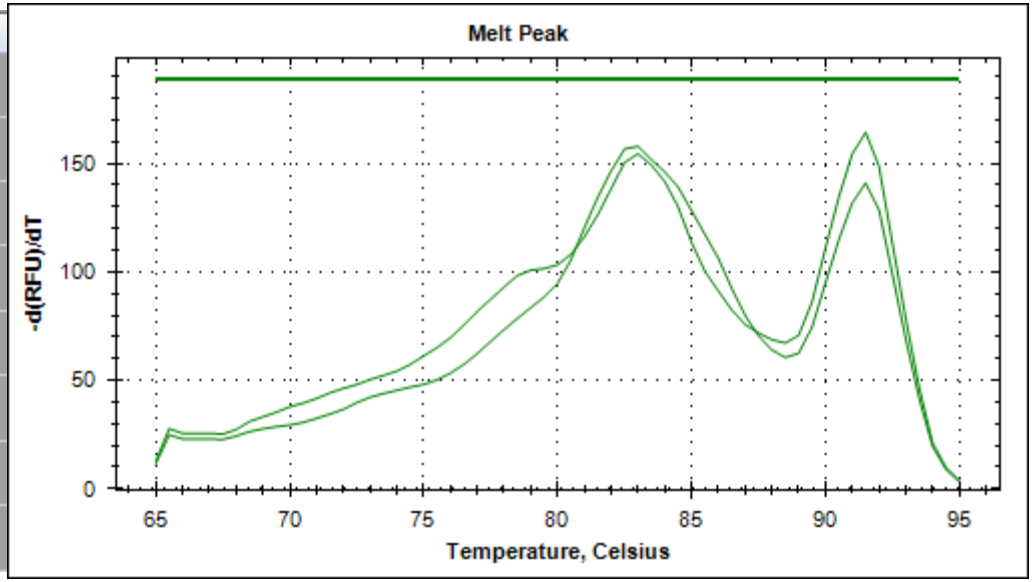
G	51.2			Unk	Unk
H	50.0			Unk	Unk



Overcome non-specific amplification

		1	2	3	4
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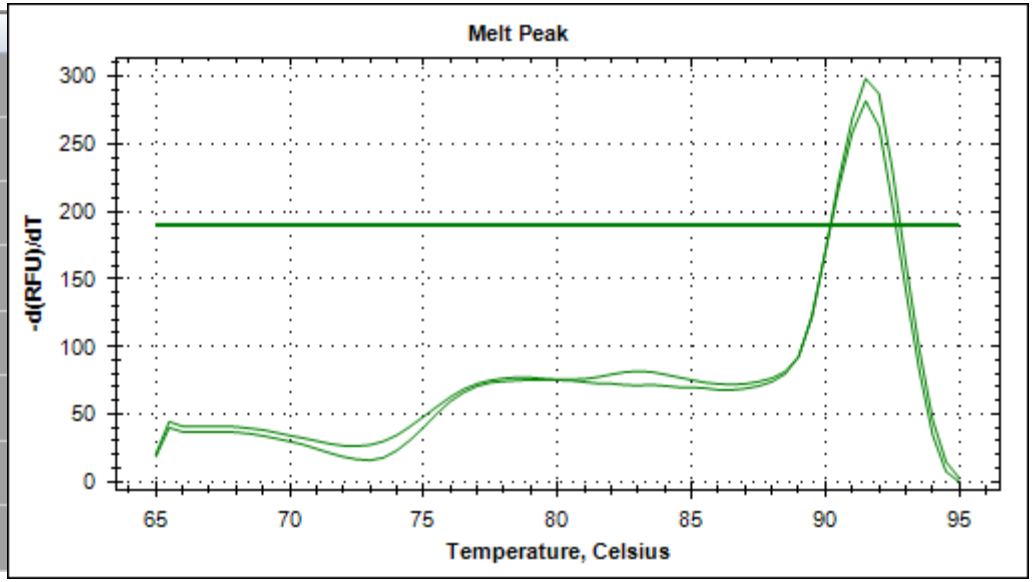
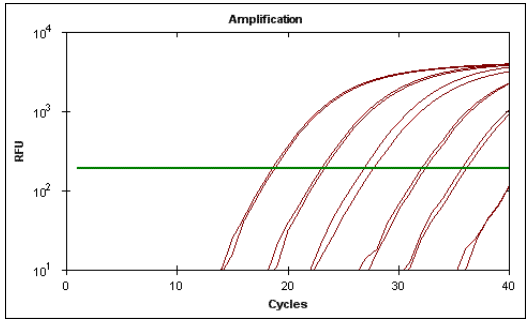
B	67.0			Unk	Unk
C	64.9			Unk	Unk
D	61.4			Unk	Unk
E	57.0			Unk	Unk
F	53.5			Unk	Unk
G	51.2			Unk	Unk
H	50.0			Unk	Unk

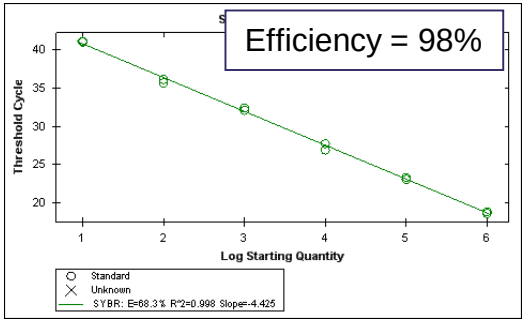


Gradient Optimization of Annealing Temperature

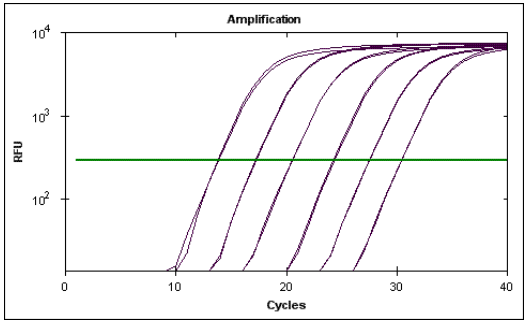
- Serial dilutions run at 8 temps, ranging from 55°C to 68°C
- Reactions at 62°C annealing have low Cts and highest reaction efficiency

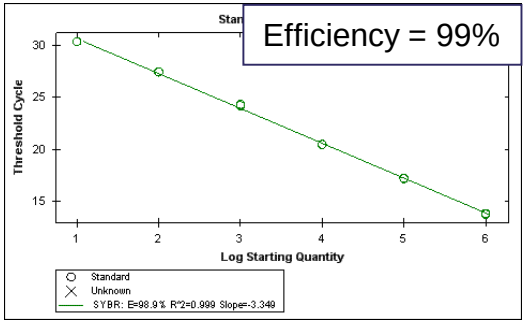
67°C



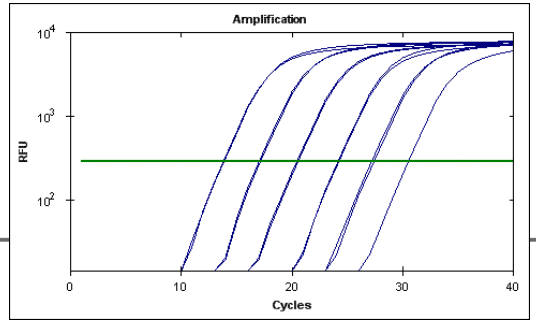


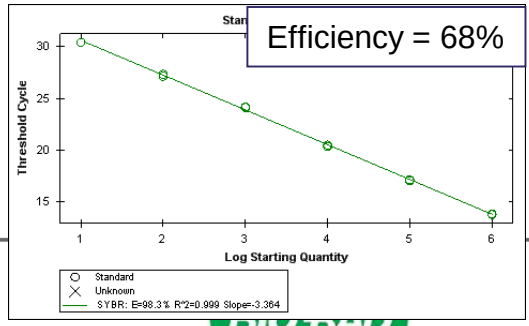
62°C





56°C





Use of Synthetic Templates

Synthetic templates allow for

- Sample independent validation of assay performance
- Comparison across plates (inter-run calibrators)
- Compare across labs

Nucleic Acids Research, 2009, 1–9
doi:10.1093/nar/gkp721

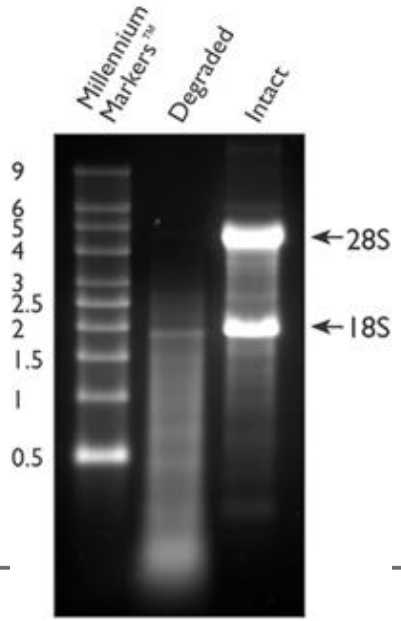
External oligonucleotide standards enable cross laboratory comparison and exchange of real-time quantitative PCR data

Joëlle Vermeulen[1], Filip Pattyn[1], Katleen De Preter[1], Liesbeth Vercruysse[1], Stefaan Derveaux[1], Pieter Mestdagh[1], Steve Lefever[1], Jan Hellemans[1,2], Frank Speleman[1] and Jo Vandesompele[1,2,*]

RNA quality

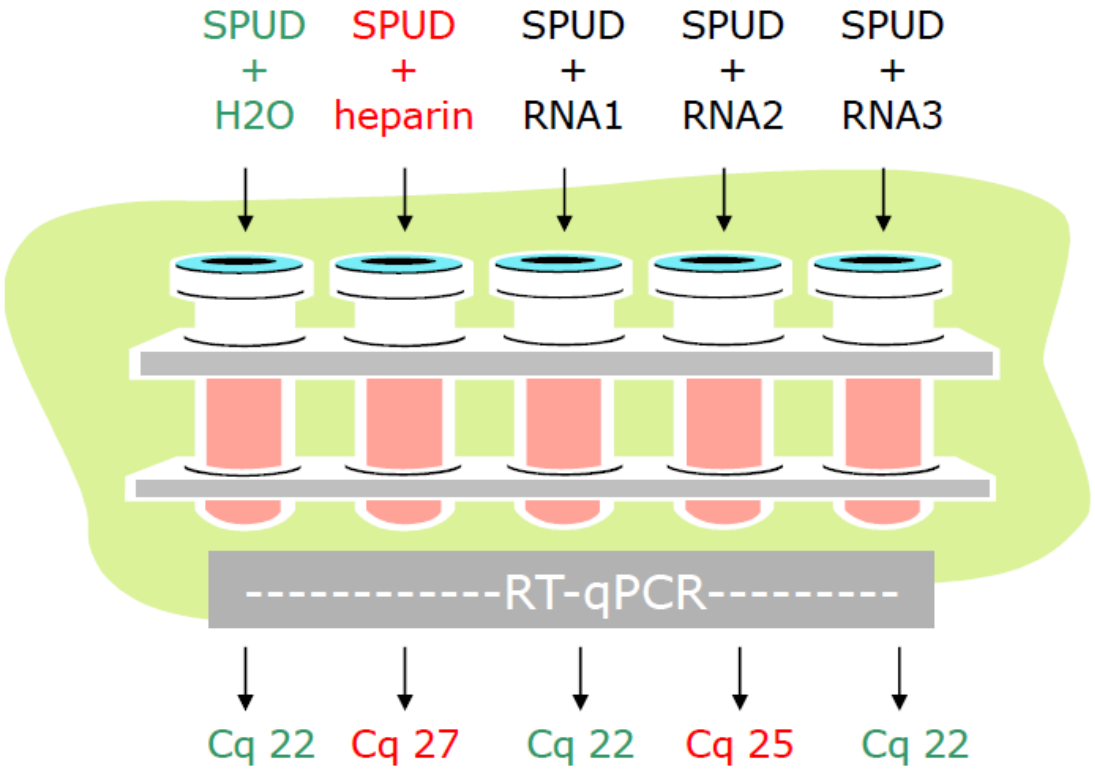
- RNA quality analysis methodology
  - Analysis A260/A280 ratio and rRNA bands on agarose gel.
  - Automated microfluidic capillary electrophoresis system.
  - 3`:5` assay
  - SPUD assay (absence of PCR inhibitors)
  - NRT PCR assay (absence of DNA contamination)

- Analyze RNA quantity and quality
- UV absorbance
  - A260/A280=2.0,
  - A260: 0.15~1
  - A260/A230=2.5
  - Gel electrophoresis analysis 28S rRNA，18S rRNA



28S:18S≈2:1

RNA quality



SPUD: detection presence of inhibitors

Nolan et al., Anal Biochem, 2006

RNA Quality Control Assay

RNA Quality Assay – Two assays (RQ1 and RQ2) that target the same transcript, but at two different locations. A significantly higher presence of the shorter transcript (RQ1) indicates the RNA is degraded. A difference in C_q less than 3 between the two assays indicates acceptable RNA quality.

RQ1

RQ1 Assay
(short transcript)

PASS = $\Delta Cq \leq 3$

RNA degradation will not negatively affect results!

RQ2

RQ2 Assay
(long transcript)

FAIL = $\Delta Cq > 3$

RNA is degraded and could be negatively affecting results – most likely need to redo sample extraction

Bio-Rad RT Kit

iScript Reverse Transcription Supermix for RT-qPCR

iScript cDNA Synthesis Kit

iScript Advanced cDNA Synthesis Kit for RT-qPCR

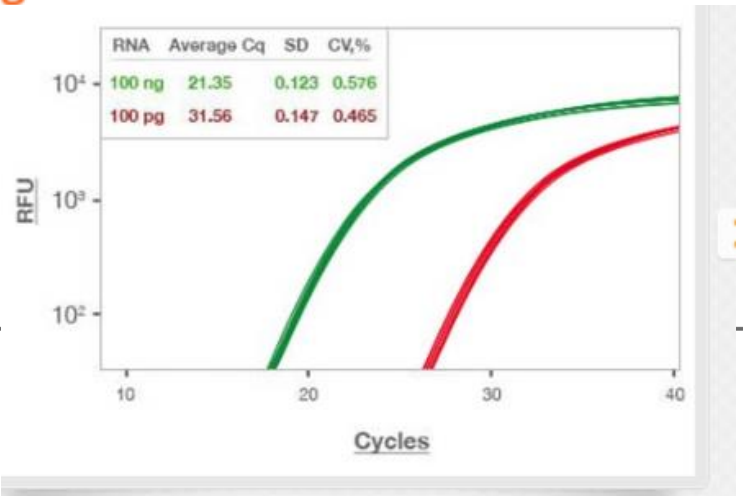
iScript gDNA Clear cDNA Synthesis Kit

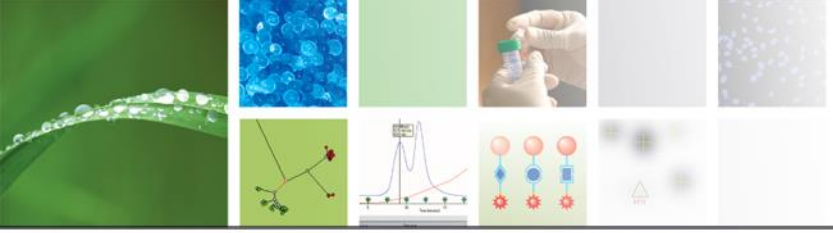
iScript Select cDNA Synthesis Kit

All Bio-Rad's iScript Kit

All Bio-Rad's iScript Kits:

- **Use an MMLV enzyme with RNase H+ activity**
- **Come with an optimal blend of oligo dTs and random primers, (*except the Select kit, which provides the primers in a separate tube*)**
- **Include a potent RNAse inhibitor to protect the RNA from degradation prior to cDNA synthesis**
- **Were benchmarked against the top selling competitor products to demonstrate superior sensitivity and dynamic range**



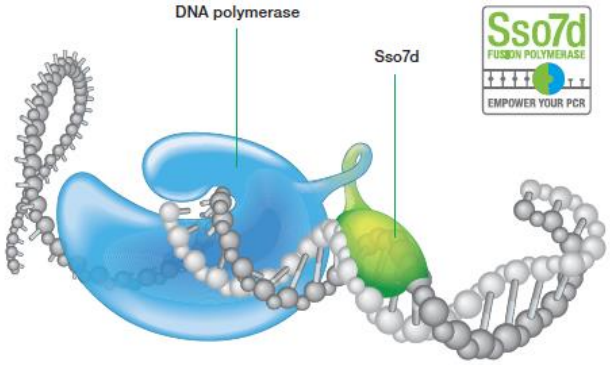


Review – Best Product for the Job

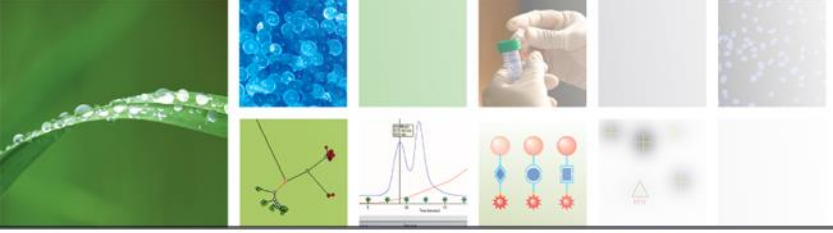
	iScript Reverse Transcription Supermix for RT-qPCR	iScript gDNA Clear cDNA Synthesis Kit	iScript Advanced cDNA Synthesis Kit for RT-qPCR	iScript Select cDNA Synthesis Kit	iScript cDNA Synthesis Kit
Features	Minimal reaction setup time	**Effective gDNA removal** before reverse transcription	Maximum RNA input for high cDNA yields	**Flexible priming options**	Reliable value solution
Cost	$$	$$$	$$$	$	$
Total input RNA Range	1 µg - 1pg	1 µg - 1pg	**7.5 µg – 100 fg**	1 µg - 1pg	1 µg – 100 fg
Kit Format	**1 tube**	3 tubes	2 tubes	5 tubes	2 tubes
Kit Contents	• 5x iScript RT Supermix • No-RT control supermix	• 5x iScript RT Supermix • No-RT control supermix • DNase • DNase buffer	• iScript Reverse Transcriptase • 5x iScript Advanced Reaction Mix	• iScript Reverse Transcriptase • 5x iScript Reaction Mix • 3 priming options	• iScript Reverse Transcriptase • 5x iScript Reaction Mix
Time to Produce cDNA	26 min	36 min	21 min	40-90 min	26 min

qPCR Supermix

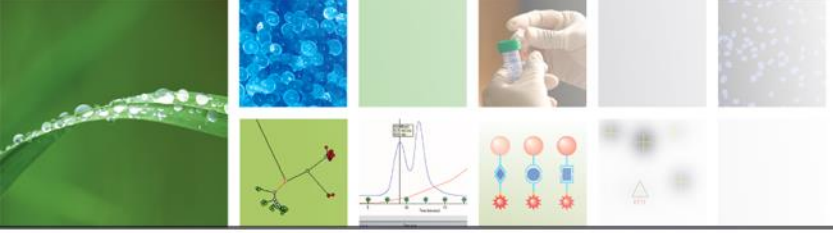
SsoAdvanced™ Universal Supermixes			iTaq™ Universal Supermixes	
				
SsoAdvanced™ Universal SYBR® Green Supermix	SsoAdvanced Universal Probes Supermix	SsoAdvanced Universal Inhibitor-Tolerant Supermix	iTaq™ Universal SYBR® Green Supermix	iTaq Universal Probes Supermix
Robust performance with demanding templates and under challenging conditions		Optimized for crude samples containing PCR inhibitors	Advanced formulation for reliable, reproducible gene expression analysis on any qPCR instrument	
★	★	★		



BIO-RAD



型號	敘述
Real-Time PCR 耗材	
八連排	
TCS-0803	Optical Flat 8-Cap Strips, 960 reactions
TLS-0851	Low-Profile 0.2 ml 8-Tube Strips without Caps, 960 reactions
TLS-0801	Low-Profile 0.2 ml 8-Tube Strips without Caps, 960 reactions
96 或 48 孔盤	
MSB-1001	Microseal 'B' Adhesive Seals, Pkg of 100
MLL-9651	Multiplate White Low-Profile 96-Well Unskirted PCR Plates, Pkg of 25
MLL-9601	Multiplate Low-Profile 96-Well Unskirted PCR Plates, Pkg of 25
HSP-9955	Hard-Shell Thin-Wall 96-Well Skirted PCR Plates, Pkg of 50
MLL-4851	Multiplate White Low-Profile 48-Well Unskirted PCR Plates, Pkg of 50



反轉錄相關試劑

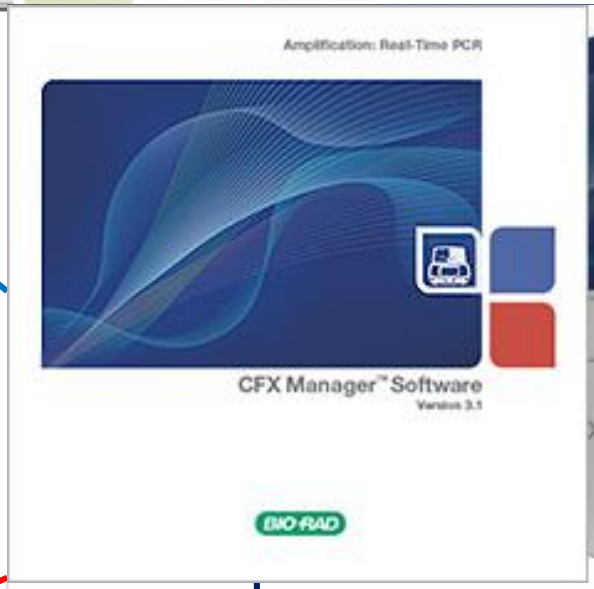
170-8890	iScriptTm cDNA Synthesis Kit, 25 reactions
170-8891	iScriptTm cDNA Synthesis Kit, 100 reactions
170-8896	iScriptTm Select cDNA Synthesis Kit, 25 reactions
170-8897	iScriptTm Select cDNA Synthesis Kit, 100 reactions
170-8892	iScriptTm One-Step RT-PCR Kit With SYBR® Green, 50 reactions
170-8893	iScriptTm One-Step RT-PCR Kit With SYBR® Green, 200 reactions
170-8894	iScriptTm One-Step RT-PCR Kit for Probes, 50 reactions
170-8895	iScriptTm One-Step RT-PCR Kit for Probes, 200 reactions

Real-Time PCR 混合液

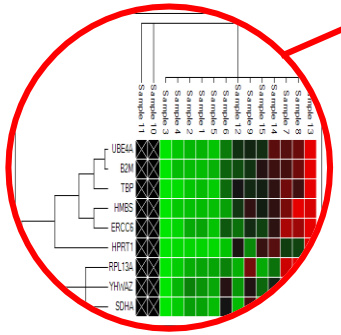
172-5848	iQTm Multiplex Powermix, 50 reactions
172-5849	iQTm Multiplex Powermix, 200 reactions
170-8860	iQTm Supermix, 100 reactions
170-8862	iQTm Supermix, 500 reactions
170-8864	iQTm Supermix, 1,000 reactions
170-8880	iQTm SYBR® Green Supermix, 100 reactions
170-8882	iQTm SYBR® Green Supermix, 500 reactions
170-8884	iQTm SYBR® Green Supermix, 1,000 reactions
170-8885	iQTm SYBR® Green Supermix, 2,000 reactions
172-5200	SsoFast™ EvaGreen® Supermix, 200 reactions
172-5201	SsoFast™ EvaGreen® Supermix, 500 reactions
172-5202	SsoFast™ EvaGreen® Supermix, 1,000 reactions
172-5203	SsoFast™ EvaGreen® Supermix, 2,000 reactions
172-5230	SsoFast Probes Supermix, 200 reactions
172-5231	SsoFast Probes Supermix, 500 reactions
172-5232	SsoFast Probes Supermix, 1,000 reactions

Industry-leading CFX Manager Software

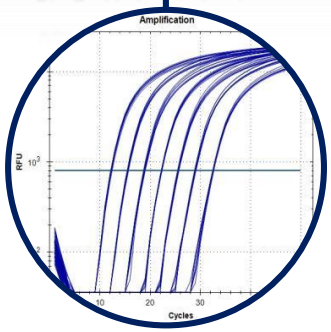
- User-friendly



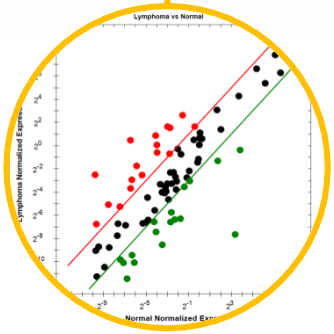
- Effective Wizard



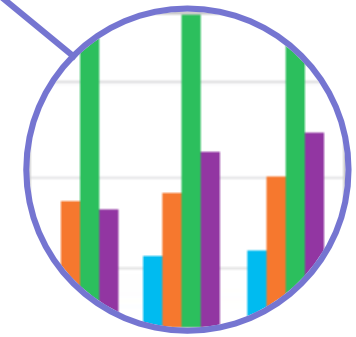
- Concise Interface



Data Collecting and Analysis



Powerful Function modules



- Rigorous algorithm

Life Science Group

Reduce Anxiety!

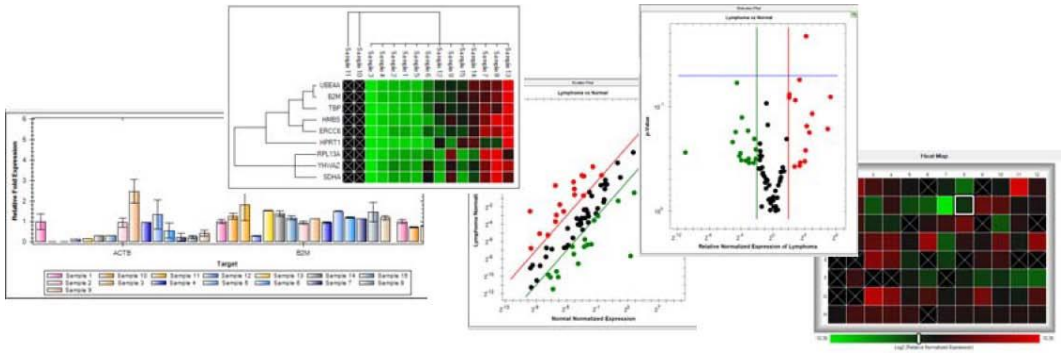
BIO-RAD

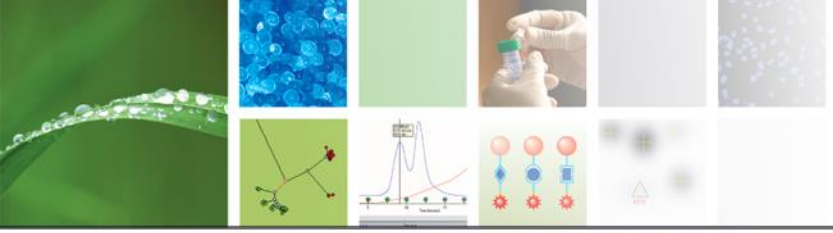
CFX Manager software

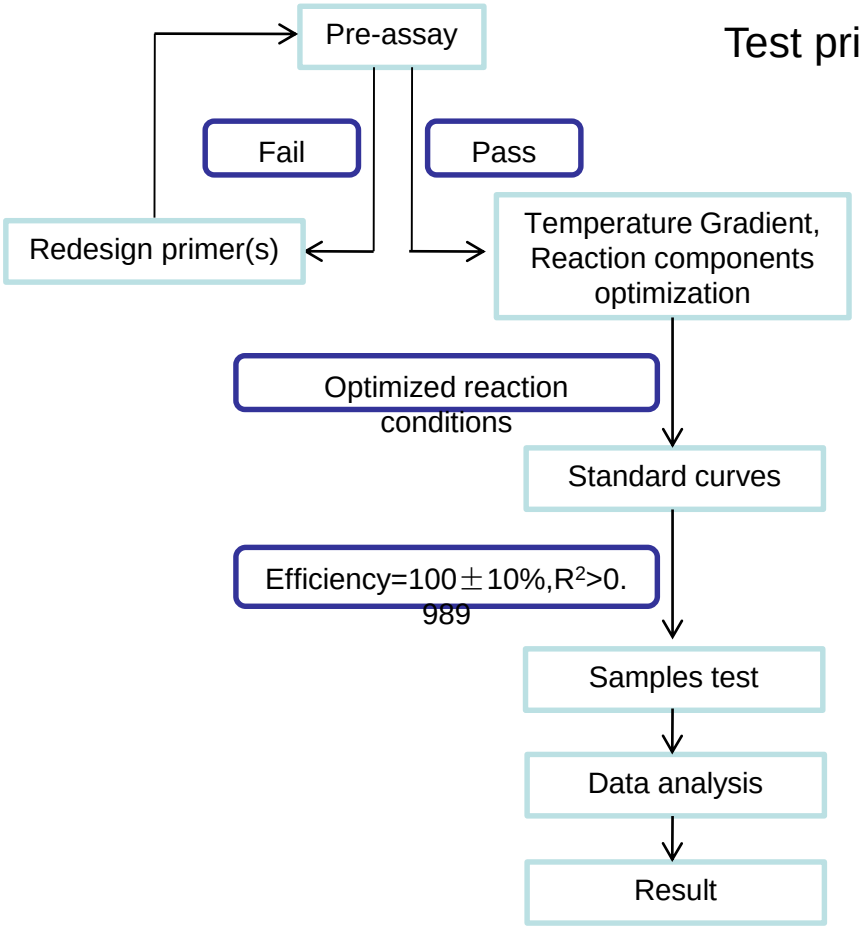
- Absolute quantification
- Relative quantification (Gene expression analysis)
- Melt curve analysis
- Genotyping
- QC
- Run information
- Qualification Plate Run

CFX Manager software

- Sample group
- PCR efficiency correction
- Normalization with multiple reference genes
- Gene Study (combination of multiple plates)
- Screening best reference genes by M value
- Inter-run-calibration
- Comply with MIQE guideline
  - — RDML export RDML
- Visualize your data
  - — Bar chart
  - — Cluster gram
  - — Scatter plot
  - — Volcano plot
  - — Heat Map
  - — Results table







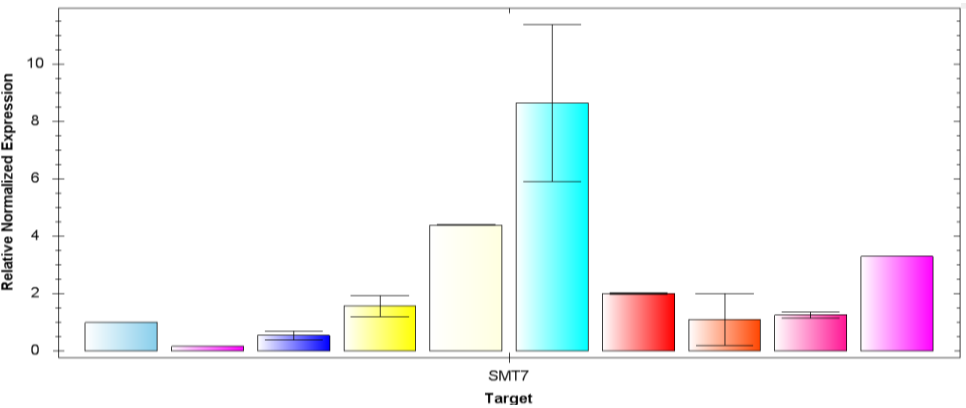
Test primers of both target gene(s) and reference gene(s)

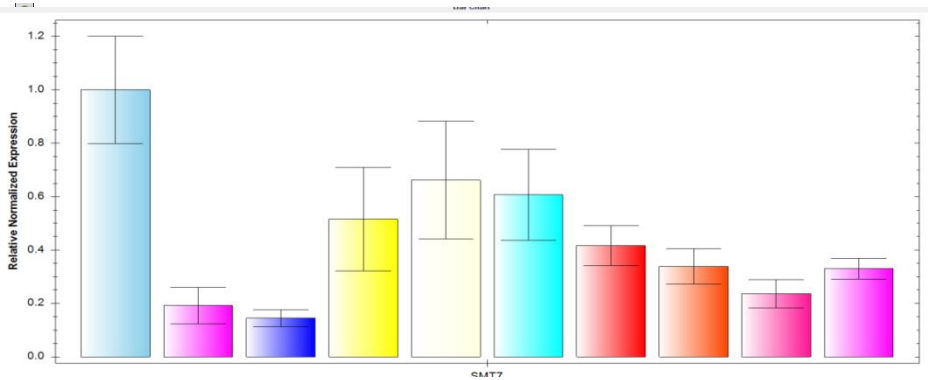
For each gene involved respectively

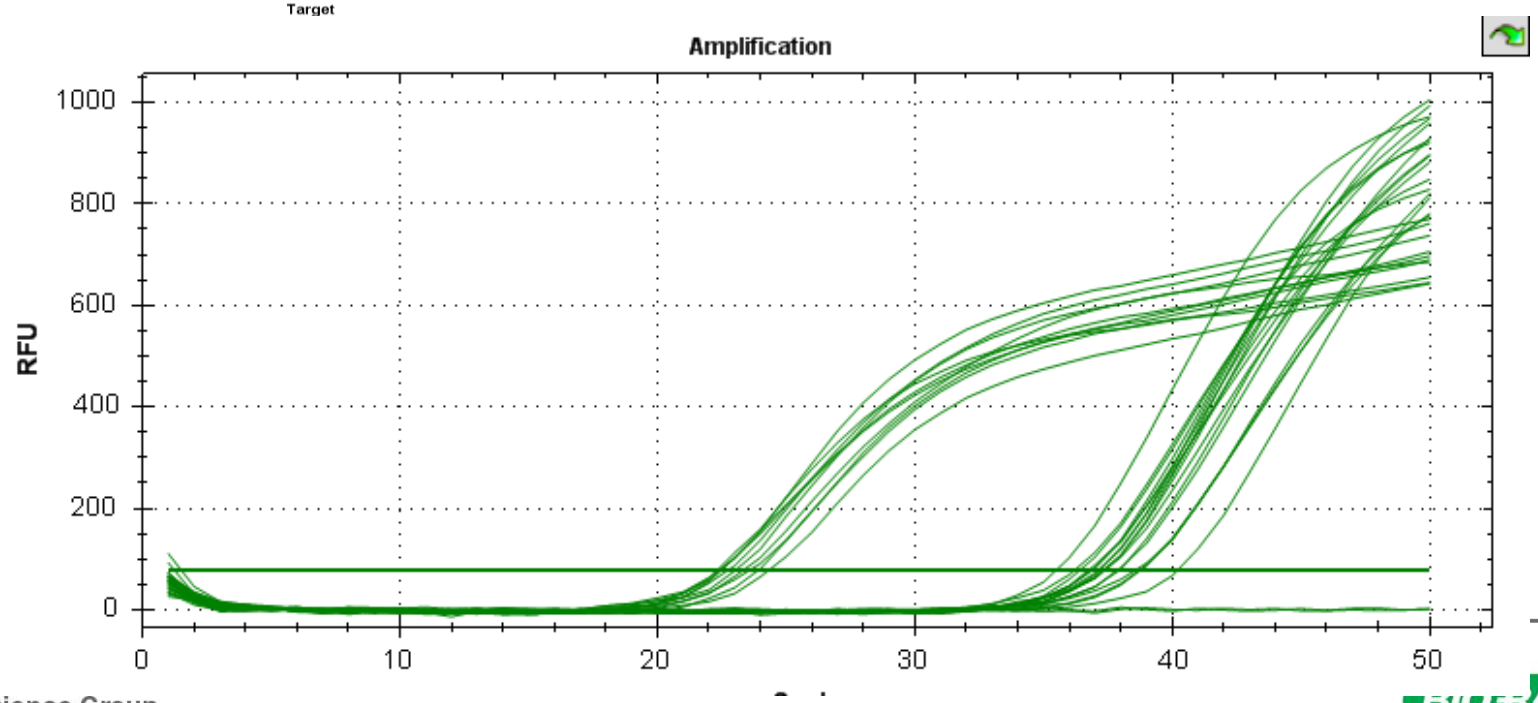
Inter-run calibrators

Correct analysis model

Challenge of q-PCR

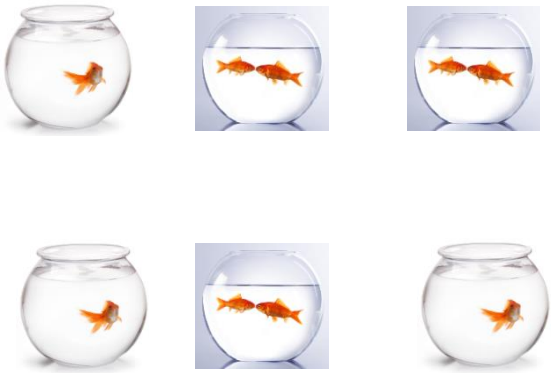


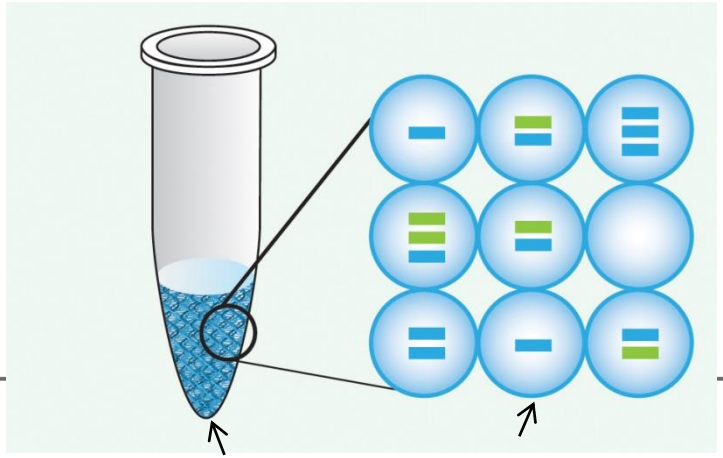




What about an new method - Digital PCR

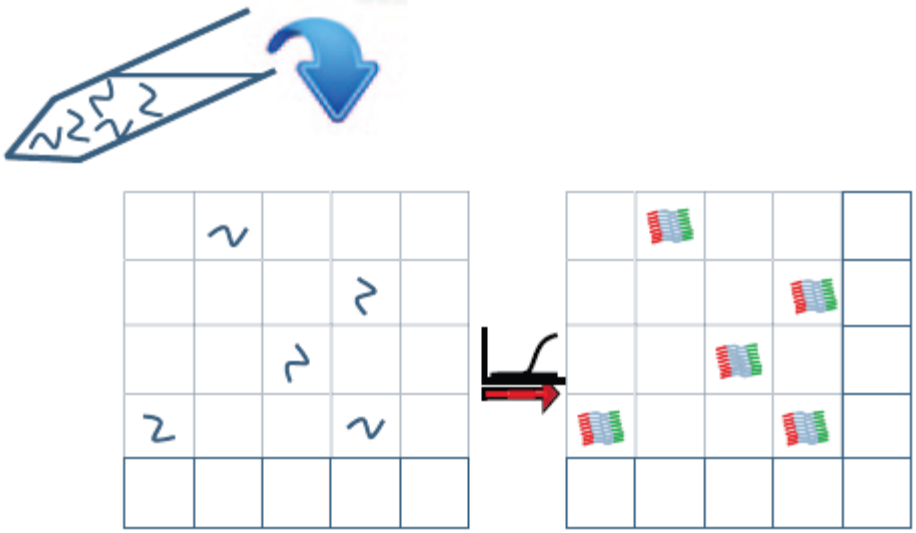
PCR reaction that is partitioned.



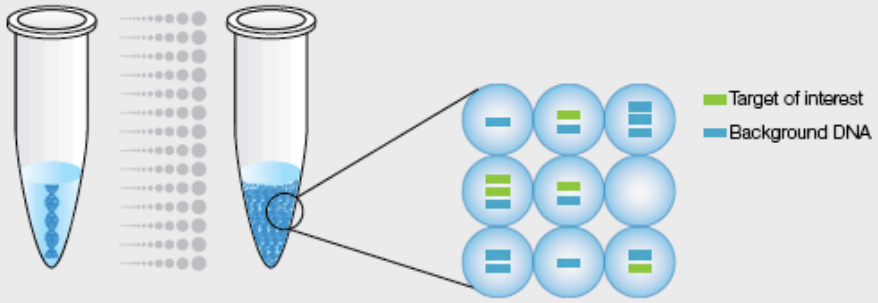


Many thousands
of discrete measurements

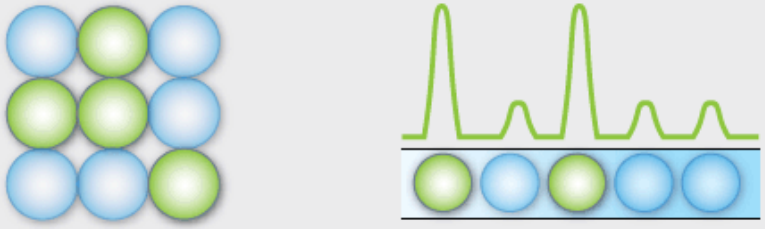
dPCR Principle



- End point (0's or 1's)
- Less sensitive to PCR efficiency
- No standard curve
- More tolerant to PCR inhibitors



The sample is partitioned into 20,000 droplets, with target and background DNA randomly distributed among the droplets.



After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target DNA was present or not present after partitioning. Each droplet provides an independent digital measurement.

"X" target copies

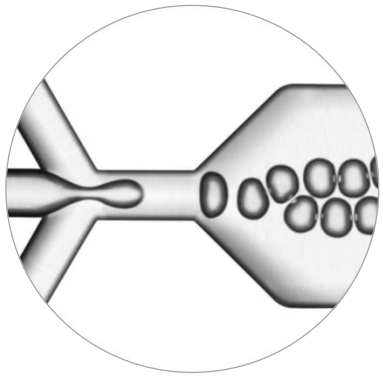
Positive and negative droplets are counted for the sample and the software calculates the concentration of target DNA as copies per microliter.

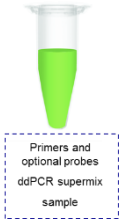
ddPCR improves precision, sensitivity and reproducibility

BIO-RAD

Workflow of Droplet Digital PCR

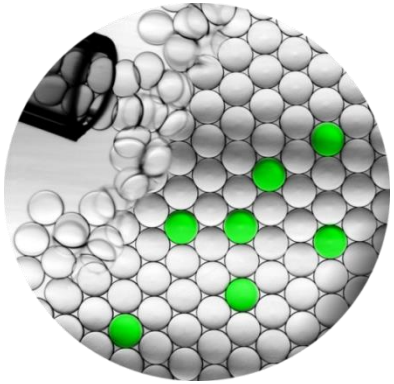
1. Make Droplets





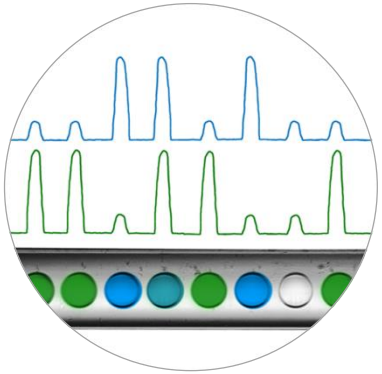
Droplet Generator

2. Cycle Droplets



Bulk PCR
Thermal Cycler

3. Read Droplets



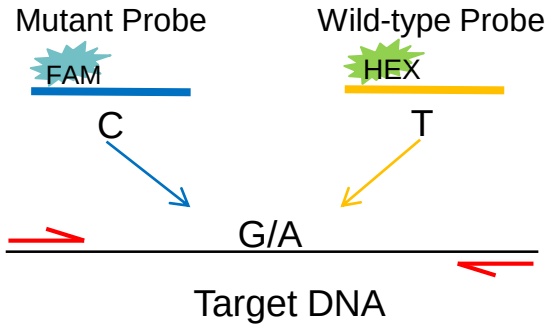
Droplet Reader

"X" target copies

Results

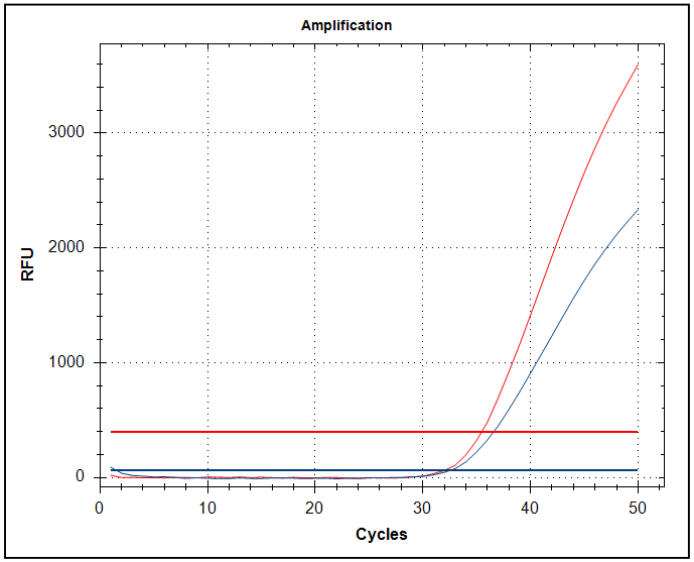
- Readout: copies/µl
- Dynamic range: 1–100,000 copies/well (~330 ng human genomic DNA)

ddPCR assays are more easy to design and validate

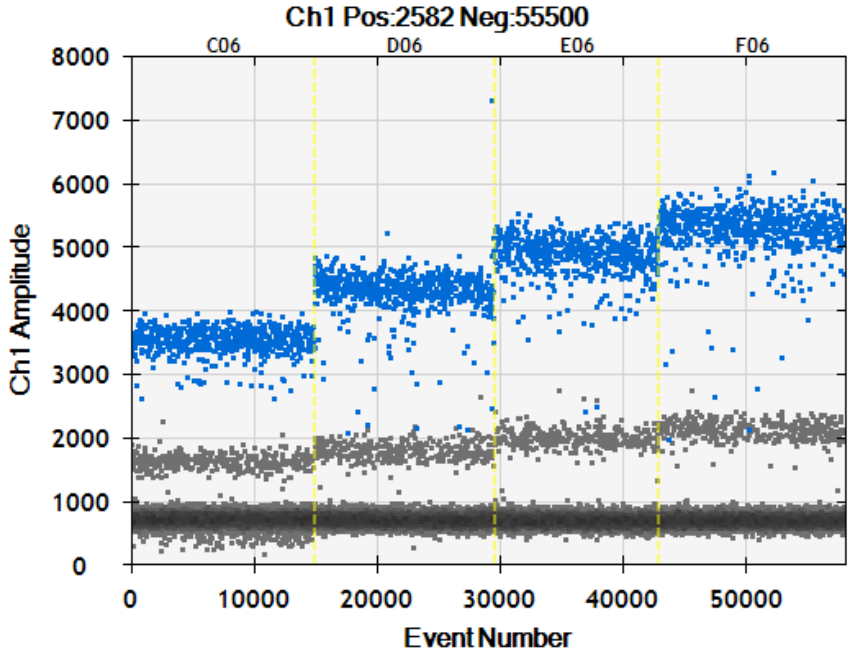


More Tolerance to:
1.Non-specific binding
2.Primer dimer
3.PCR inhibitor

Non-specific cross reaction



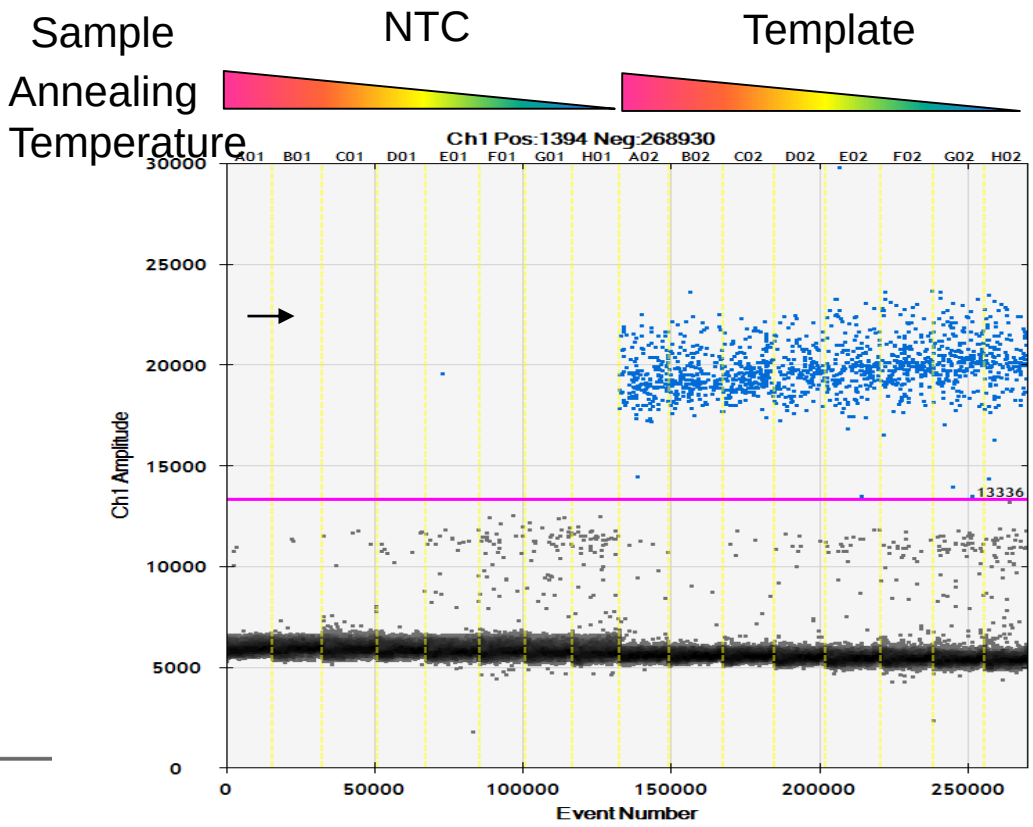
qPCR



ddPCR

Temperature Gradient: EvaGreen

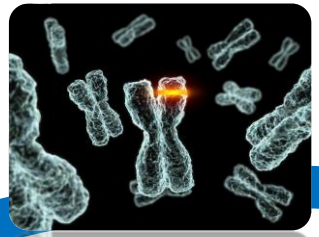
- Primer dimers can be visualized with EvaGreen chemistry
- Primer dimer frequency increases at lower annealing temperature and are visible in NTCs



More Tolerance to:
1.Non-specific binding
2.Primer dimer
3.PCR inhibitor

Primer Dimer

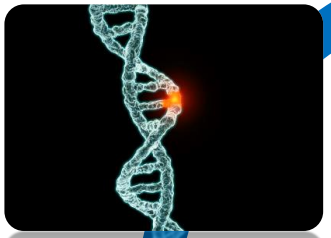
Applications



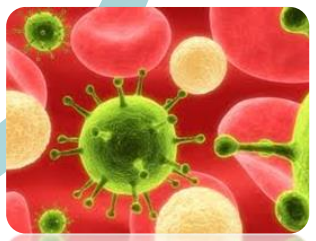
Mutation Detection

Stem Cell Analysis

Copy Number Variation



Virus Detection



NGS Cross Validation

Gene Expression
mRNA, small RNA, lncRNA

Q&A

E-mail: charlene_lu@bio-rad.com
Phone: 0987-792-623