Maximize Your Reverse Transcription-qPCR (RT-qPCR) Assays

Carl A. Strayer, PhD February 2012

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Outline

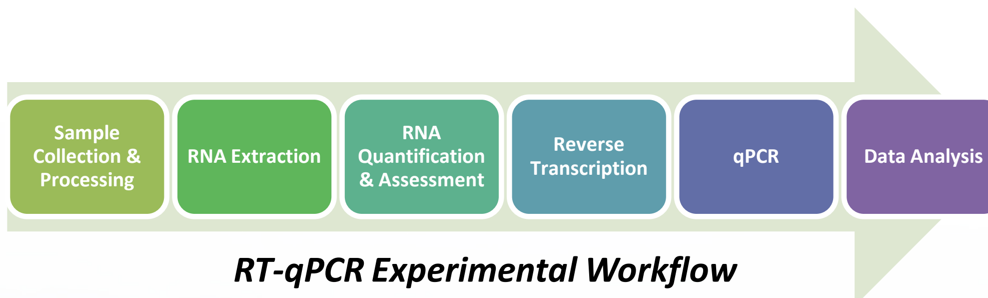
I. Fundamentals of Real-Time PCR

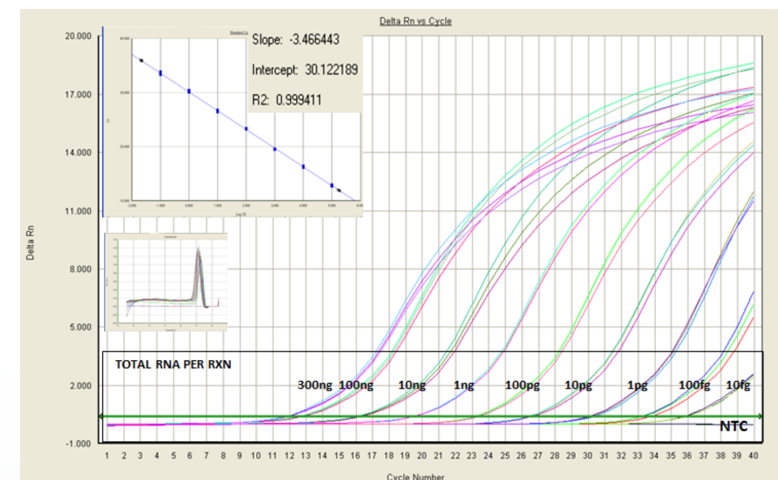
- What is Real-Time PCR?
- Real-Time PCR Metrics & Analysis

II. RT-qPCR Assay Design

- Primer Design
- Assay Validation

III. Tips & Troubleshooting





What is Real-Time PCR?

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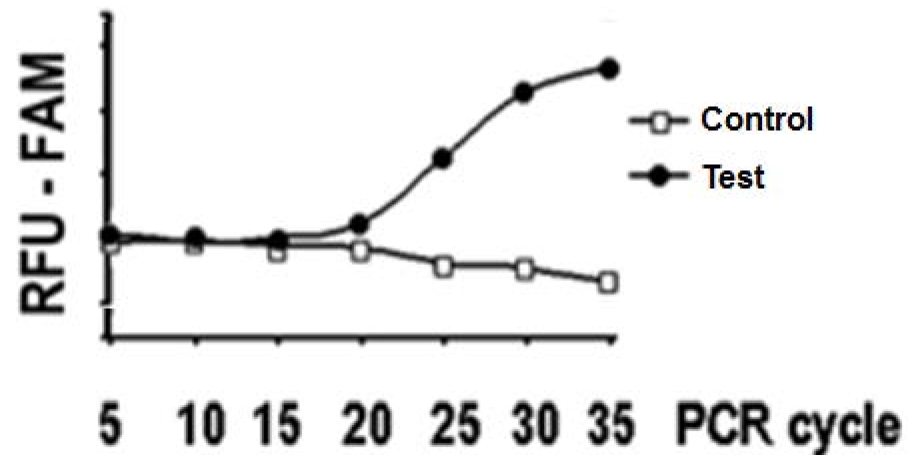
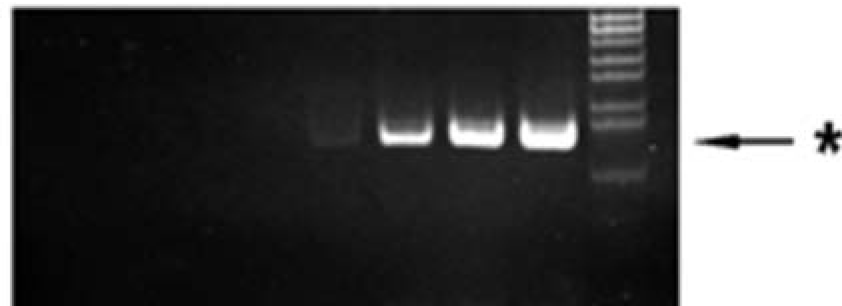
Real-Time PCR

Product formation measured
at each cycle,
during the reaction

Endpoint PCR

Product formation measured
after reaction is
complete





Adapted from Sherrill et al., JACS 2004

Real-Time PCR Chemistries

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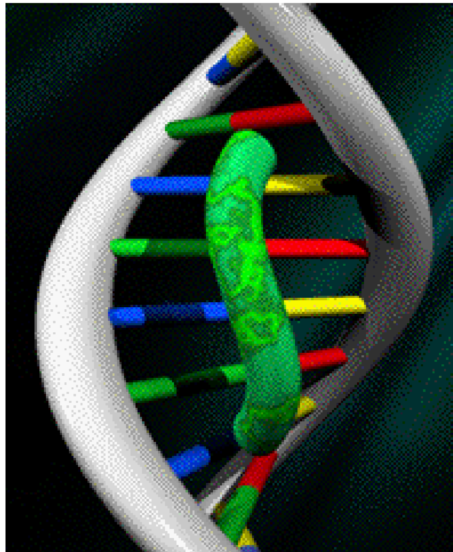
A fluorescent **Reporter** is used to detect product formation

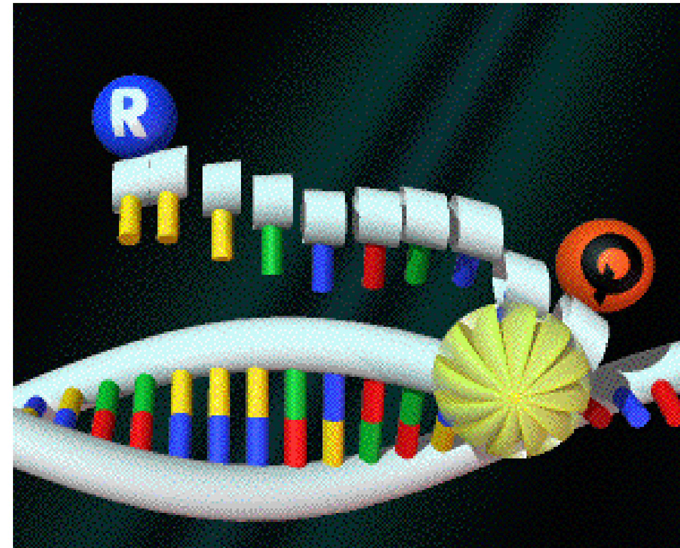
- *part of the reaction mix*
- ***two general types…***

dsDNA binding dye



or

Labeled primer or probe

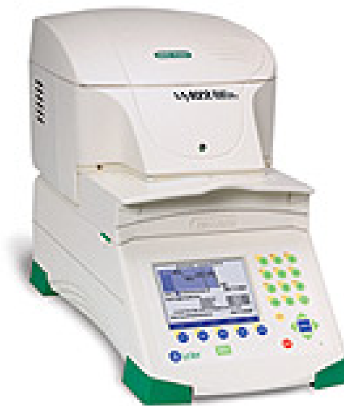


Real Time PCR Instruments

Thermal cycler + fluorescence detection module

Many manufacturers, many models, for example…

ABI 7500
Real Time System



Bio-Rad MyiQ2

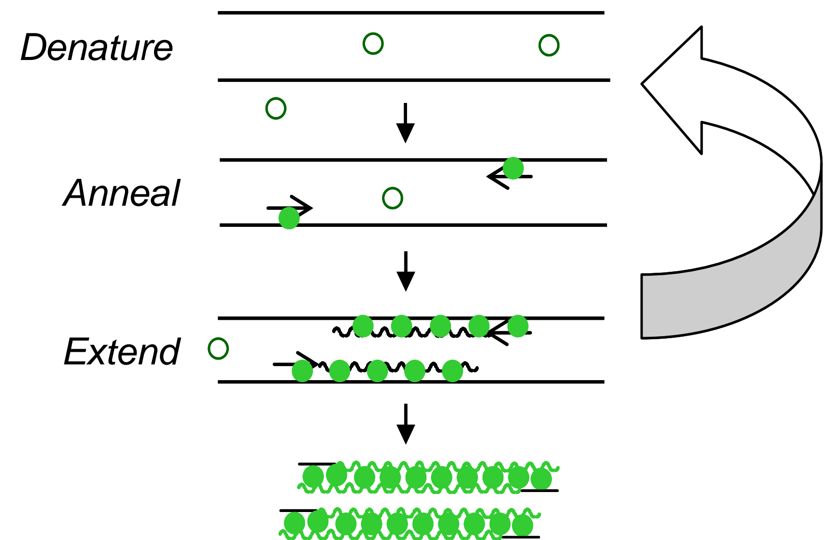
Stratagene Mx3005P®

Hardware differences *- determine reporter compatibility, multiplexing capability, cost*

Excitation source	Detection method	Fluorescence Filters
Lamp, laser, LEDs	*CCD Camera or PMT*	*Type & number*

Real-Time PCR Chemistries – Dye-based

- ***dsDNA-binding dye is included in PCR mastermix***
- *Standard primers used*
- *Dye associates with PCR product*



- ***Free Dye -> low fluorescence***
- ***Bound Dye -> high fluorescence***

As more PCR product is produced, more dye is bound

Fluorescence is proportional to the amount of product

SYBR® Green is familiar…
…improved dyes now available…

New generation of Dye-based Real-Time systems offer performance advantages

GoTaq® qPCR Master Mix

- *BRYT Green® Dye*
- *Hot-start Taq*
- *Optimized reaction buffer*

- ***BRYT Green®*** is a new dsDNA binding dye developed by Promega
- Spectra nearly identical to SYBR® Green I, detected using same filters

Delta Rn vs. Cycle

Delta Rn

12.000
10.000
8.000
6.000
4.000
2.000
0.000
-2.000

Higher Fluorescence

0.01ng
0.1ng
1ng
10ng
100ng

Earlier C_q's

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

Cycle Number

8277TB

GoTaq® Master Mix

Company A

100ng
10ng
1ng
0.1ng
0.01ng

- ***Higher signal***
- ***Greater sensitivity***
- ***Earlier C_q's***

Real-Time PCR Chemistries – Label-based

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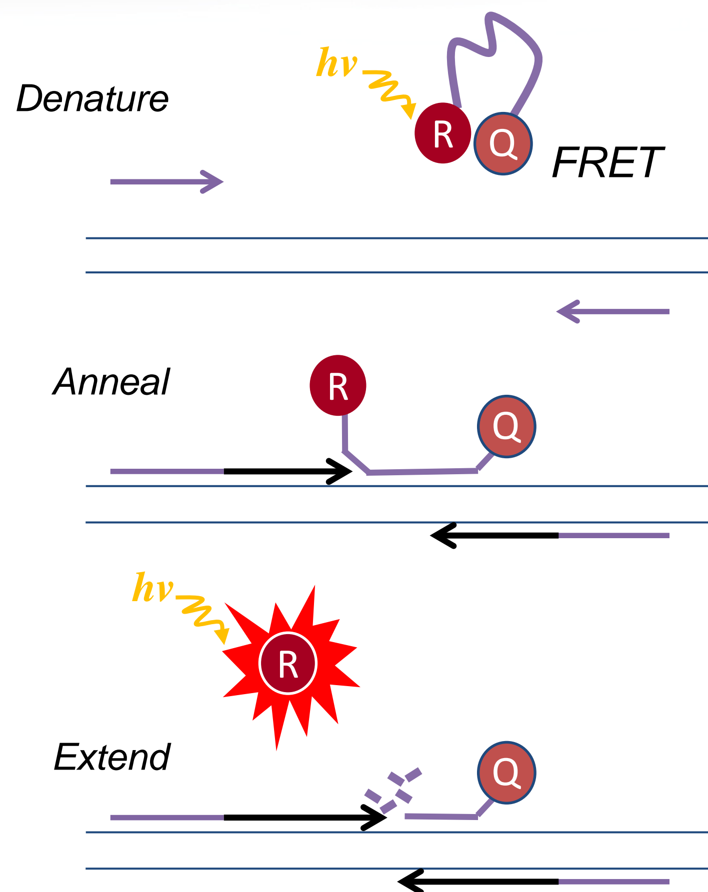
- *Primer or probe is synthesized with reporter*
- *Product formation alters fluorescence of the reporter*

TaqMan® is the most familiar type:

- *2 PCR primers + 1 probe*
- *Probe labeled with reporter & quencher*
- *Primers & probe anneal to target*
- *During extension, 5' nuclease activity of Taq degrades probe*

Free probe -> FRET occurs
Degraded probe -> reporter un-quenched

Fluorescence is proportional to the amount of product

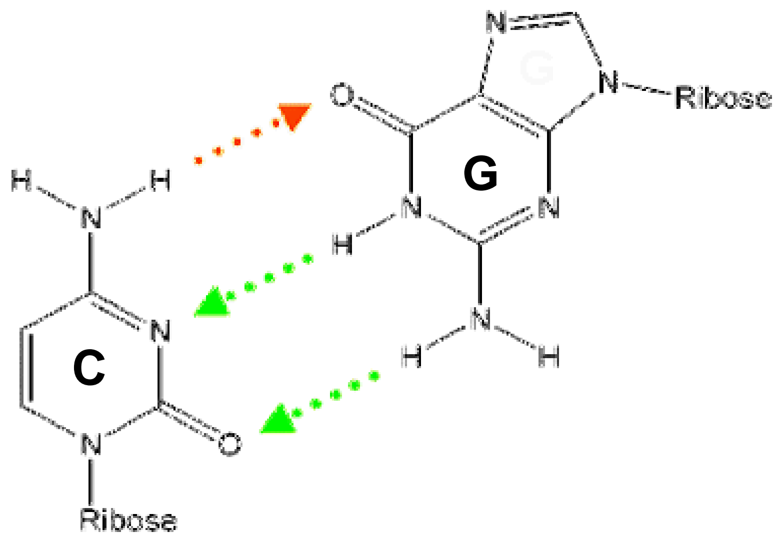


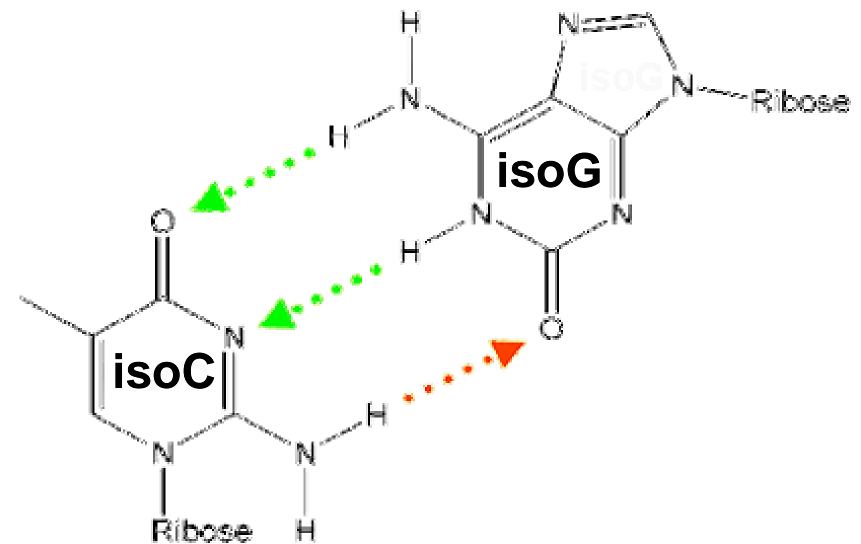
As with dye-based chemistry, other options available…

Plexor® Technology - Novel Label-based Chemistry

Iso-C & Iso-G dNTPs

- Pair only with each other - not with A·C·G·T·U
- Recognized by DNA Polymerase





Johnson, S.C., et al. (2004) Nucleic Acids Res. ***32****, 1937-41.*

Plexor® Technology - Novel Label-based Chemistry

2 Primer Method

- 1 standard primer
- 1 primer with iso-C base & reporter at 5´end

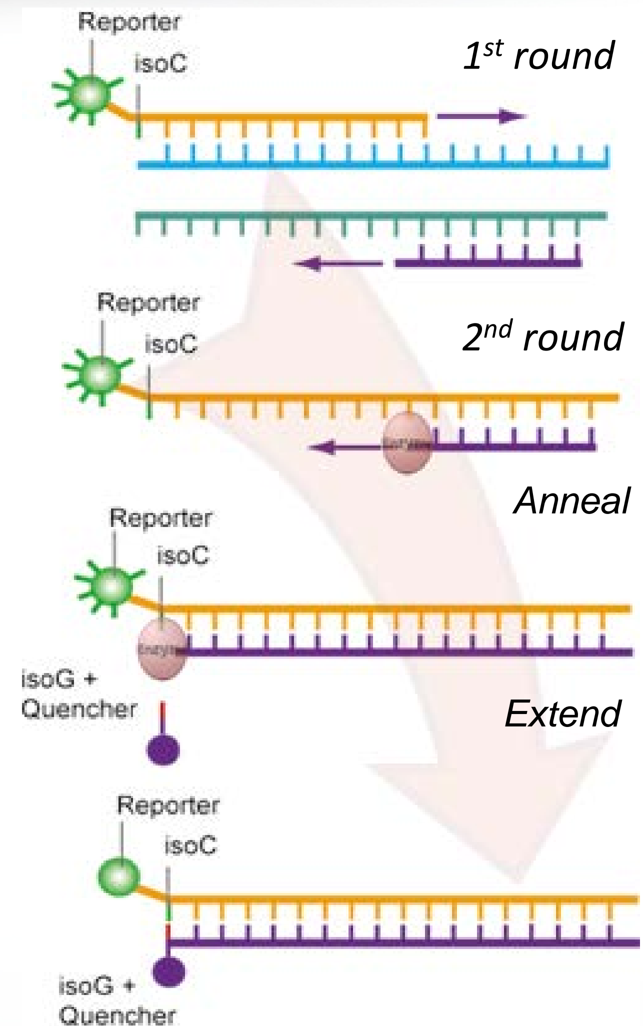
Amplification Master Mix Contains

- Standard dNTPs
- Quencher-labeled iso-dGTP

Free primer-reporter & quencher -> no FRET

Incorporation of primer & quencher -> FRET occurs

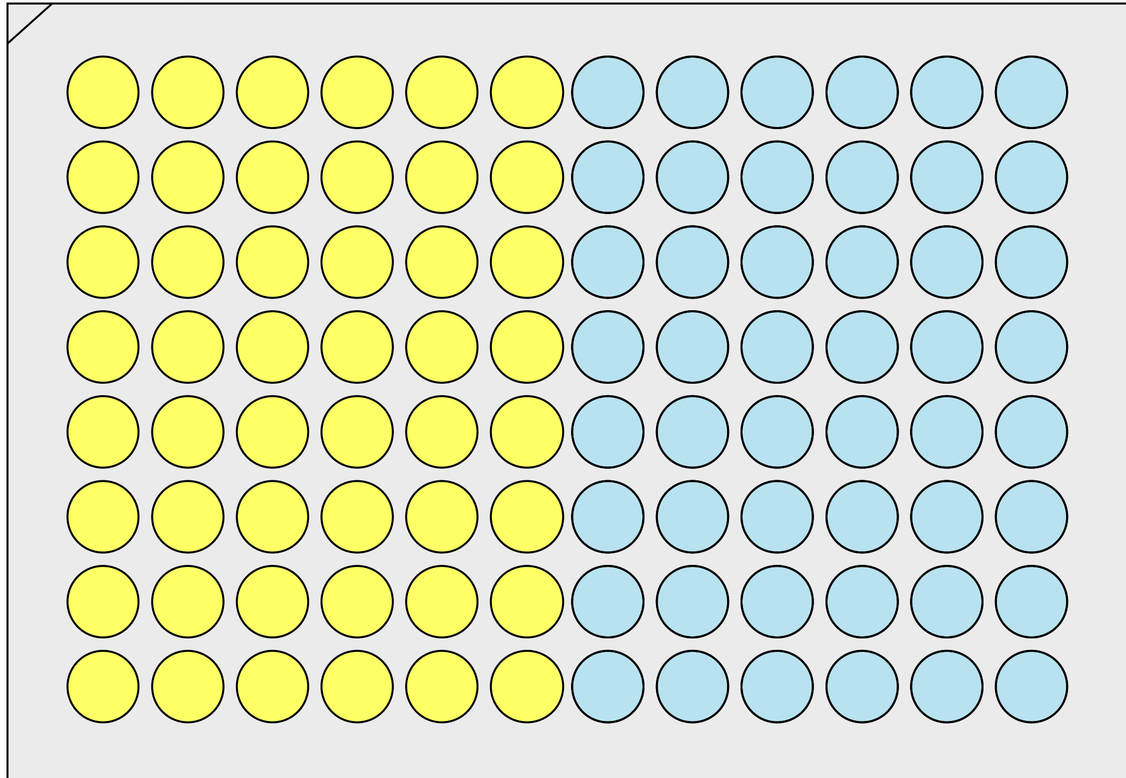
Fluorescence is <u>inversely</u> proportional to the amount of product



Sherrill, C.B., et al. (2004) J. Amer. Chem. Soc. ***126****, 4550*

Label-based methods allow multiplexing

Experimental target



Normalizer

Label-based methods allow multiplexing

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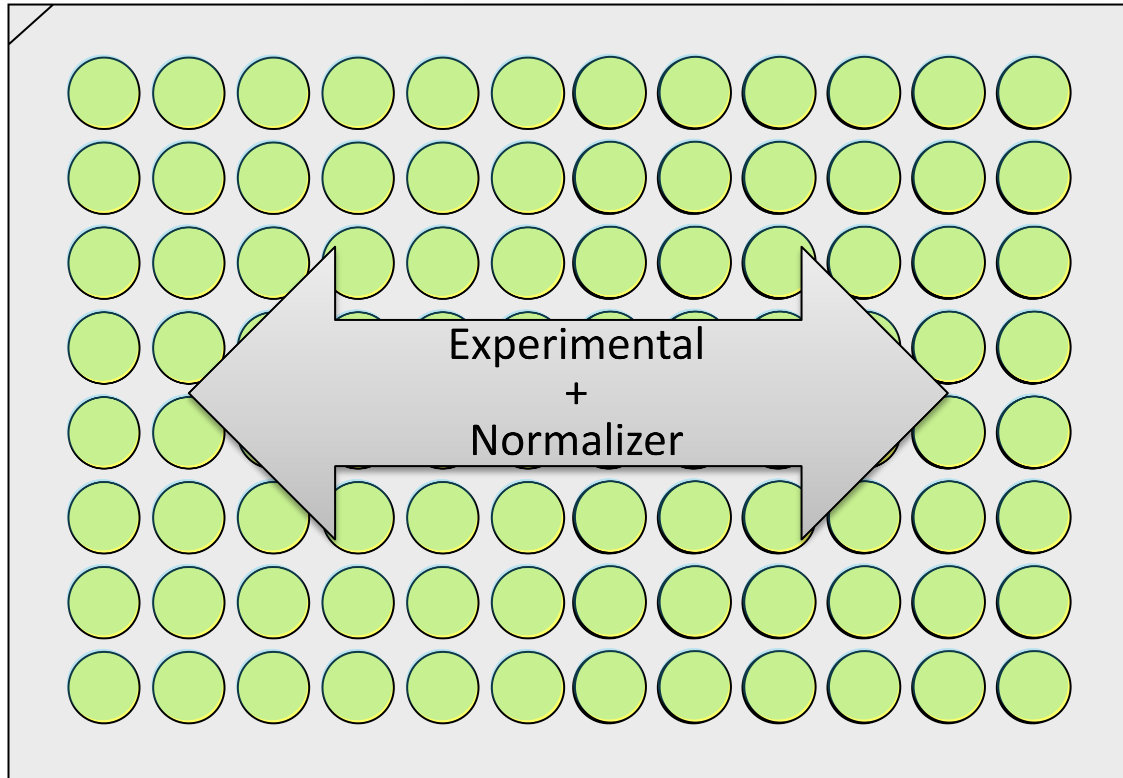

- ***Better data -*** *Targets & Normalizer in same reaction*
- ***Economical -*** *More samples analyzed per well/plate*
- ***Conserves sample***

Real-Time PCR Metrics & Analysis

Primary output is the Amplification Curve

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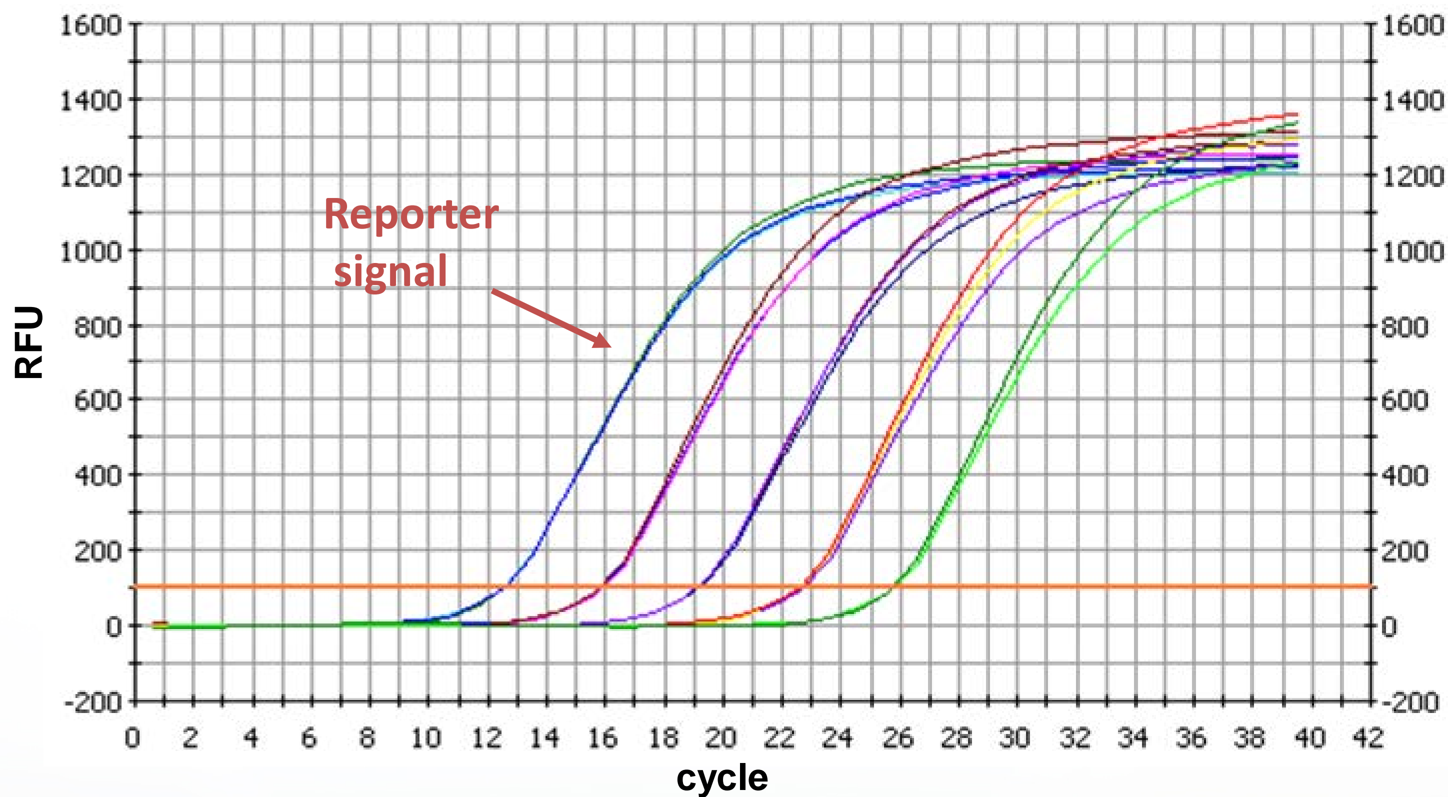
Amplification Curve – shows accumulation of product as PCR progresses

- ***reporter*** – fluorescent dye or label used to monitor PCR product formation
- ***R*** – raw fluorescence of reporter (RFU = relative fluorescence unit)



Primary output is the Amplification Curve

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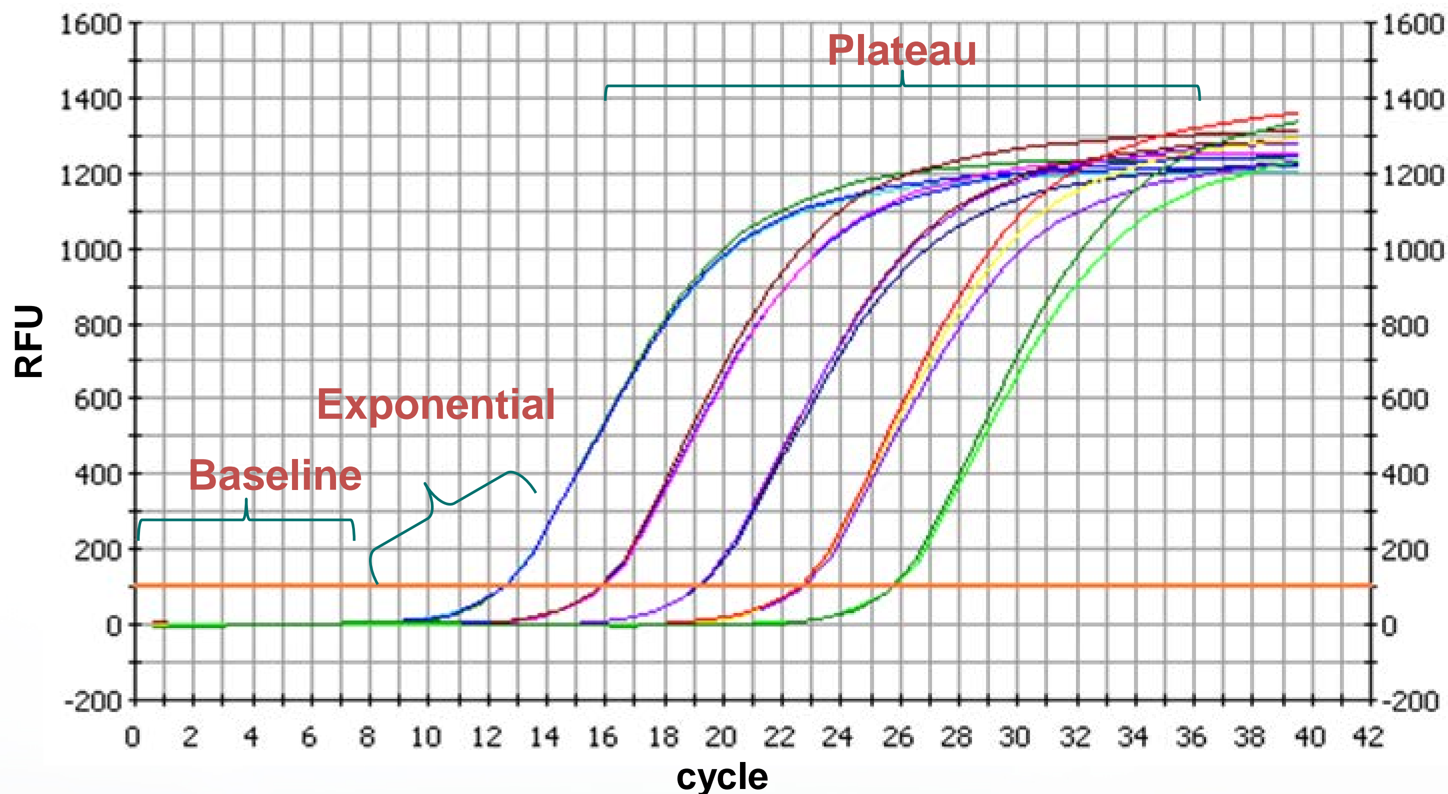
Amplification Curve – shows accumulation of product as PCR progresses

- ***baseline*** **–** initial reporter fluorescence, before significant product formation occurs
- ***exponential phase*** **–** stage of reaction when product is doubling with each cycle
- ***plateau phase*** **-** stage of reaction when rate of product formation is diminishing



Analysis of the amplification curves gives C_q value

C_q = quantification cycle – Cycle number at which amplification curve crosses amplification threshold *(aka C_t)* – this is the "take-away" metric…

C_q value is inversely proportional to amount of starting template

RFU: 1600, 1400, 1200, 1000, 800, 600, 400, 200, 0, -200

cycle: 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42

C_q

amplification threshold

Steps in the analysis of amplification curves

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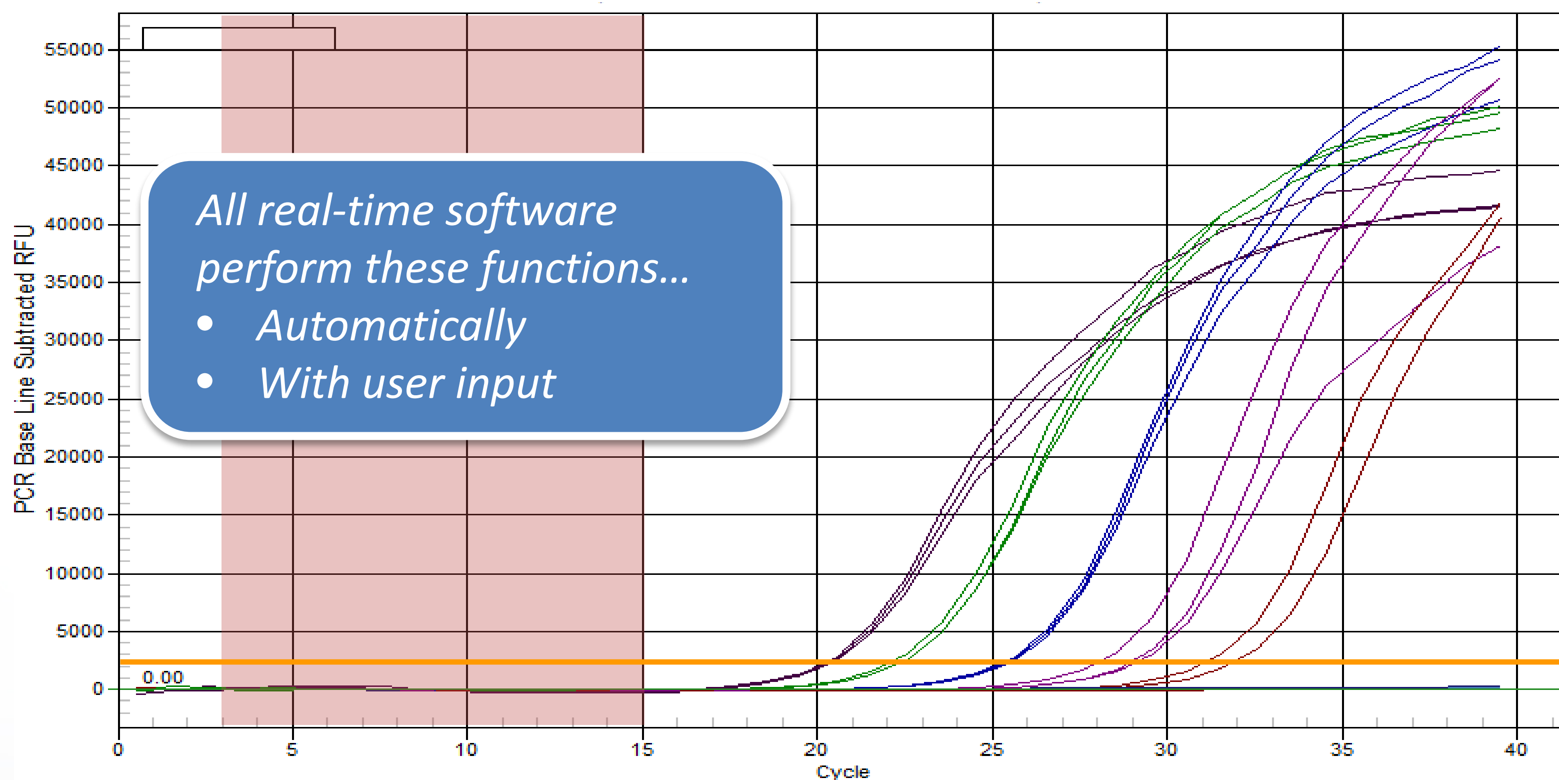
- Passive reference normalization is applied (R_n)
- Baseline regions are defined for each amplification curve
- Curves are baseline-corrected (subtracted & de-trended) (ΔR_n)
- Threshold is set (function of noise in baseline regions for all samples)



Graphs of amplifications curves can be re-scaled to reveal more detail

Standard plot of amplification curves allows you to see the baseline phase & plateau phase, but can't really tell anything about the exponential phase!

Amplification

Plateau

"Linear"

Exponential

Baseline

amplification threshold

RFU

0
1000
2000
3000
4000
5000
6000
7000

0
10
20
30
40

Cycles

Graphs of amplifications curves can be re-scaled to reveal more detail

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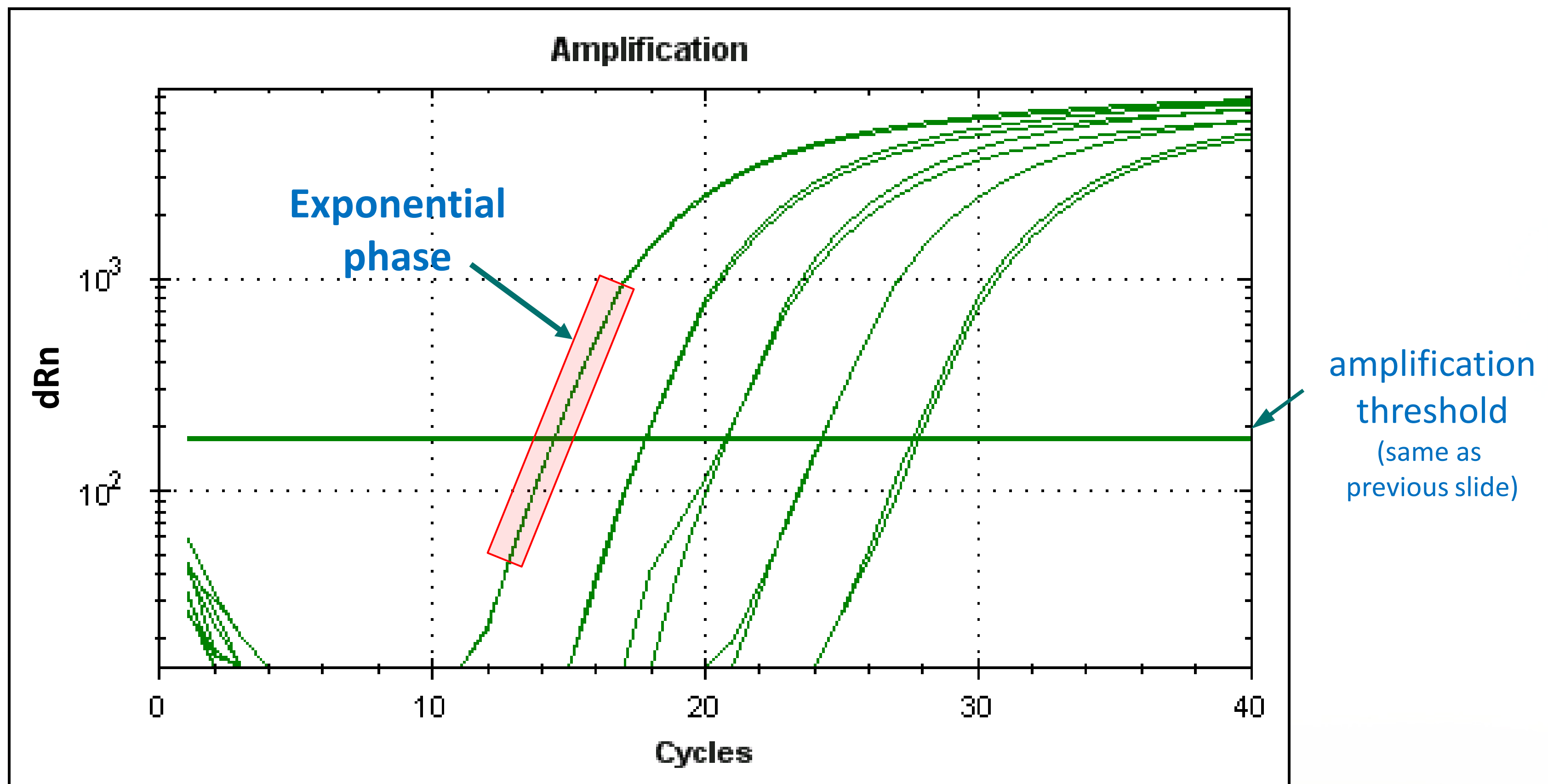
Semi-log plot of amplification curves emphasizes exponential phase…

- Allows better visualization of amplification threshold crossing (C_q)



Graphs of amplifications curves can be re-scaled to reveal more detail

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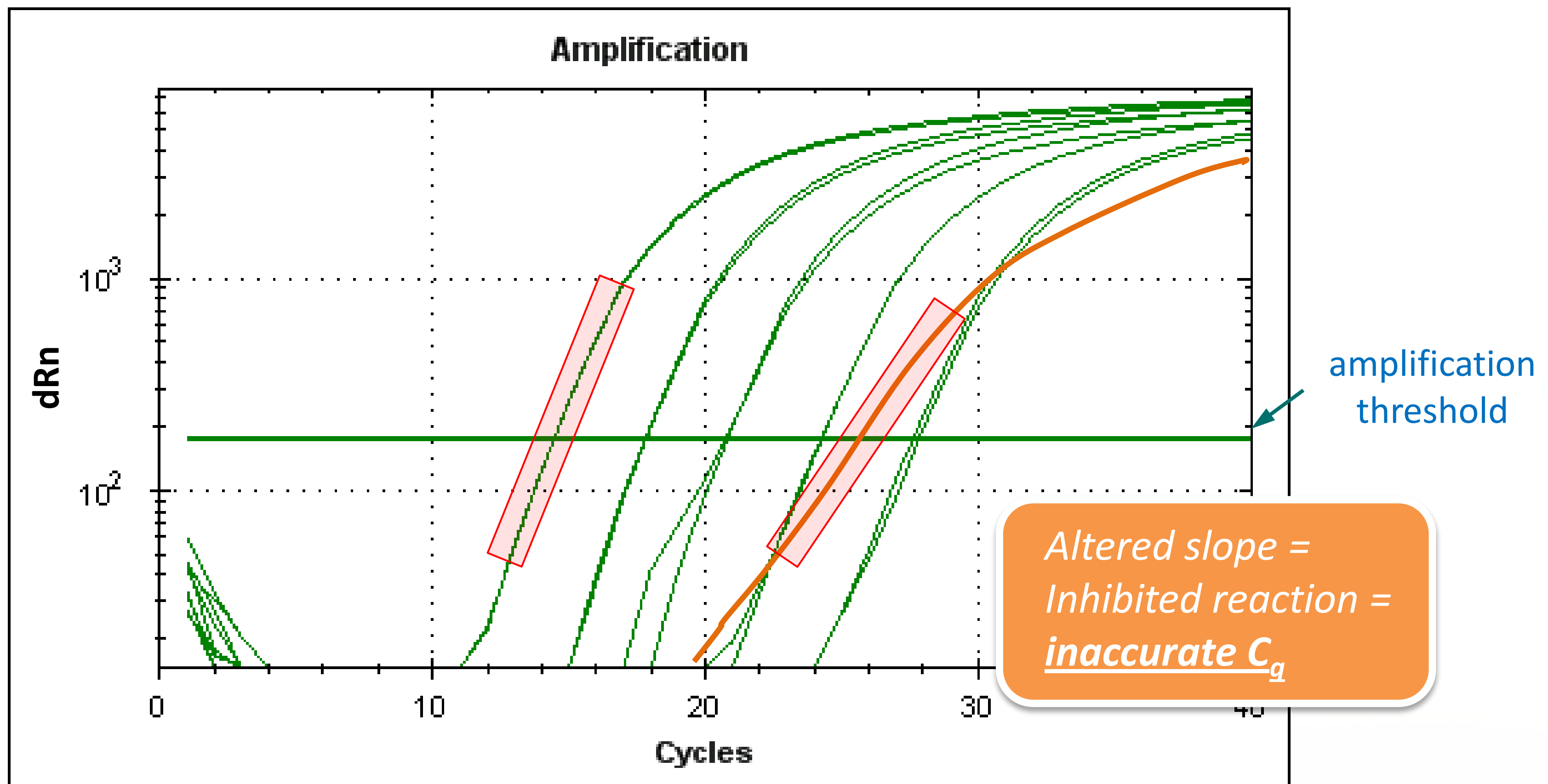
Semi-log plot of amplification curves emphasizes exponential phase…

- Provides information about efficiency of individual reactions



Experienced users utilize both views of amp curves

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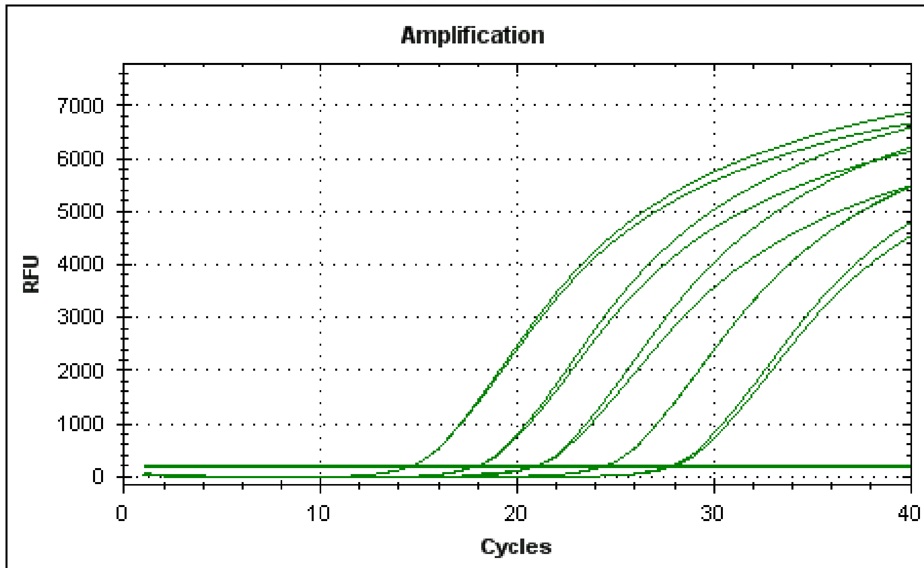
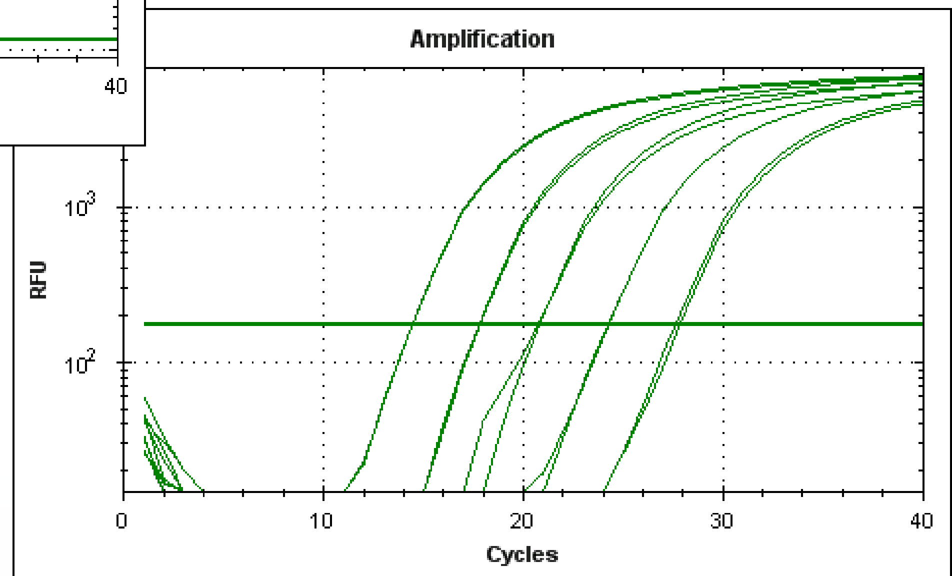


Check baseline settings in standard plot…



…check threshold setting & amplification curve quality in semi-log plot

Converting C_q values to quantity - Two approaches

Now you have C_q values – how do you use them?

Absolute (Standard Curve) Quantitation

- Use C_q values to determine amount in unknown samples based on standard curve
- Normalize the amount of target relative to…
  - internal reference (another target that is always at the same level, e.g. GAPDH, beta-actin, 18S rRNA, amplified in same or parallel reaction)

Relative Quantitation

- Compare the C_q values of target in test sample versus control samples - ΔC_t
- Can also normalize the amount of target in each sample relative to internal reference (e.g., GAPDH, beta-actin, 18S rRNA) - $\Delta\Delta C_t$

Standard Curve Quantitation

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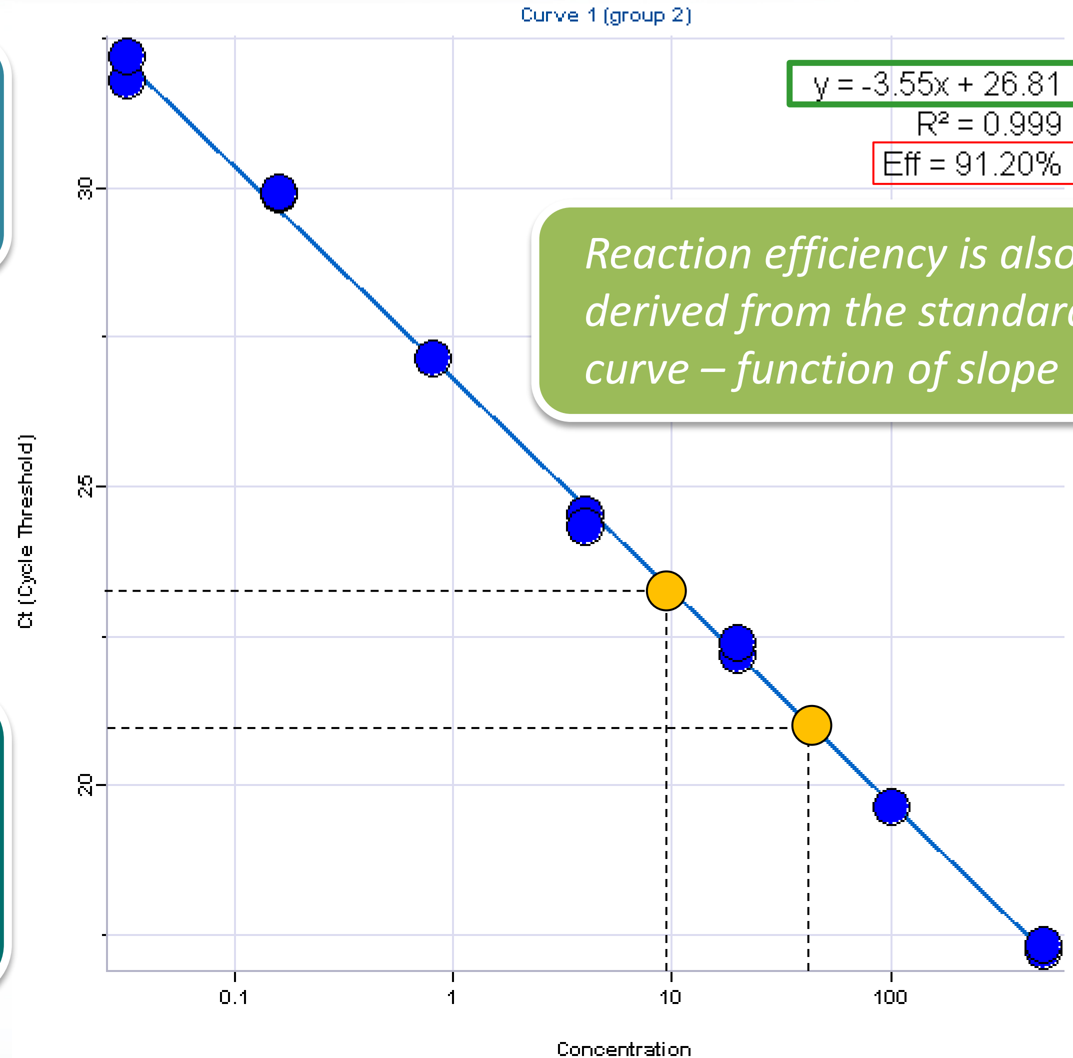
Standard curve is made by plotting C_q vs (log) concentration



Reaction efficiency is also derived from the standard curve – function of slope

Concentrations of unknowns are derived from C_q based on standard curve

Relative Quantitation

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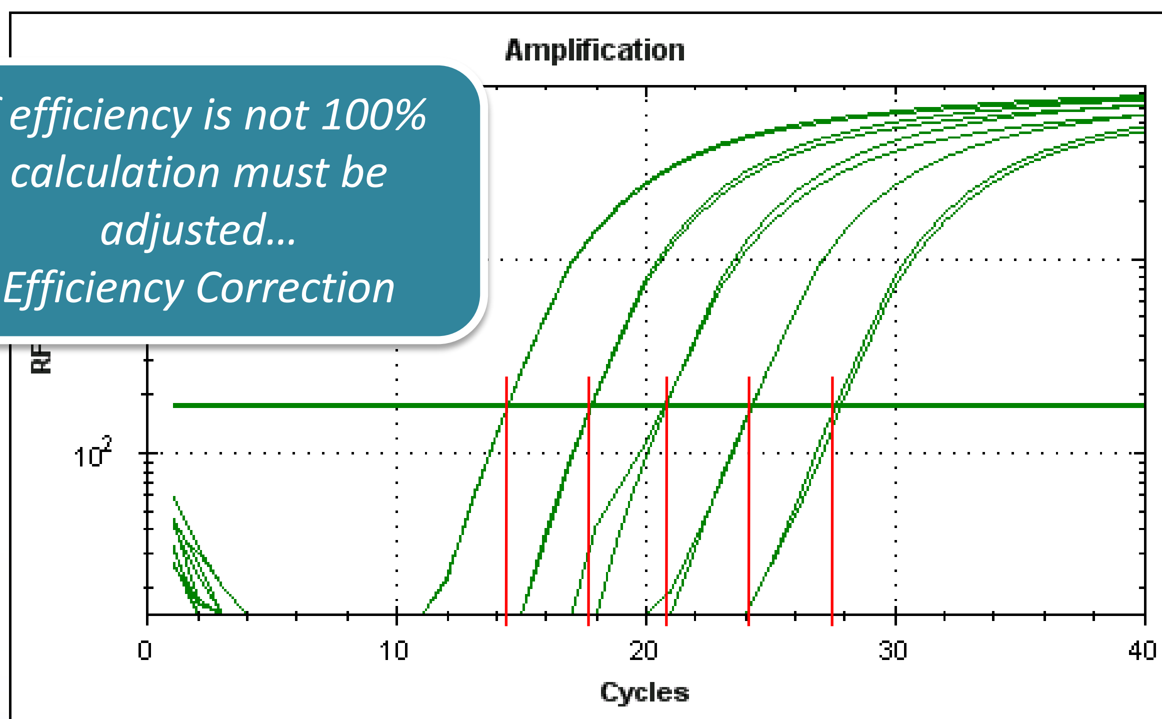
- Based on tenet that in PCR each template is replicated at each cycle…
- Therefore, product formed after *n* cycles = 2^n (assuming 100% efficiency)
- *The relationship works in reverse*… if two samples have a C_q difference of 1 (threshold is reached 1 cycle apart), then they have 2^1 = two-fold difference in starting template concentration



ΔC_q (n cycles)	Difference in concentration (2^n)
3.3	10
5	32
10	1024
15	~32k
20	~1M

Real Time PCR Output may include Melt Data

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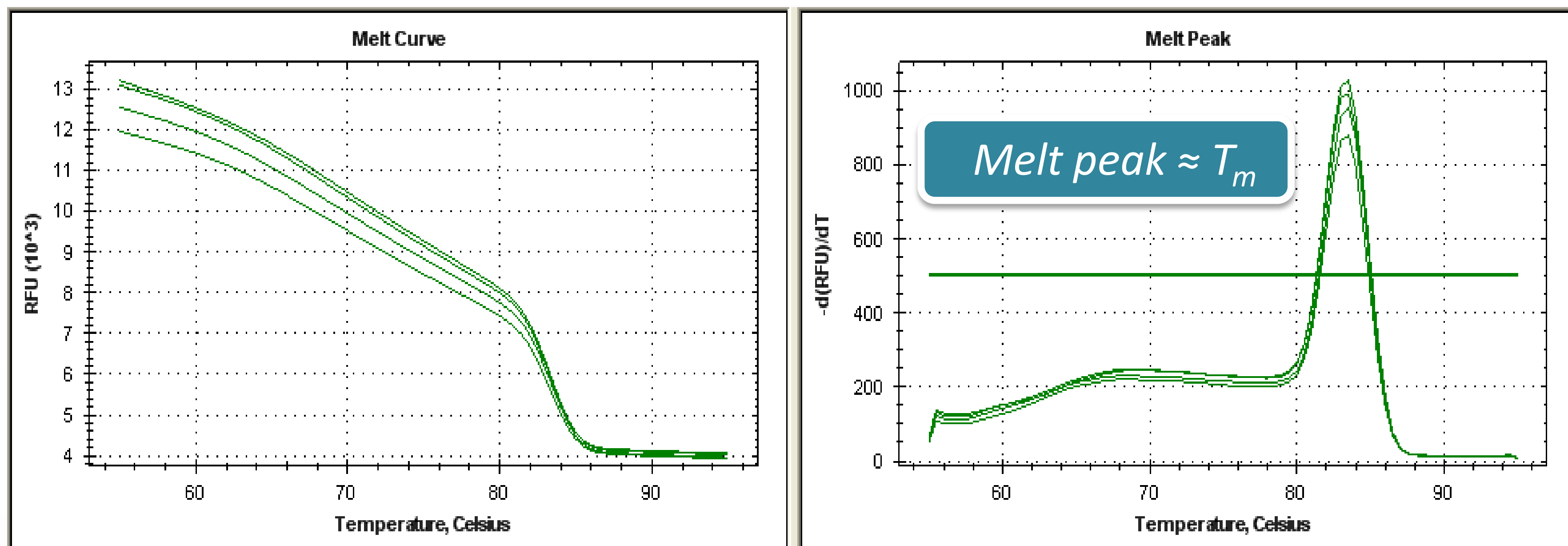
- Produced in a second, linked thermal profile performed after amplification
- Product is heated slowly, signal is continually measured
- As dsDNA amplicon denatures, signal changes

Provides qualitative information about PCR products – primarily, number & size

Graphed as **Melt Curve** (RFU vs T, *left panel*) or **Melt Peak** (dRFU/dT vs T, *right panel*)



Real Time PCR Melt Data as qPCR Control

What to look for in melt data…

- Melt peak for **samples & positive controls** should be at **similar Temp (T_m)**

Likely primer dimer

dF/dT

10

5

0

70

75

80

85

90

ºC

Real Time PCR Melt Data as qPCR Control

What to look for in melt data…

- Melt peak for **samples & positive controls** should be at **similar Temp (T_m)**
- **No secondary peak or shoulder -** indicates secondary (non-specific) product formation

dF/dT

10
5
0

70
75
80
85
90

°C

Real Time PCR Melt Data as qPCR Control

What to look for in melt data…

- Melt peak for **samples & positive controls** should be at **similar Temp (T_m)**
- **No secondary peak or shoulder -** indicates secondary (non-specific) product formation
- *Melt peaks height of samples may not be the same – that's not necessarily bad*

dF/dT
10
5
0
70
75
80
85
90
ºC

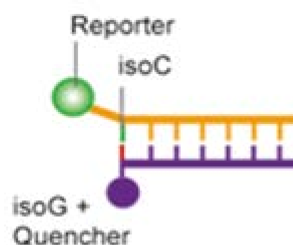
Norm. Fluoro.
0.4
0.3
0.2
0.1
0.0
Threshold
5
10
15
20
25
30
35
Cycle

late amplification,
lowered delta RFU

Real Time PCR Output – Melt Data

Promega

Some Real-Time PCR Chemistries can produce melt data…

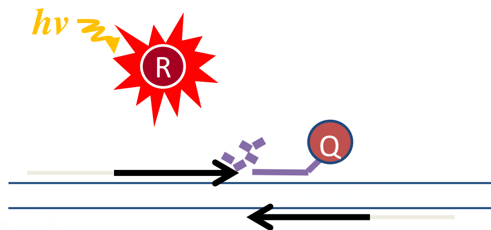


dsDNA dye & Plexor®

fluorescence signal modulation is reversible

Some Real-Time PCR Chemistries can't …

In this case, gel analysis is crucial during validation



TaqMan® assay

because signal generation is irreversible…

melt analysis is *NOT* possible

RT-qPCR Assay Design

Which reverse transcription (RT) strategy?

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- **<u>1-step RT-qPCR</u>**: RT & qPCR in one tube, one reaction set-up.
  - *Gene-Specific Primers (GSP) used for both RT & qPCR*
    - *Reverse primer is the RT primer*
  - *1 aliquot of RNA sample is consumed for 1 qPCR reaction*
    - *With dye-based chemistry this is necessarily a monoplex*
    - *With label-based chemistry can multiplex >1 target*
  - *May be the most sensitive approach*

- **<u>2-step RT-qPCR</u>**: RT reaction done separately from qPCR
  - *Oligo-(dT) &/or random primers used for RT (GSP used for qPCR)*
    - *Either primes all transcripts in an unbiased way (theoretically)*
    - *Random primers will prime all RNA; Oligo-(dT) only poly-(A) RNA (mRNA)*
  - *1 aliquot of RNA can be used for multiple qPCR reactions,*
    - *to quantify multiple targets; or for technical replicates*

RT-qPCR primer design considerations

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- Target sequence is from the correct organism
- RefSeq is used (or validated mRNA)

NCBI mRNA accession pre-fixes
NM_*, XM_* = reference mRNA

- Paralogs, or conserved motifs in other genes
- Species or strain variation (SNPs or INDELs)
- Amplicon (or primer) should span Exon:Exon junction
- Alternate transcripts
- RT primer position

  if Oligo-d(T) used, 3'-target may be more sensitive
- Size of amplicon

  75-125 bp is typical range

RT-qPCR Primer Design Resources

Pre-designed qPCR Primers:

Primer Bank - http://pga.mgh.harvard.edu/primerbank/

RTPrimerDB - http://www.rtprimerdb.org/

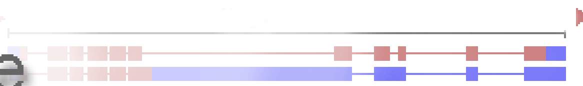
Primer design software:

Primer3 - http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

Primer-BLAST - http://www.ncbi.nlm.nih.gov/tools/primer-blast/

IDT PrimerQuest - http://www.idtdna.com/Scitools/Applications/Primerquest/

Sequence Resources



- Gene Name & Aliases
- Reference Sequence
- Gene organization
- Evidence Viewer – cDNA alignments

Validation of RT-qPCR Primers is Essential

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- *You have a new primer design – what next?*

BLAST® - *in silico check to see that they are specific!*

If there are matches to unintended genes, evaluate:

- *Match at 3' end?*
- *Percent identity*
- *Predicted T_m of interaction*

- *The in silico analysis looks good, & you order them – now what?*

Experimental Validation! - *Test them on a dilution series of positive control sample…*

- *Determine efficiency*
- *Determine linear dynamic range*

***This is essential …
even if primers are from a bank or previously published!***

MIQE is a valuable reference

***M**inimum **I**nformation for Publication of **Q**uantitative Real-Time PCR **E**xperiments*

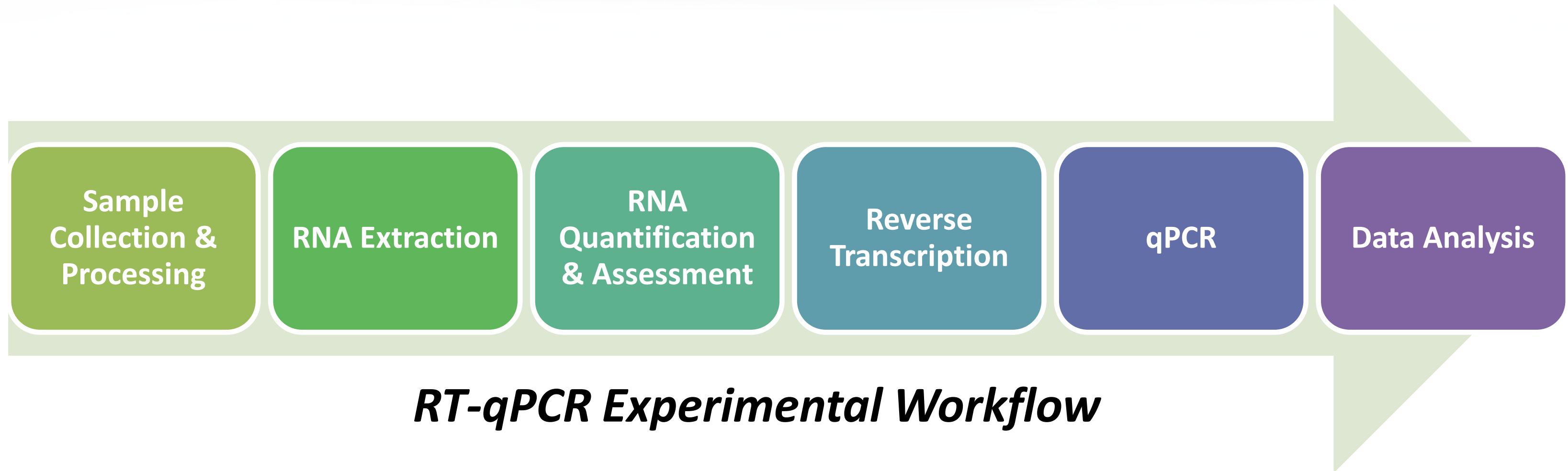
Stephen Bustin et al. (2009) Clinical Chemistry, 55:4

- Recommendations & rational for:
  - *qPCR experimental design, validation, execution, controls, analysis, & presentation.*
  - *Sample handling, nucleic acid extraction, & characterization (quantification & integrity)*
  - *Real-Time PCR terms & nomenclature.*

MIQE
http://www.rdml.org/miqe.php
- *pdf of paper*
- *Checklist*

Tips & Troubleshooting

RT-qPCR Tips & Troubleshooting



RT-qPCR Experimental Workflow

- Success depends on proper tools & technique *at each step*
- Problems in any step of the experiment can cause experimental failure, or worse, *inaccurate* results

Proper handling of samples is the first step to ensure good RNA yield & quality

Degradation & loss of RNA often occurs during sample collection & processing

- Temperature abuse of samples before/during /after collection
  - *Process or store immediately*
  - *Snap freeze on liquid N or dry ice*
- Dissection takes too long
  - *If dissection is difficult, do gross dissection first (quickly)… then fine dissection in a preservative, or after preservation (e.g., RNAlater®)*
- Sample dimensions too large – takes too long to freeze & thaw
  - *Cut into smaller chunks during dissection, before further processing*
- Insufficient tissue disruption
  - *Rotor-stator is generally the best approach*
  - *Dounce homogenizer or blue pestle - may need to chop/mince first*

Consider all parameters when choosing – and using - RNA extraction method

Yield – often the primary consideration

- *Most methods give similar yield - organic extraction often perceived as best…but often at a cost*
- *Exceeding processing recommendations usually does not increase yield (& may compromise purity)*

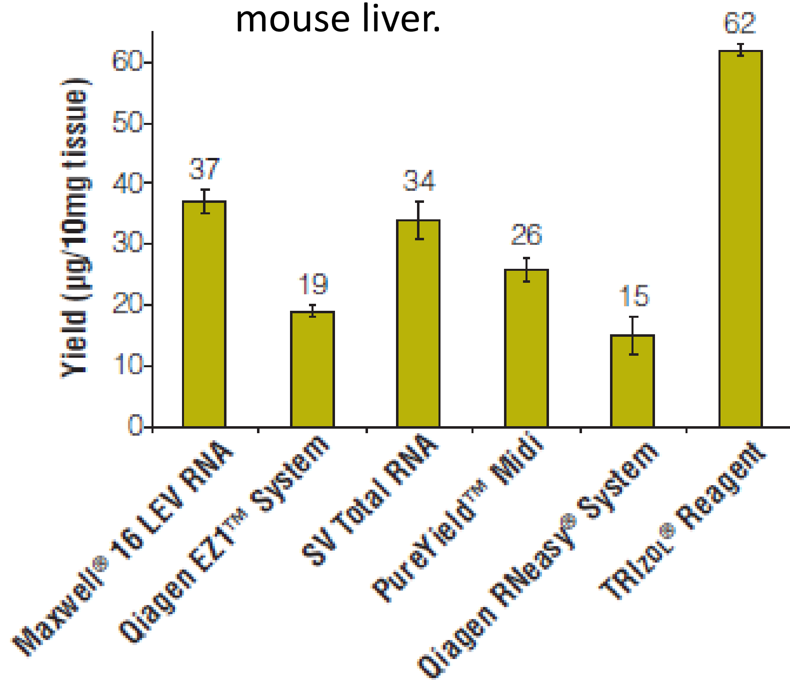
gDNA removal – high amounts can cause quantitation error; even low amounts can cause problems in qPCR

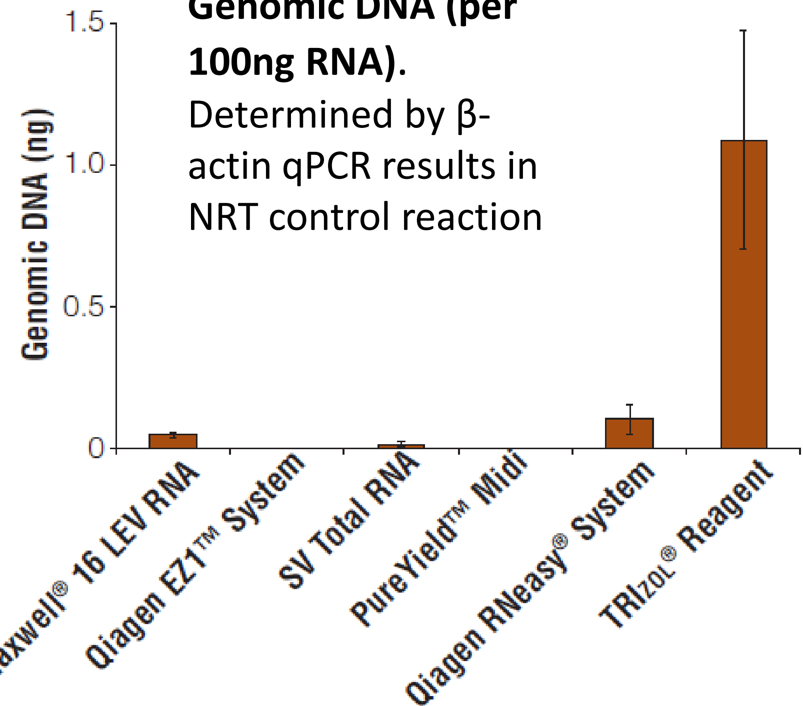
- *gDNA contamination is often an issue with organic separation methods*
- *DNase can be added after any method*
- *Efficient removal is important, but impossible to remove 100% (& may not be necessary)*

Inhibitor carryover – may lead to variation in Cq's rather than reaction failure.

- *Can be an issue with organic separation methods*
- *Exceeding processing recommendations can compromise purity*

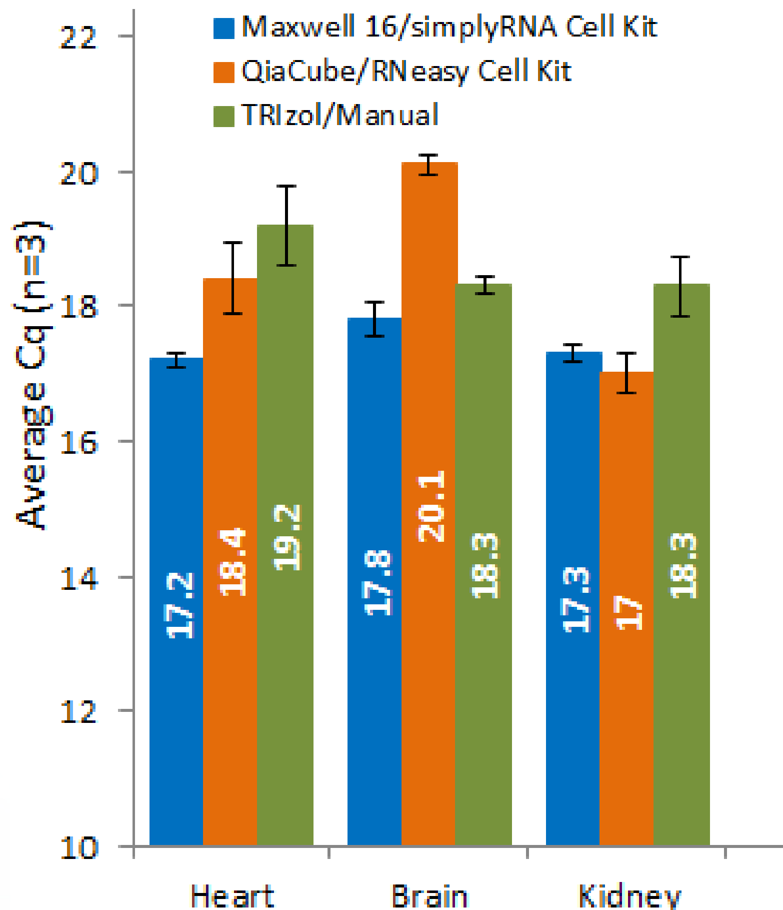
Increased yield at the cost of purity is a poor trade





Schagat et al., 2008 Promega Notes 99 "RNA Purification Kit Comparison: Yield, Quality and Real-Time RT-PCR Performance"

Increased yield at the cost of purity is a poor trade



Lower purity may result in higher, more variable C_qs

What is advantage in 2- fold difference in yield In an assay with 10^6-fold range?

Beware the blind pursuit of maximum yield!

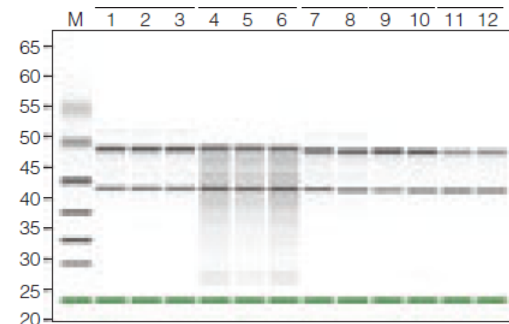
There are many methods to evaluate RNA

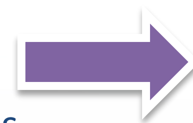
Quantification - Ideally, RNA input amount should be similar in each RT reaction

- Direct absorbance – A260; abs ratios can provide information about purity
- Fluorescent dye – greater sensitivity & dynamic range

Quality assessment –

- Gel or Bioanalyzer -
  - *Crucial to assess integrity*
- Absorbance ratios
- Spike experiment
  - *For inhibitors*
- No RT qPCR control (NRT)
  - *For gDNA contamination*
  - *Not as critical if primers span introns*





ΔC_q	Difference in concentration
3.3	10
5	32
10	1024

RT reaction considerations

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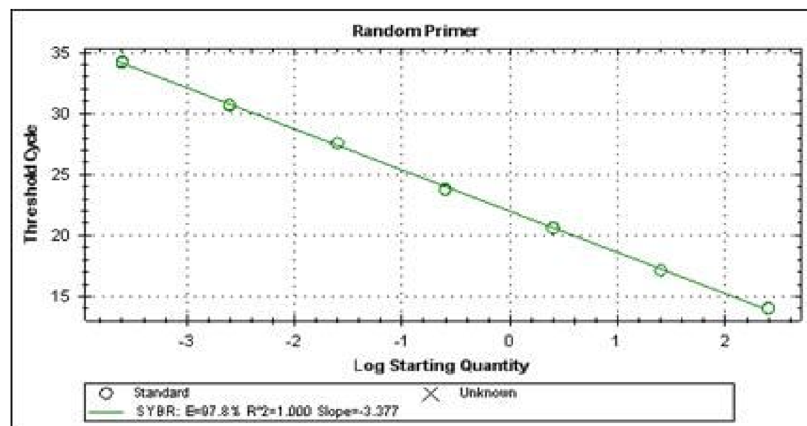
RNA amount – current RT systems have very good range & proportional yield; however, it is still desirable to have similar amounts of total RNA within each rxn

Oligo-(dT) or random primer amount - need not necessarily be adjusted relative to mRNA input amount…

- *Total Human RNA*
- *Diluted 1ug/ul to 1pg/ul - 1ul per rxn*
- *500ng random primers per rxn*
- *GAPDH primers*

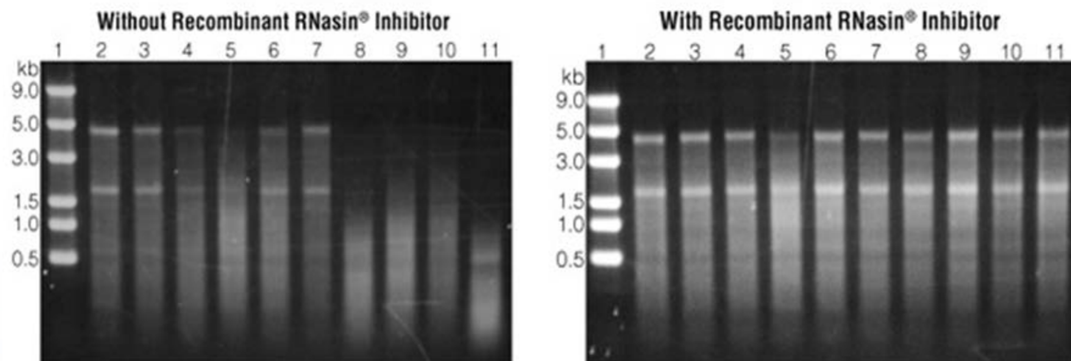
GoScript® 2-Step RT-qPCR System



Avoid RNase contamination post-purification

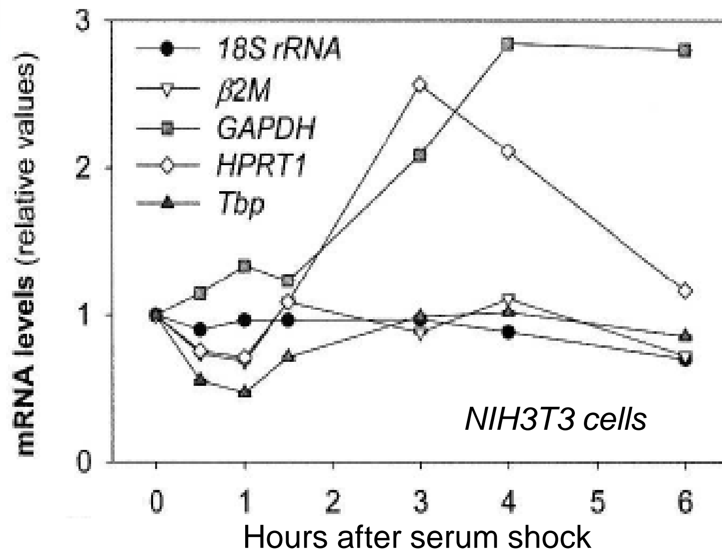
- *Follow best practices, e.g., gloves, barrier tips, etc*
- *Use **RNase inhibitor** in RT reaction*

RNasin® *Is included in GoScript® & GoTaq® Systems*



Stability of normalization target must be verified

Promega

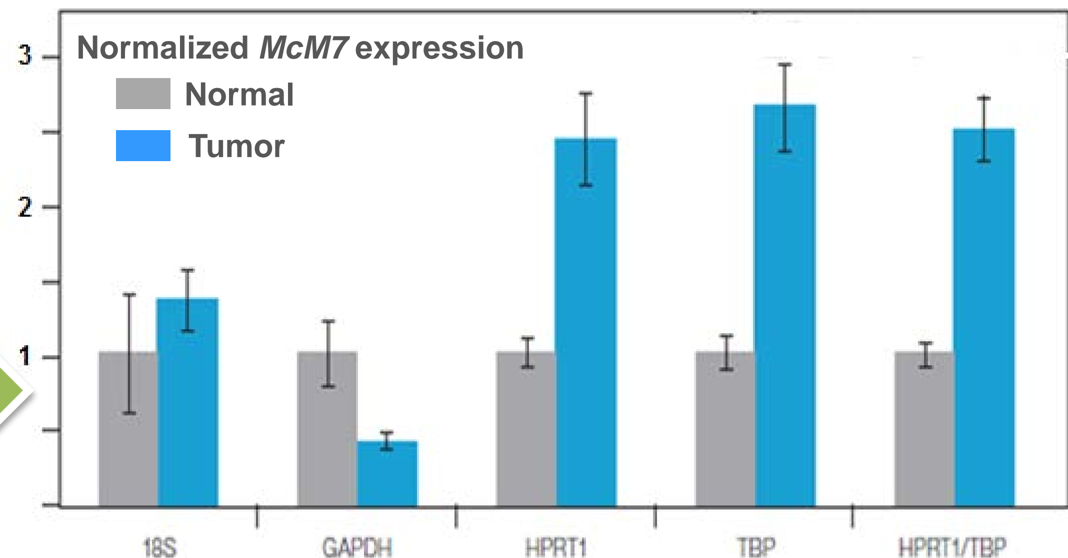


Garabino-Pico, E. et al. (2007) RNA

Commonly used normalizers care not always constitutive

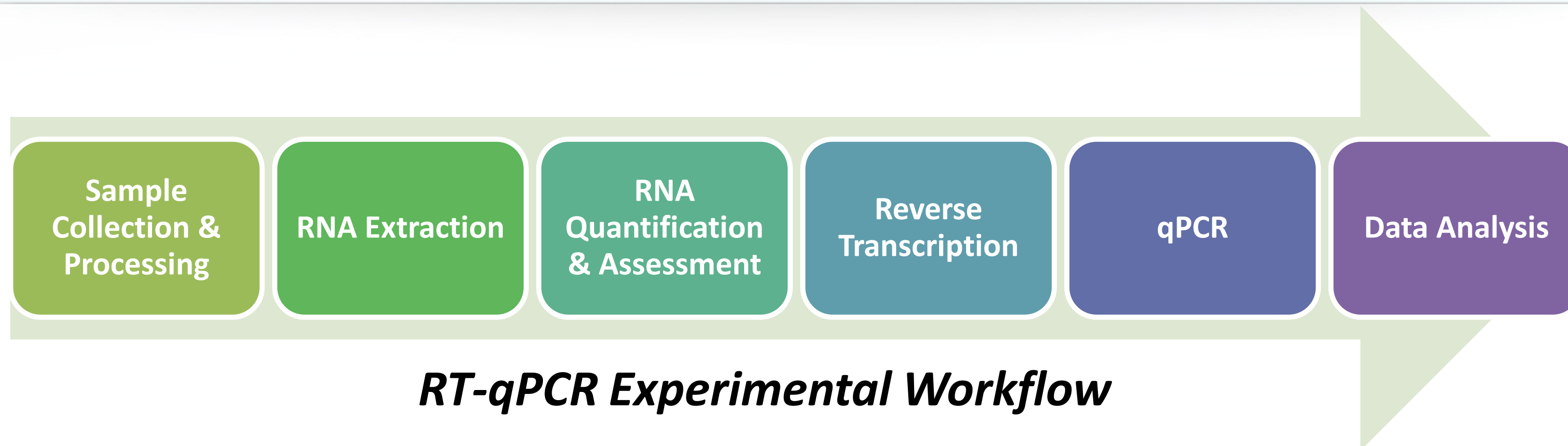
- ***MIQE suggests two normalizers***
- ***Software exist to evaluate - geNORM***

Using the wrong normalizer can qualitatively change the results



Adapted from Taylor, S. (2011) Bio-Rad tech note 6245

Promega Products for RT-qPCR



RNA Extraction

- SV Total RNA Isolation System
- Maxwell® 16 Instrument
- Maxwell® 16 simplyRNA Purification Kits

RNA Quantification

- QuantiFluor™ RNA System
- GloMax®-Multi+ Detection System

Reverse Transcription & qPCR

- GoScript™ Reverse Transcriptase
- GoTaq® qPCR Master Mix
- GoTaq® 1-Step & 2-Step RT-qPCR Systems
- Recombinant RNasin® Ribonuclease Inhibitor
- Plexor® qPCR & RT-qPCR Systems

Questions? ***Ask a Scientist!***

[***http://www.promega.com/support/***](http://www.promega.com/support/)

Good luck with your experiments!