Dealing with Amplification Inhibitors: Reagent Choice Matters

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Promega Corporation

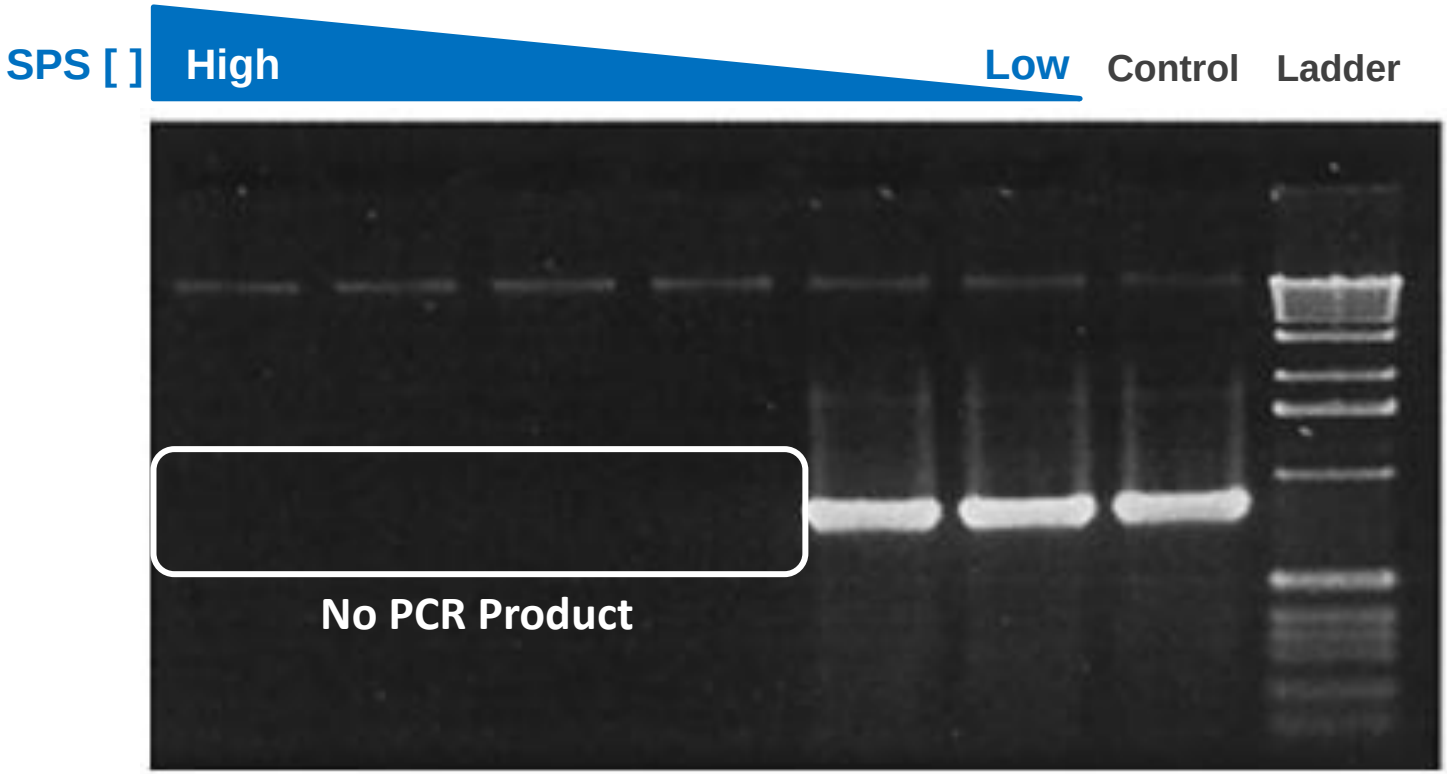
Outline
Helping You Deal with Amplification Inhibitors

- A couple of examples
- Real time PCR (qPCR) refresher
- Overview of PCR and reverse transcriptase inhibitors
    - Sources
    - Modes of action
    - Practical impacts
    - Common examples
- Strategies for dealing with inhibitors
- Choice of reagents for downstream applications

Inhibitors May Impact Any PCR or RT-PCR Assay

Example: Sodium Polyanetholesulfonate (SPS)

End-point PCR



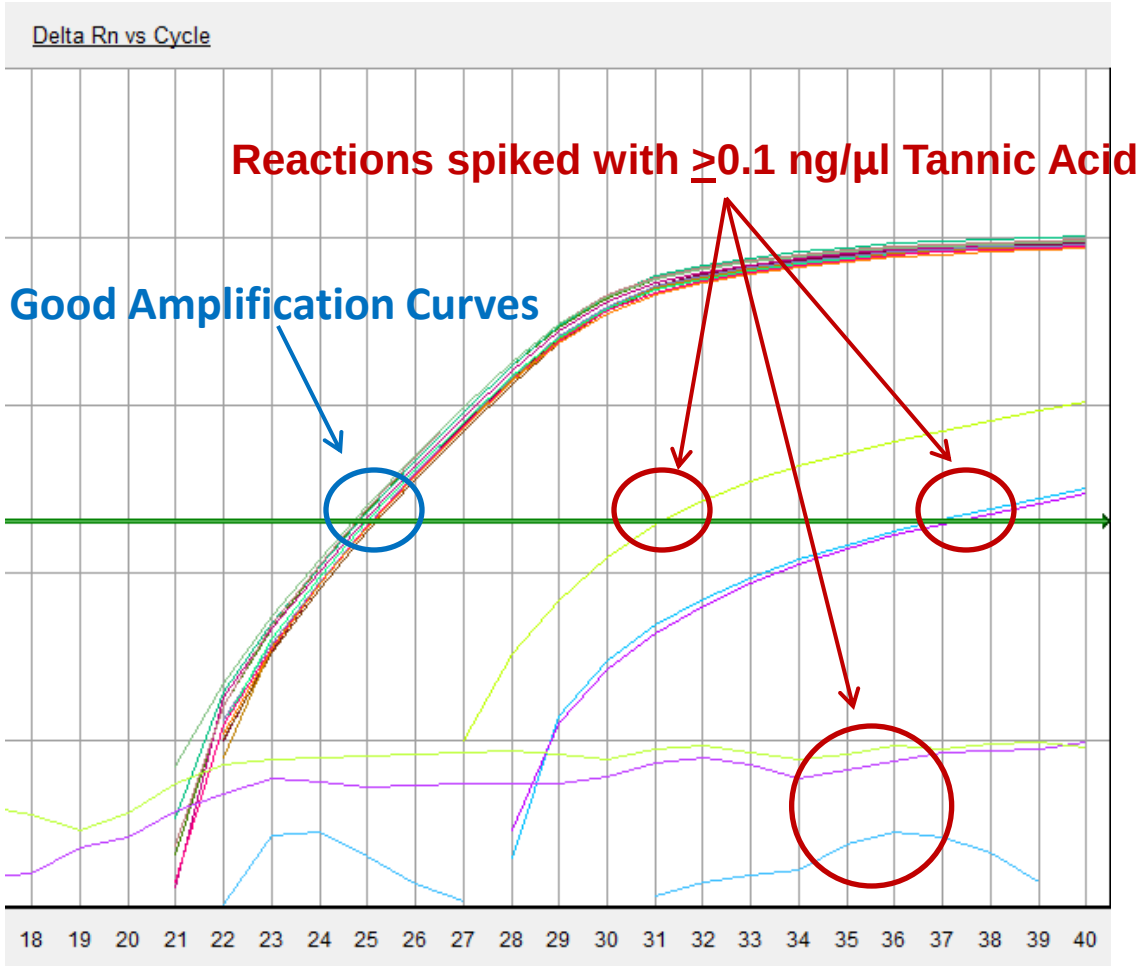
Example: amplification of bacterial 16s rRNA gene inhibited by SPS

Inhibitors May Impact Any PCR or RT-PCR Assay
Examples of Inhibition

Real-Time qPCR

Amplification of human GAPDH gene inhibited by Tannic Acid

"Good Curves" = no inhibitor or Tannic Acid ≤ 0.01 ng/uL



Terminology

For this talk:

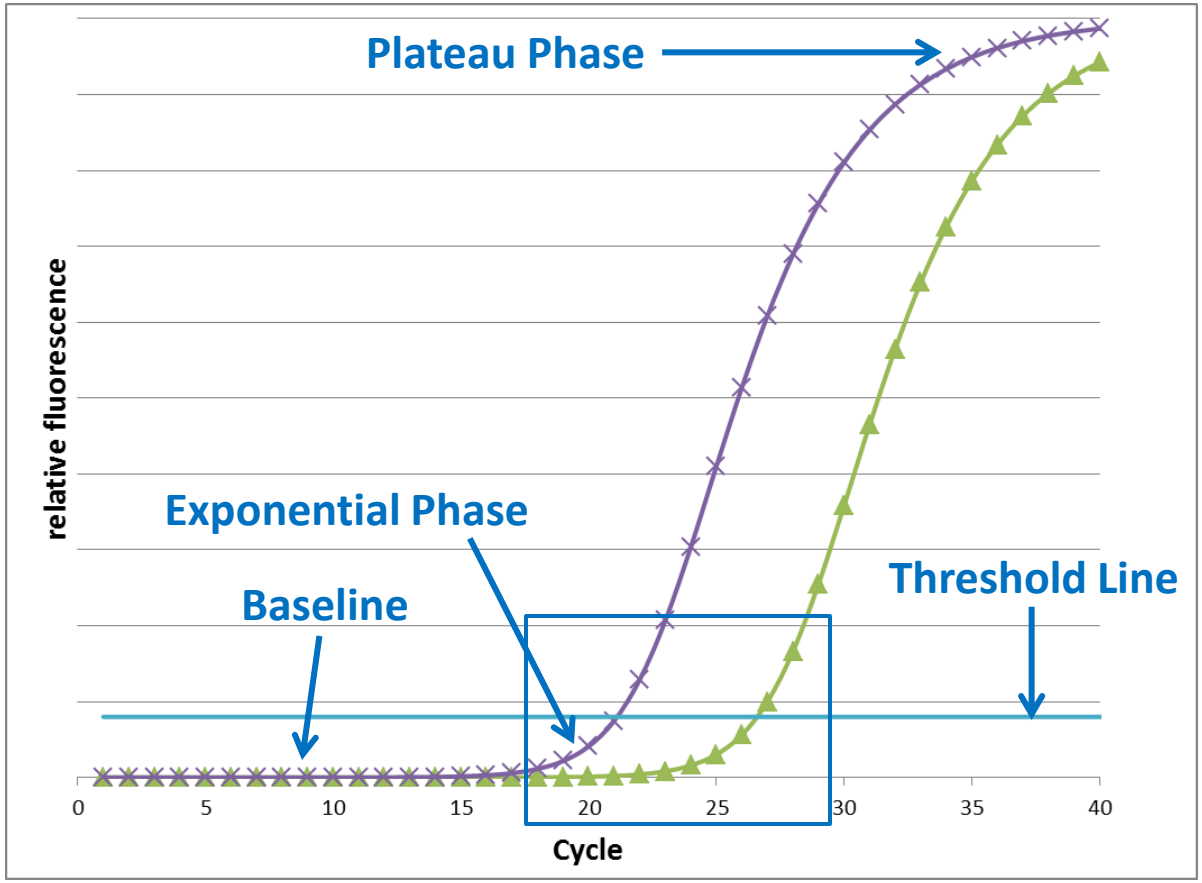
RT-PCR = “Reverse Transcription-PCR”

qPCR = “Real-Time PCR”

RT-qPCR = “Reverse Transcription Real-Time PCR”

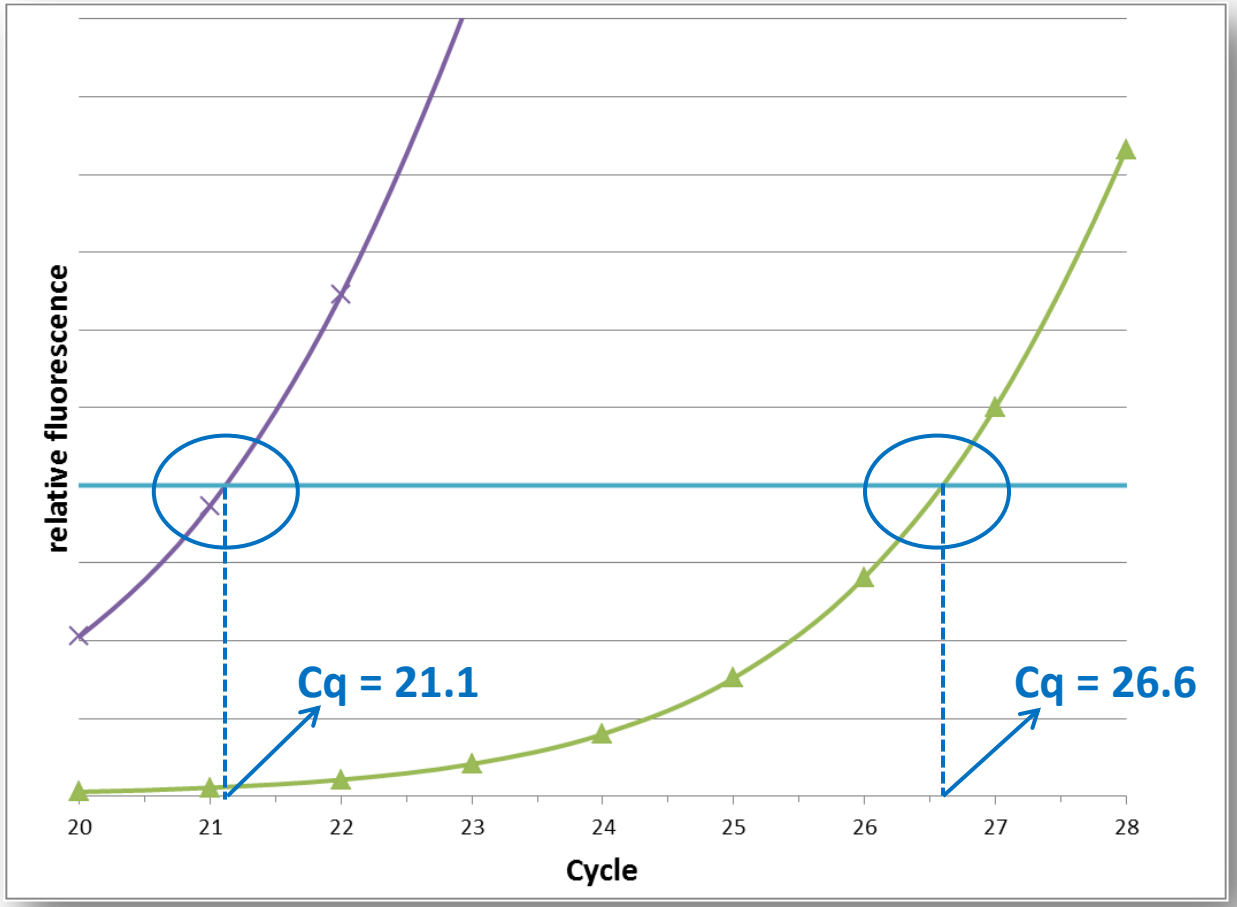
qPCR Refresher: Amplification Curves

There are Three Phases of Amplification



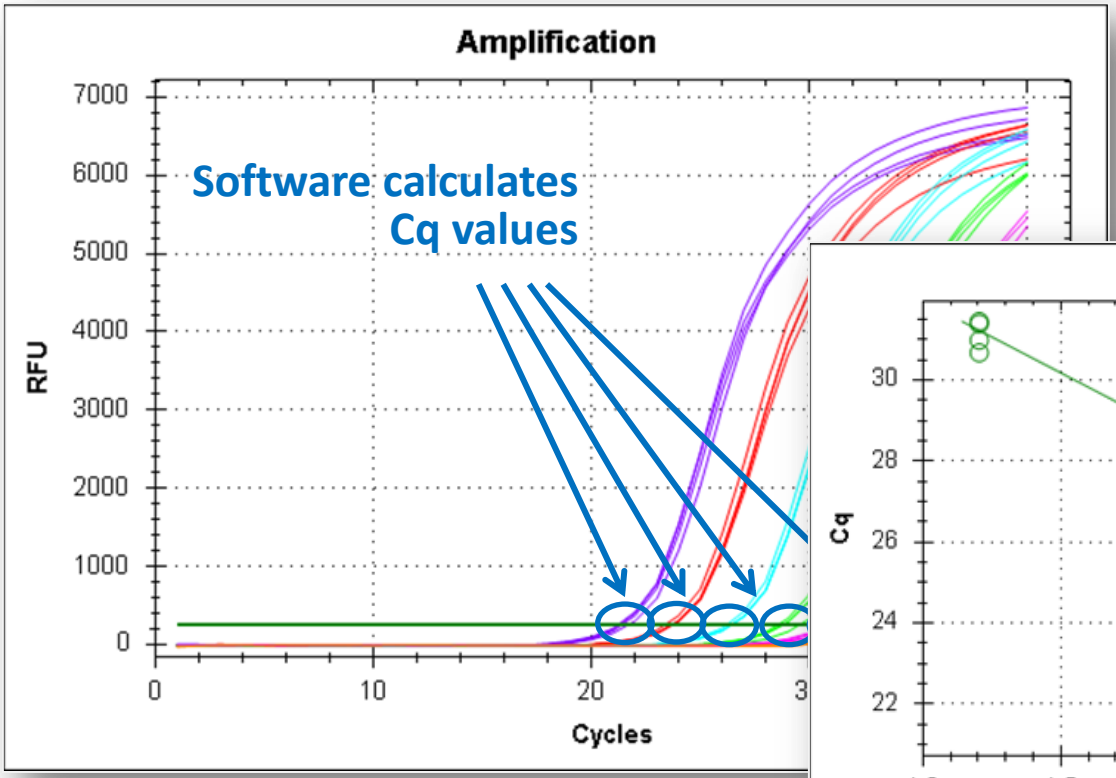
qPCR Refresher: Quantification Cycle (C_q, aka C_t)

Cq is Inversely Proportional to Input Template Amount



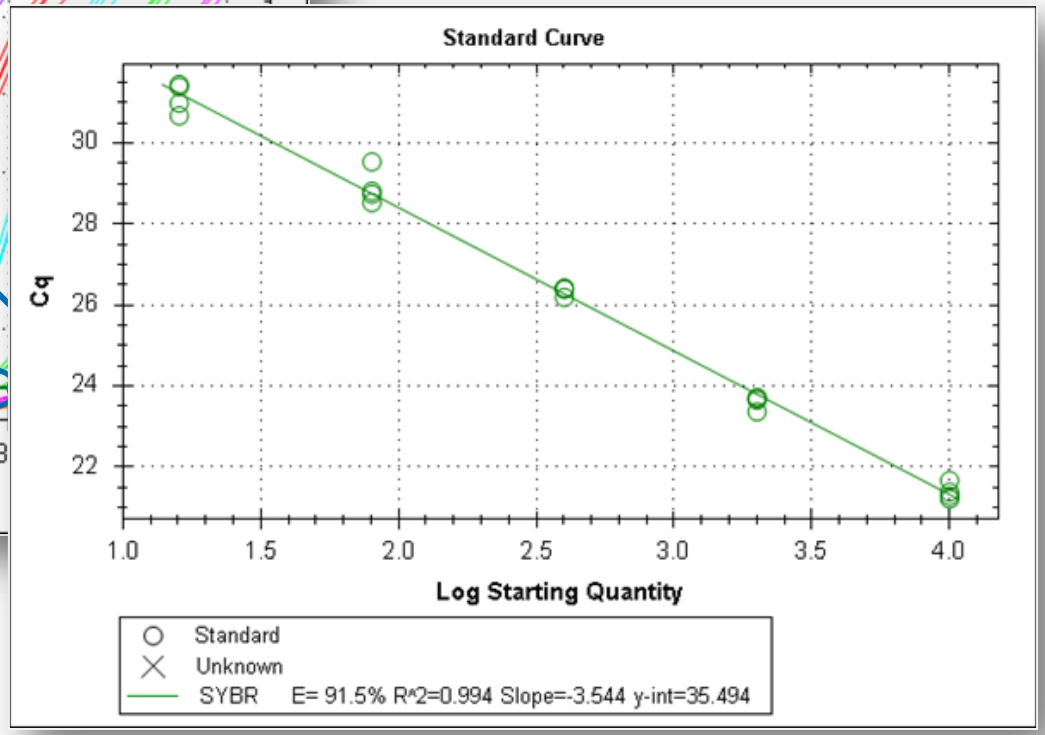
qPCR Refresher: Standard Curve

Slope of a Standard Curve Indicates PCR Efficiency

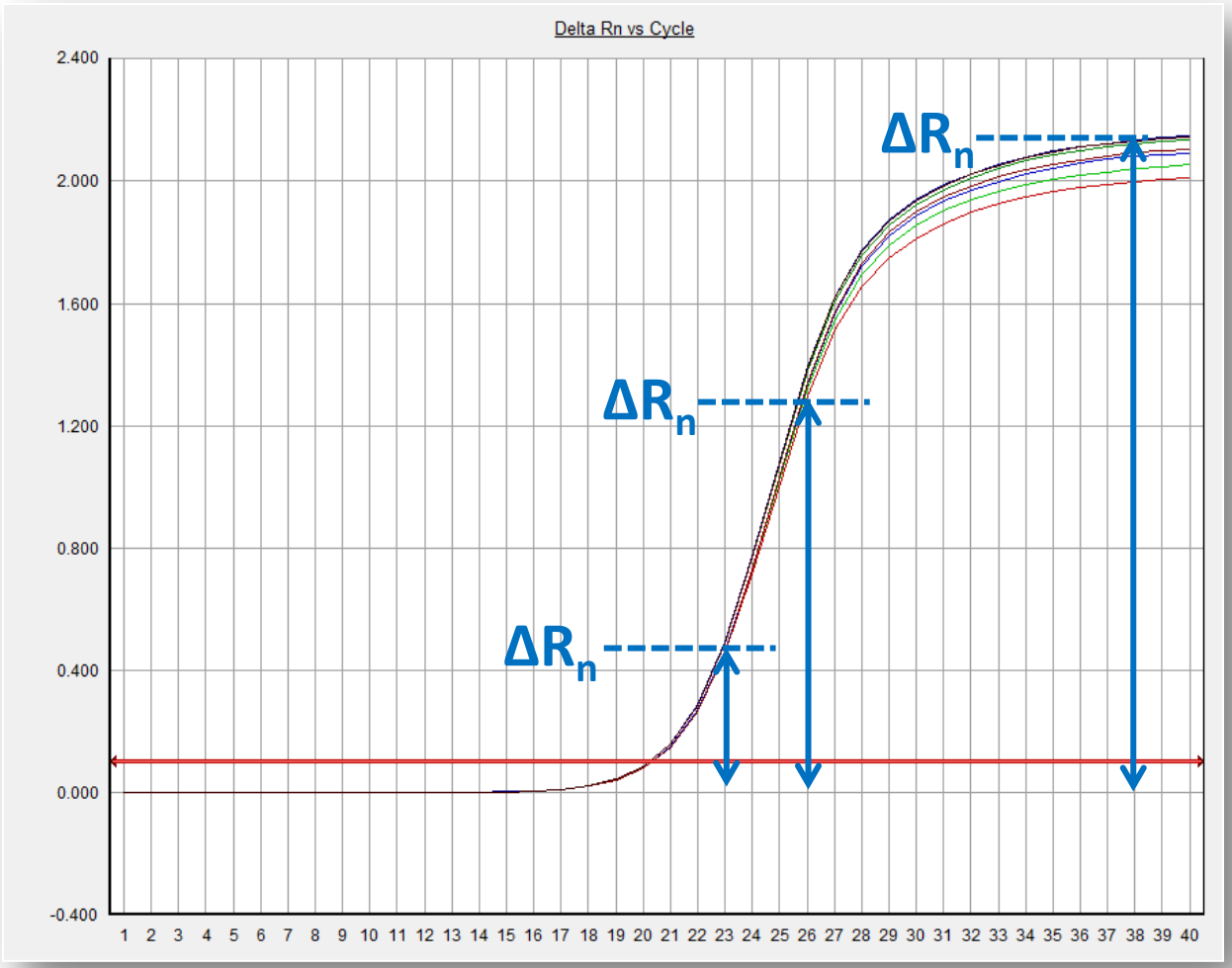


Amplify standards of known DNA concentration

Plot Cq values against log of the DNA concentration



qPCR Refresher: ΔRn = Change in Signal During Amplification (Compared to Baseline)



Overview of PCR and Reverse Transcriptase Inhibitors

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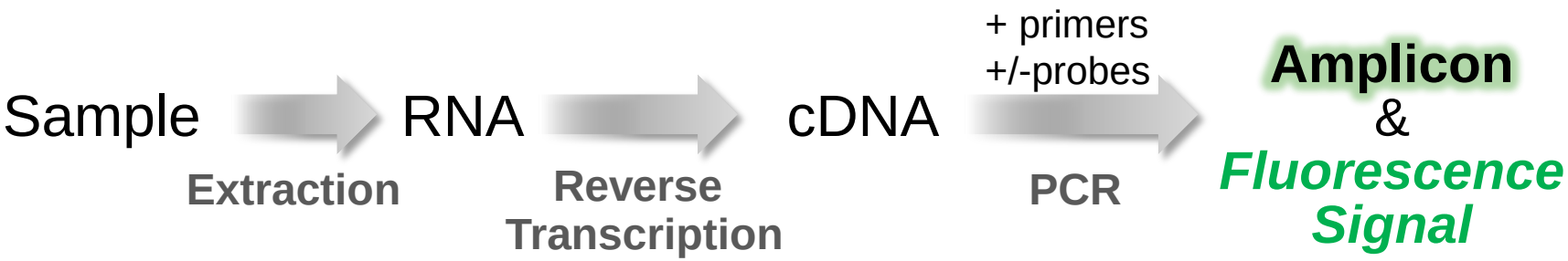
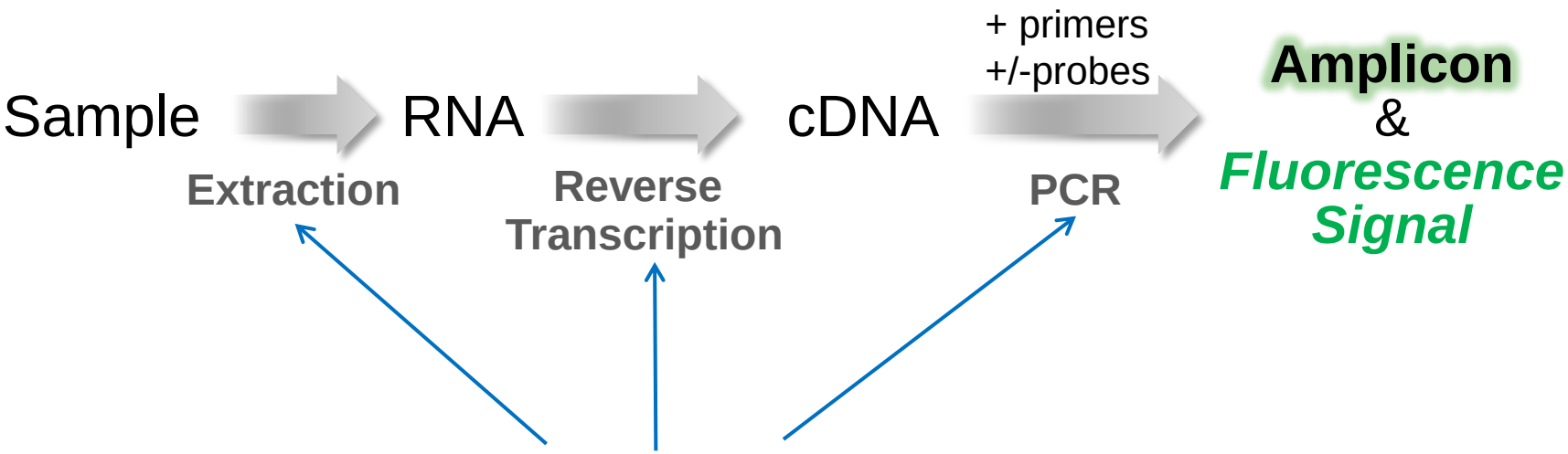
Modes of Inhibition
Many Steps in an RT-PCR Experiment Are Susceptible



- RT-PCR workflow has multiple steps and stages
- Most of these are potential targets for inhibitors
- Some inhibitors exert their effects at multiple points in the workflow

Modes of Inhibition

Absorption of Nucleic Acids to Surfaces

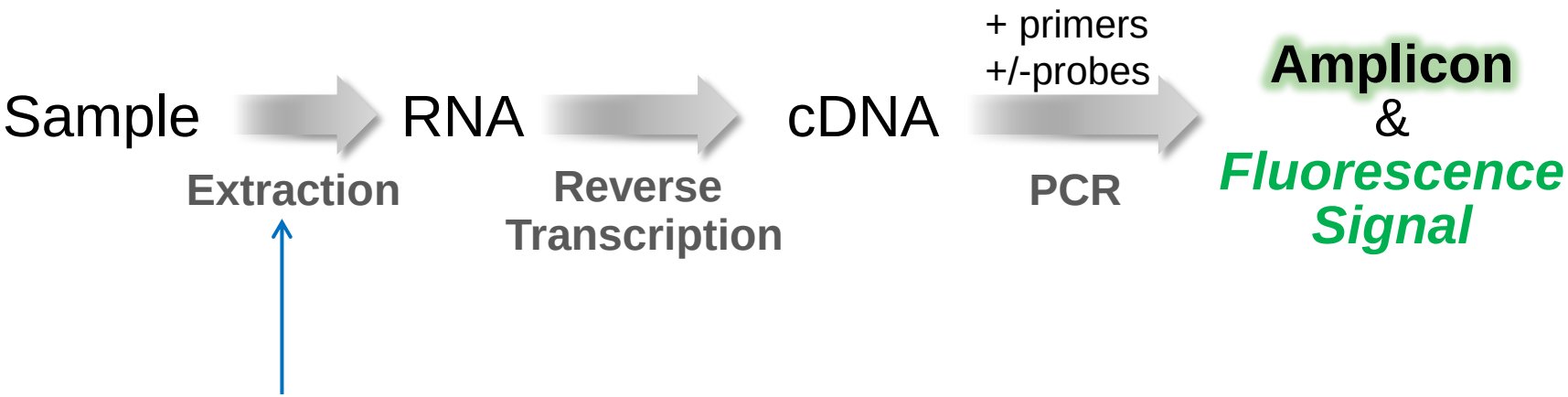


Absorption of nucleic acids to surfaces

- Storage tubes, reaction tubes, etc.
- Polymeric surfaces
- All can lead to loss of sample/template

Modes of Inhibition

Multiple Compounds May Inhibit Extraction

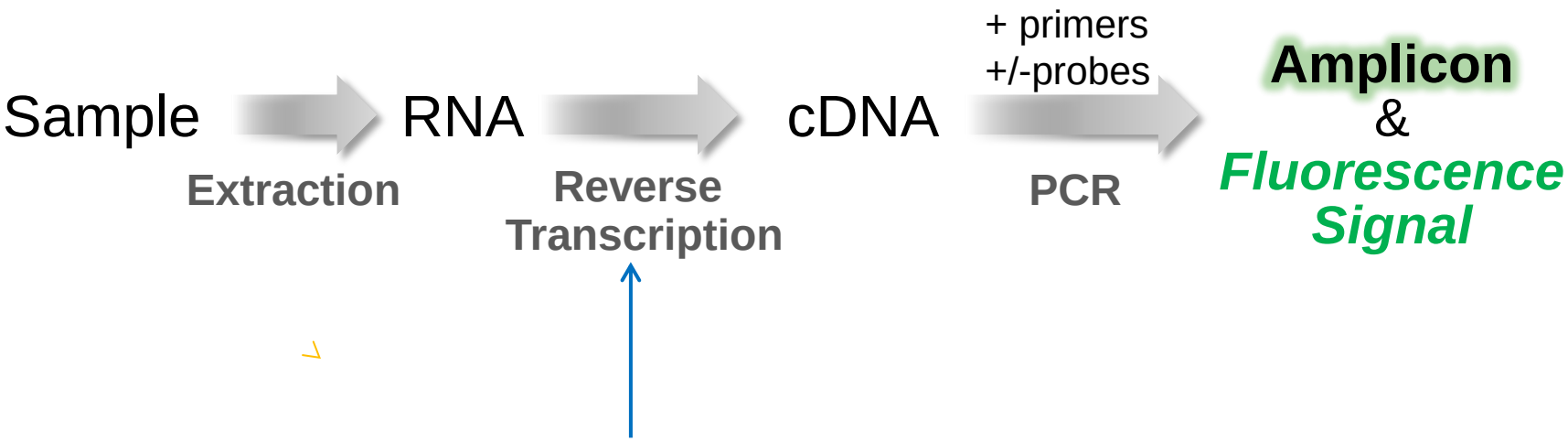


Interference with extraction

- Phenols-crosslink RNA
- Polysaccharides reduce efficiency of pellet re-suspension

Modes of Inhibition

RT is also Susceptible to Inhibition

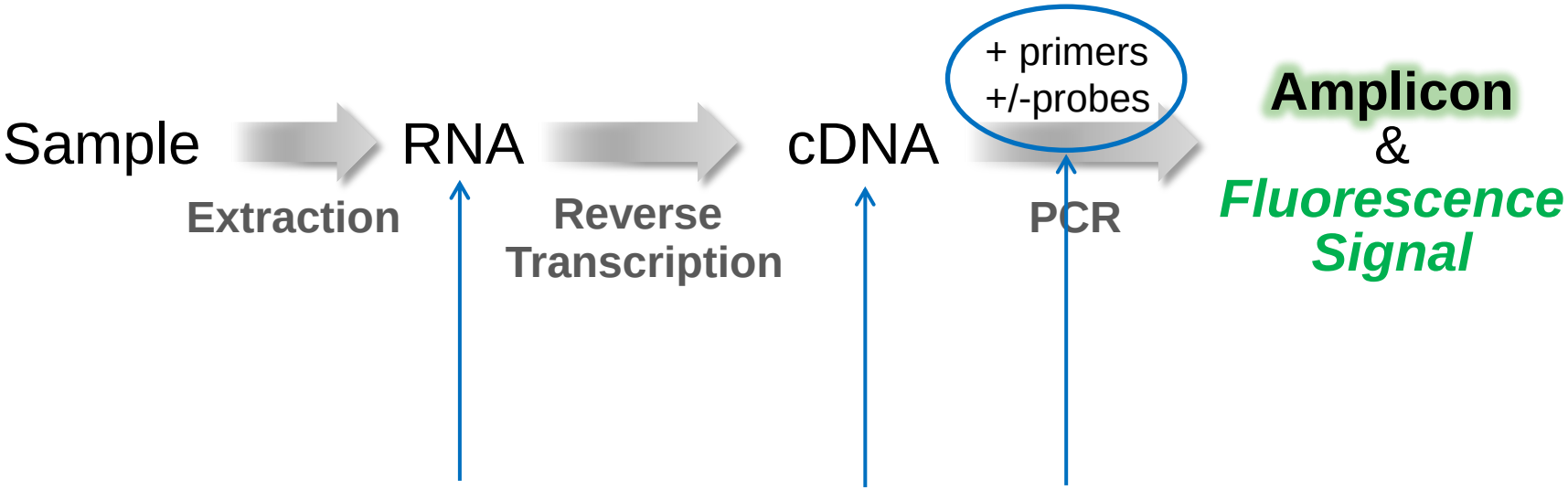


Inhibition of reverse transcriptase

- Melanin
- Alcohols (e.g. ethanol)
- Guanidine, urea
- RNA secondary structure, GC-richness

Modes of Inhibition

RNases, DNases Degrade Templates & Components

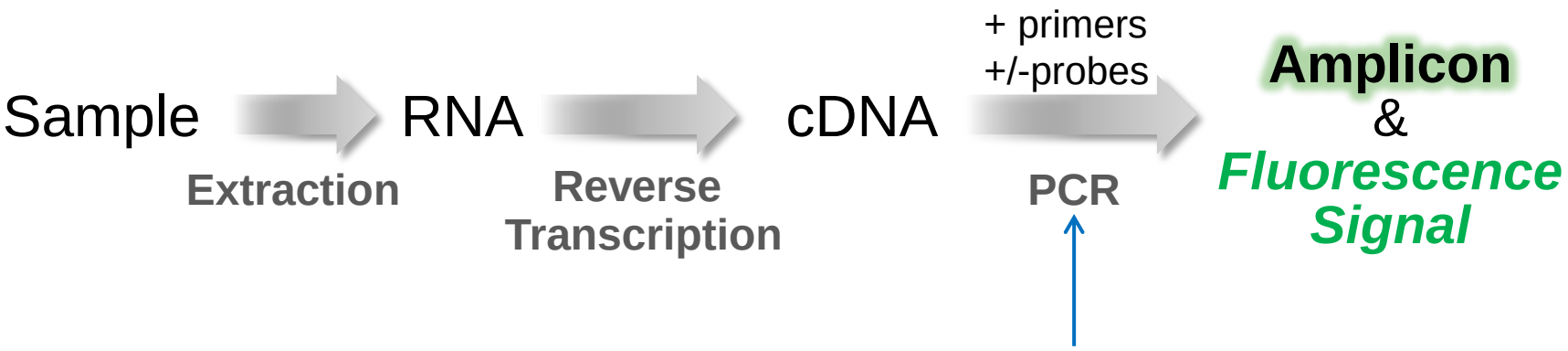


Degradation of template and primers by nucleases

- RNases and DNases

Modes of Inhibition

Molecules/Compounds May Interfere with Annealing

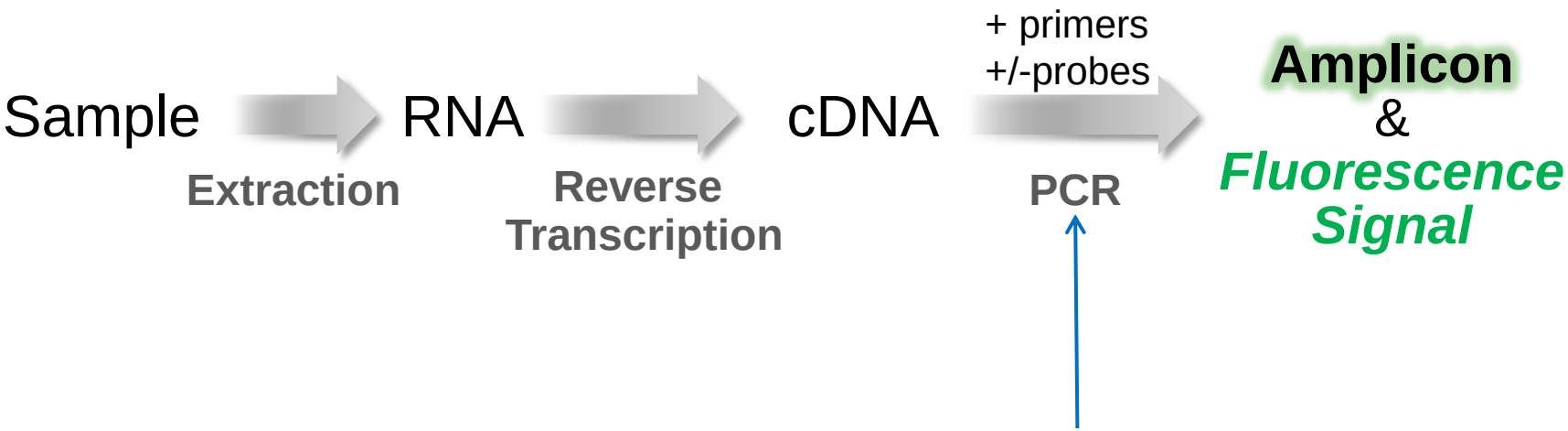


Interference with annealing

- Compete with primers for template binding sites
- Might be overcome by better primer design

Modes of Action

Inhibition of *Taq* DNA Polymerase Activity

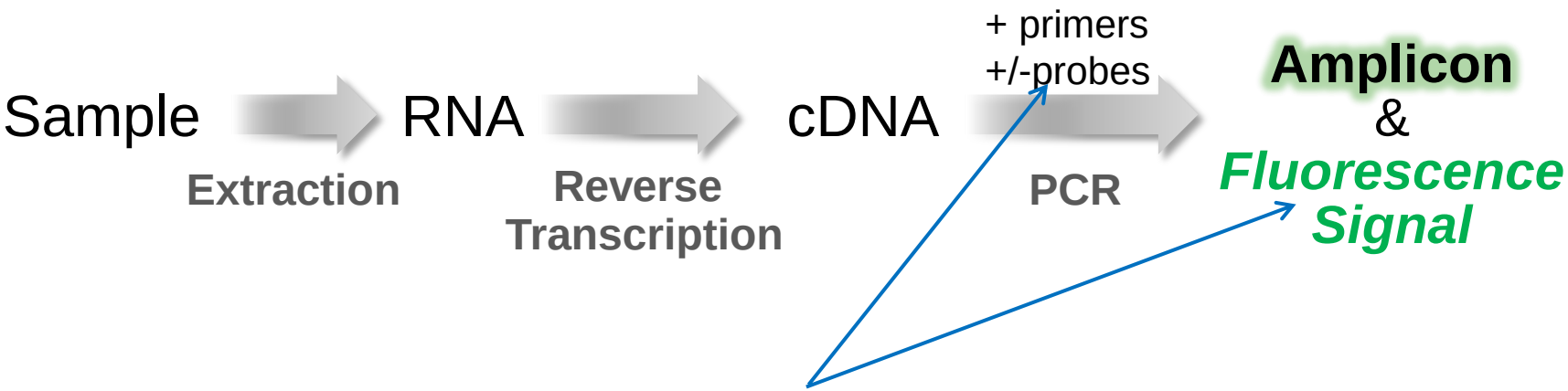


***Taq* DNA polymerase inhibition**

- Direct interactions with *Taq* polymerase
- Also-interactions with co-factors
- **Net result**: reduced *Taq* activity

Modes of Action

Compounds May Reduce Fluorescent Signals



Interference with fluorescence

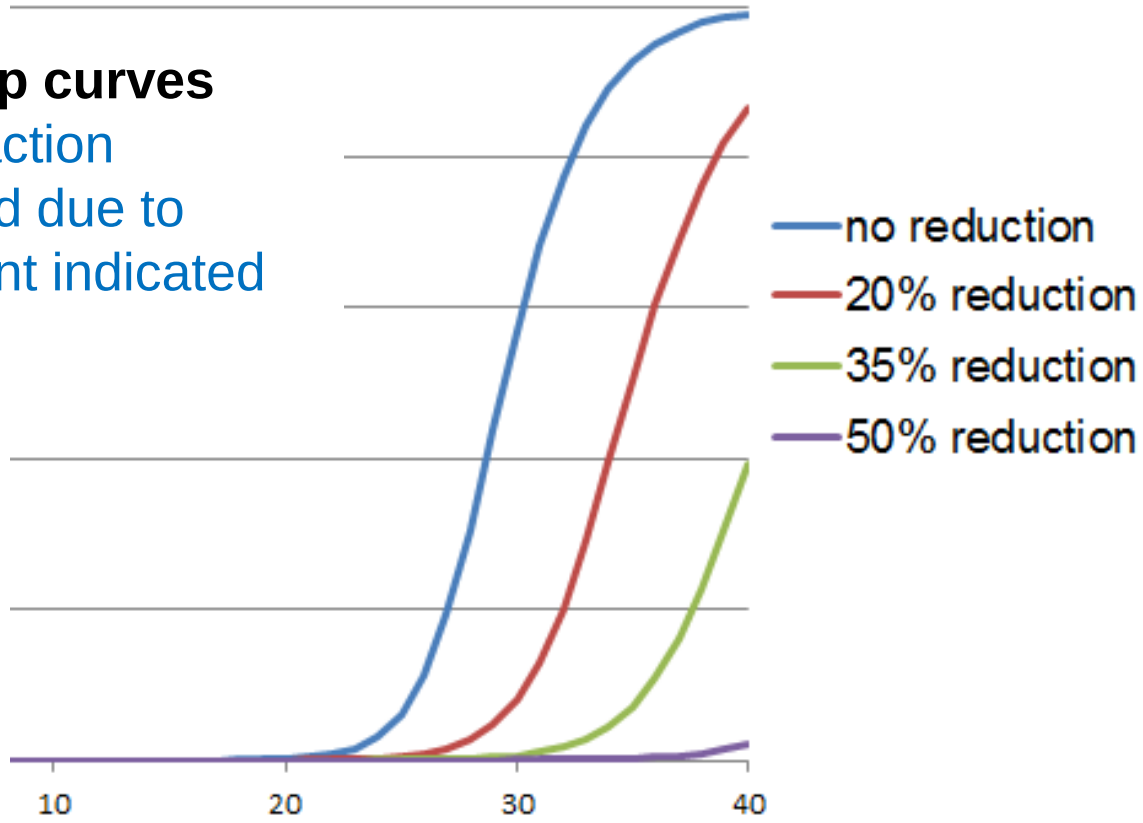
- Physical interactions with the probe or dsDNA binding dye
- Compete with dsDNA binding dye for binding to amplicons
- Increase background fluorescence

Inhibition of Taq Activity

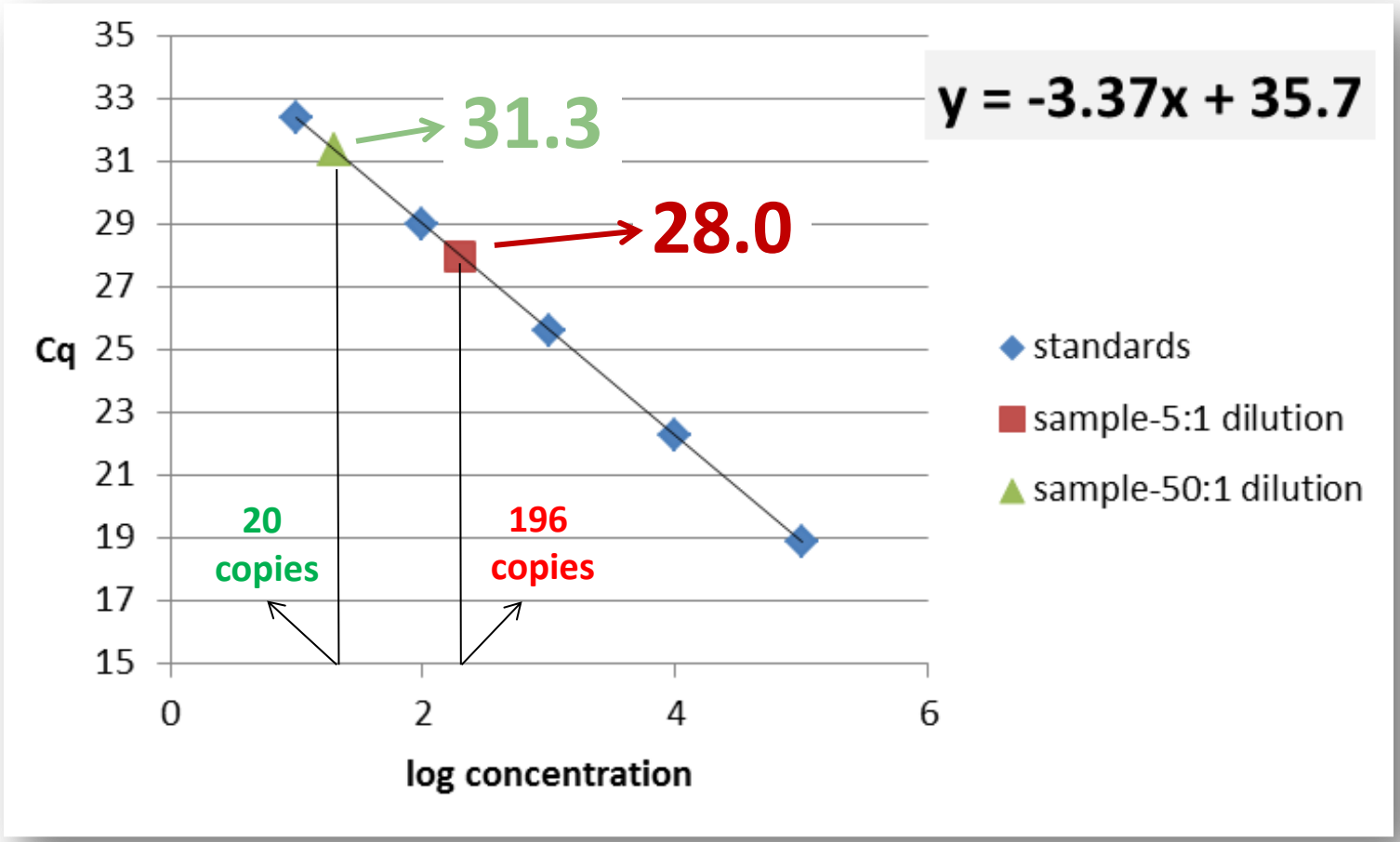
Inhibitors Increase the Cq Values

Hypothetical qPCR amp curves

- 2000 target copies/reaction
- PCR efficiency lowered due to inhibitors by the amount indicated



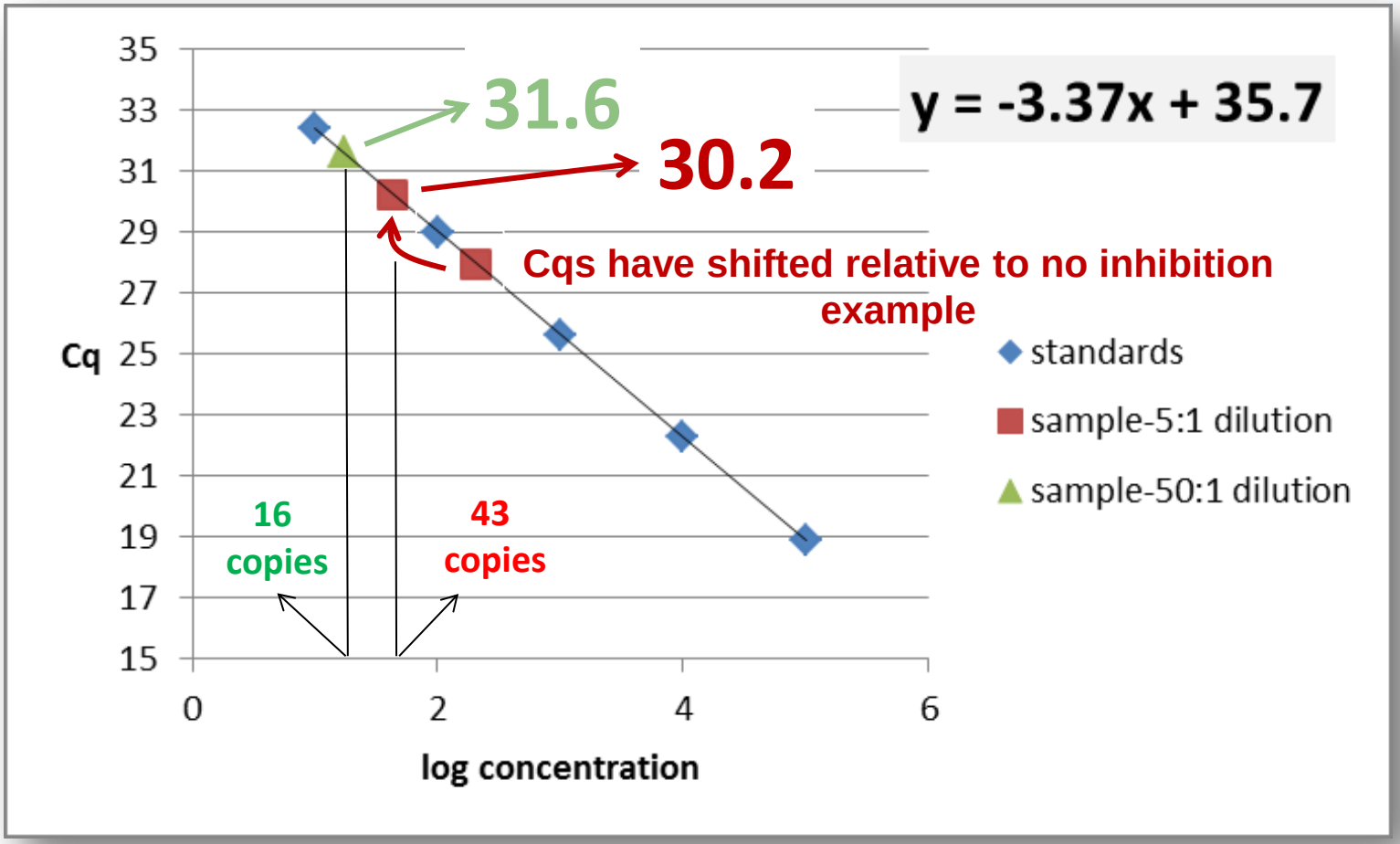
Testing for Inhibition Using Sample Dilution and Comparison of Cq Values



No Inhibition: Calculated sample concentrations are ~10X different as expected based on dilution

Sample with a PCR Inhibitor

Cq difference between 5:1 and 50:1 Dilutions is abnormally low



Inhibition: 5:1 dilution has “less template” than expected with; <10X difference with 50:1

Summary of Cq Differences for Detecting PCR Inhibitors

	no inhib.	w/ inhib.
Cq-5:1 dilution	28.0	30.2
Cq-50:1 dilution	31.3	31.6
Cq difference	3.3	1.4

Remember:

- For 10X difference in starting temple, ΔCq should be approx. equal to PCR slope
- In this example, ΔCq between 5:1 and 50:1 dilutions should be ~3.3-3.4 cycles

Clues a Sample Contains an Inhibitor

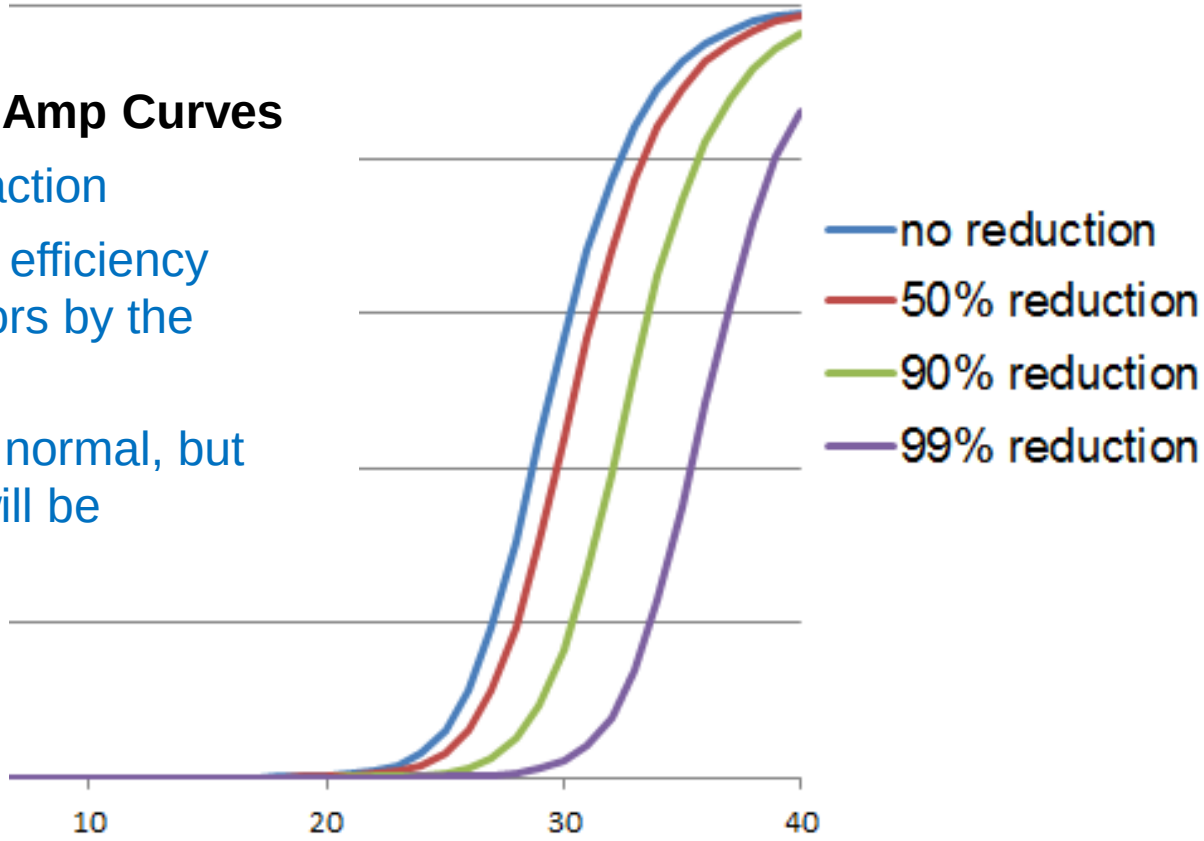
- Cqs are shifted
- Cq of more concentrated sample is shifted more (compressed)
- *Difference in Cqs is greatly reduced compared to expected difference indicating presence of an inhibitor*

Inhibition of Reverse Transcriptase Activity

RT Inhibitors will Increase RT-qPCR Cqs

Hypothetical RT-qPCR Amp Curves

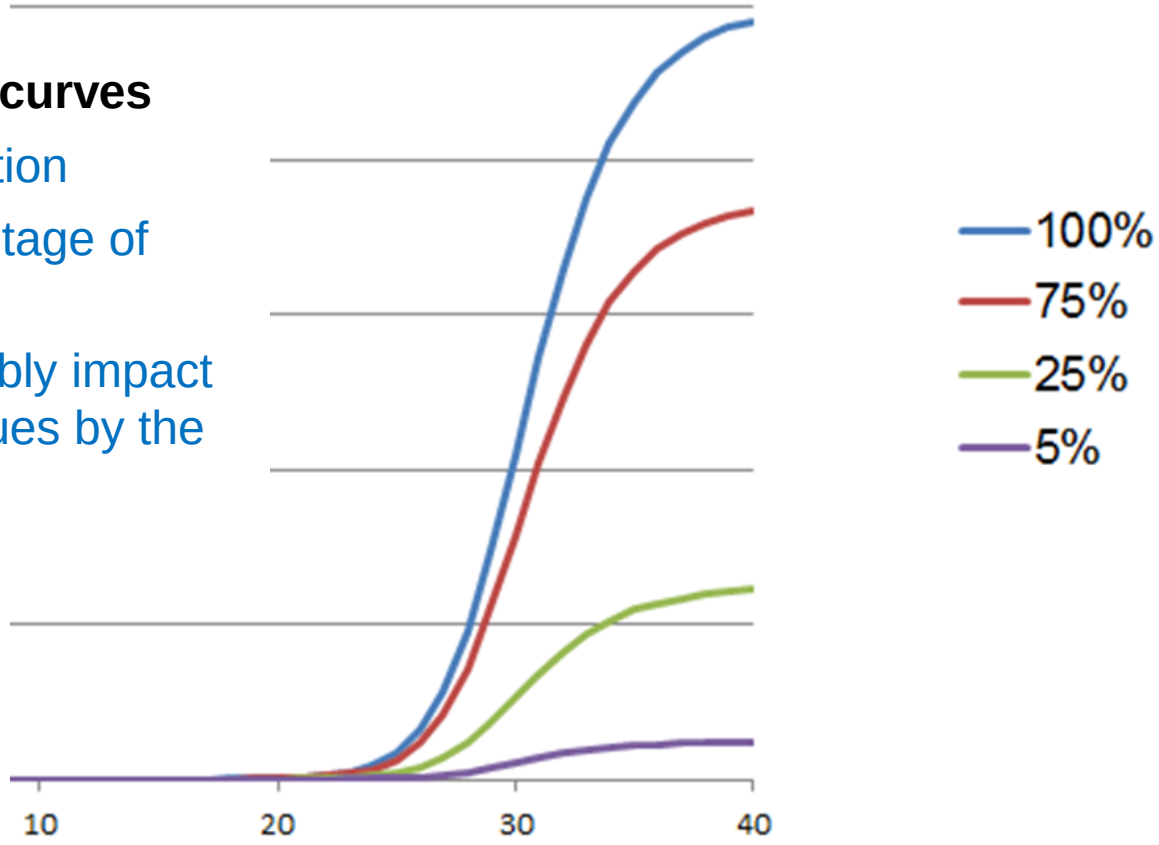
- 2000 target copies/reaction
- Reverse transcriptase efficiency lowered due to inhibitors by the amount indicated
- PCR curves may look normal, but target concentration will be underestimated



Interference with Fluorescence Production
qPCR Curves will Flatten

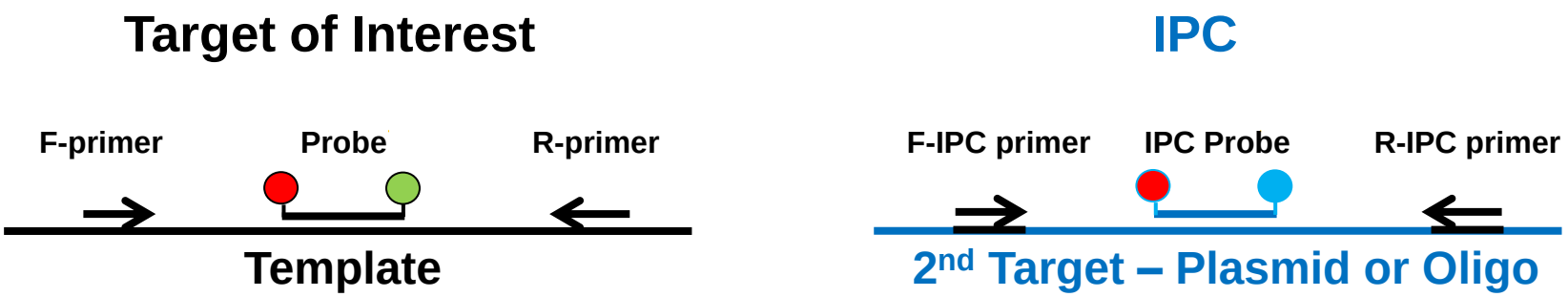
Hypothetical qPCR amp curves

- 2000 target copies/reaction
- Fluorescence as percentage of normal is indicated
- Flatter curves will probably impact determination of Cq values by the software



Internal PCR Control (IPC)

Oligo/Plasmid Target Added to All PCR Rxn (Multiplex)

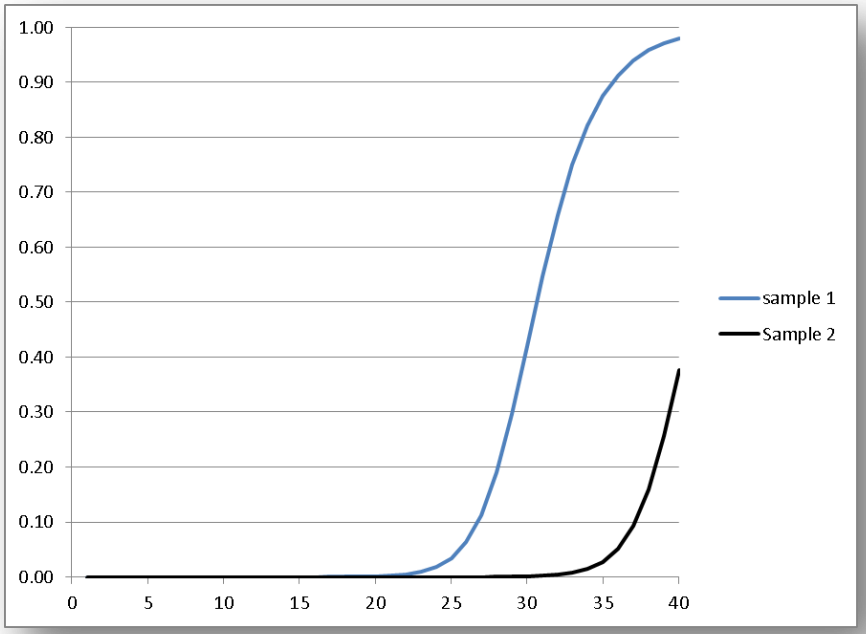


IPC Concepts

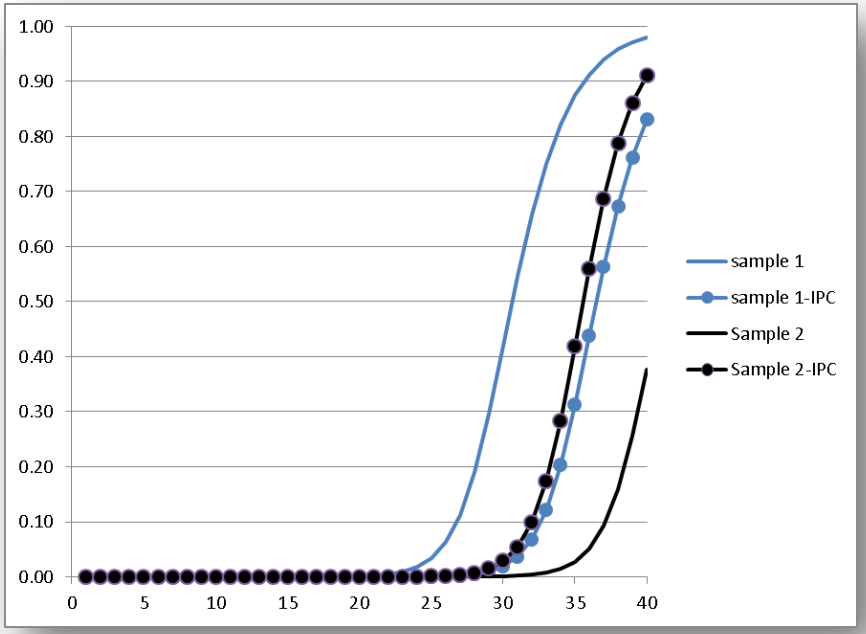
- IPC amplicon: Different sequence but similar amp efficiency
- IPC probe: Different reporter dye to allow multiplexing
- IPC primers: May be same as used for the main target
- IPC added to all reactions at low copy number
  - Minimize impact on sensitivity and dynamic range of target assay

Internal PCR Control Example

Differentiating Low Copy Number from Inhibition



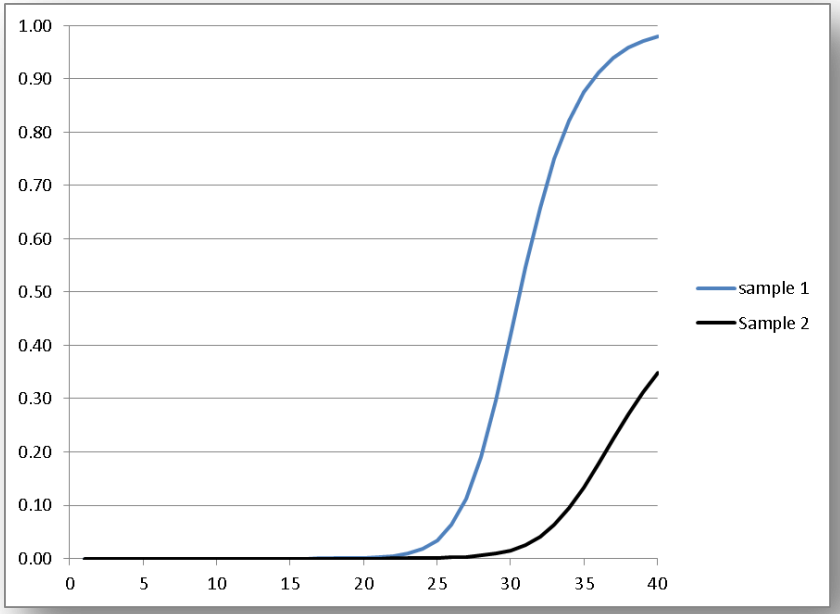
- Sample 2 has late Cq
- Low copy number template (real) or is an inhibitor present?



- IPCs amplified similarly in both samples
- late Cq for sample 2 is due to low copy number, not an inhibitor

Internal PCR Control Example

Differentiating Low Copy Number from Inhibition



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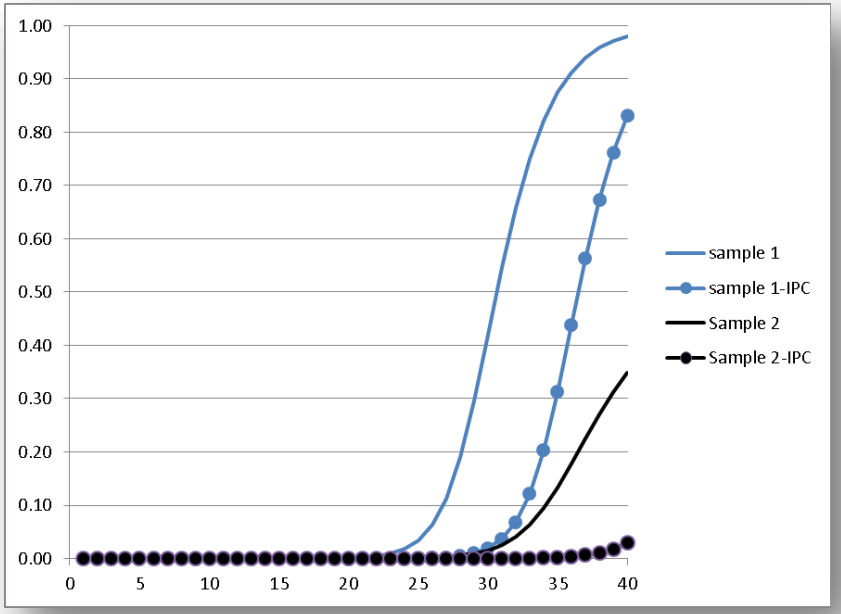


- IPC in Sample 2 amplified poorly
- Inhibitor is present
- Can't trust data obtained for Sample 2

Sources of Inhibitors
Part of Original Sample or Introduced During Handling

- Present in sample matrix
  - May be inherent in the sample type
  - Many starting materials have high levels of inhibitors
- Introduced during sample handling, nucleic acid isolation, or reaction set-up

Sample Matrix Examples

Plant Materials

- Phenols/polyphenols (Tannic acid)
  - Cross-link nucleic acids, impede re-suspension of pellets
  - Chelate metal ions
- Polysaccharides
  - Sequester nucleic acids, inhibit *Taq* or reverse transcriptase

Soil

- Humic substances (fulvic and humic acid)
  - From dead organic matter
  - Crosslink or adsorb nucleic acids, bind/adsorb to enzymes

Sample Matrix Examples

Shellfish/Seafood/Oysters

- Shell-fish contain various strong RT-qPCR inhibitors
  - Filter feeders >> may accumulate inhibitors
- Polysaccharides may be most prominent inhibitor in seafood, reduce PCR efficiency

Cheese/Milk Products

- Proteases (eg., plasmin in milk)
  - Degraded enzymes
- Calcium ions
  - Compete with polymerase cofactors, inhibit activity

Sample Matrix Examples

Environmental samples (soil, water, etc.)

- Wide variety of inhibitors
- Soil and dead animal material contain humic and fulvic acid
  - Inhibit PCR even at low concentration
  - Bind to nucleic acids and/or enzymes

Forensic/Palaeobiology/Archaeology

- Bone dust (calcium, *Taq* inhibitor)
- Peat extract
- Clay-rich soil (adsorption of nucleic acid or enzyme)

Sample Matrix Examples

Clinical Samples (blood, muscle, stool, urine, etc.)

- Heme/Hemoglobin (blood)
  - One commercially available enzyme described as completely inhibited by <0.004% whole blood or traces of heme
- Leukocyte DNA (blood)
- IgG (blood, binds to nucleic acids)
- Anticoagulants (EDTA, sodium citrate, heparin)
  - EDTA-chelates Mg^{++}
  - Heparin-binds to nucleic acids
- Complex polysaccharides, bile salts, lipids (stool)

Inhibitors Introduced During Handling

Materials used during cell lysis or nucleic acid extraction

- Powder from gloves
- Salts
- EDTA
- Ethanol/isopropyl alcohol
- Detergents
- Denaturing reagents (guanidine etc.)
- Transport media

BOTTOM LINE

There are wide variety of potential inhibitors with multiple modes of entry into your work flow and with a wide variety of possible effects.

Strategies for Dealing with Inhibitors

Common Approaches to Reducing Inhibitor Effects
Three Broad Approaches

- **Remove the inhibitors**
  - **Use kits that don't contain possible inhibitors and therefore can't introduce them into the samples/assays**
  - **Dilute your sample!**
- **Select amplification/RT enzymes with intrinsic resistance to inhibitors**
- **Use PCR additives to negate inhibitor effects**

Removing Inhibitors
Sample Processing Approaches

Separate nucleic acid from the inhibitor

- Guanidium thiocyanate extraction
  - Effectively removes inhibitors from many sample matrices
- Phenol chloroform extraction (remove lipids)
- Immuno-capture (pull viral particles away from sample matrix)
- Column chromotagraphy with cetrimonium bromide (removes polysaccharides and degraded protein)
- Many others tricks, many of which are inhibitor-specific
- **Use kits that don't contain possible inhibitors and therefore can't introduce them into the samples/assays**

Removing Inhibitors
Sample Processing Approaches

Sample dilution!

- Decreases inhibitor concentration to level that does not effect enzymes
- Simple and can be very effective
- Always a good initial strategy
- Downsides
  - Effectiveness may depend on nature of the inhibitor
  - Since you’re diluting your target as well, may not be practical with low copy number samples

Dealing with Inhibitors
Choose a More Resistant Polymerase

- Reverse transcriptases and heat-stable DNA polymerases exhibit different inhibitor resistance characteristics
- Examples:
  - Certain *Taq* mutants and chimeric *Taq* enzymes (see literature) are more resistant
- Reagent/buffer formulations may also confer resistance to inhibitors (*more on this later*)

Dealing with Inhibitors
PCR Additives (Enhancers) Help Reduce Inhibition

Bovine Serum Albumin (BSA)

- Relieves inhibition caused by wide variety of compounds
  - Humic acid, FeCl3, hemin, tannic acid, extracts from difficult samples, etc.
- Doesn’t work for others
  - Bile salts, EDTA, SDS, etc.

T4 gene 32 protein (gp32)

- Single stranded DNA-binding protein
- Benefits are similar to those for BSA

Dealing with Inhibitors
RT and PCR Additives Help Reduce Inhibition

RNasin® RNase Inhibitor

- Inhibits common RNases preventing them from degrading RNA samples during purification, analysis

Other Enhancers

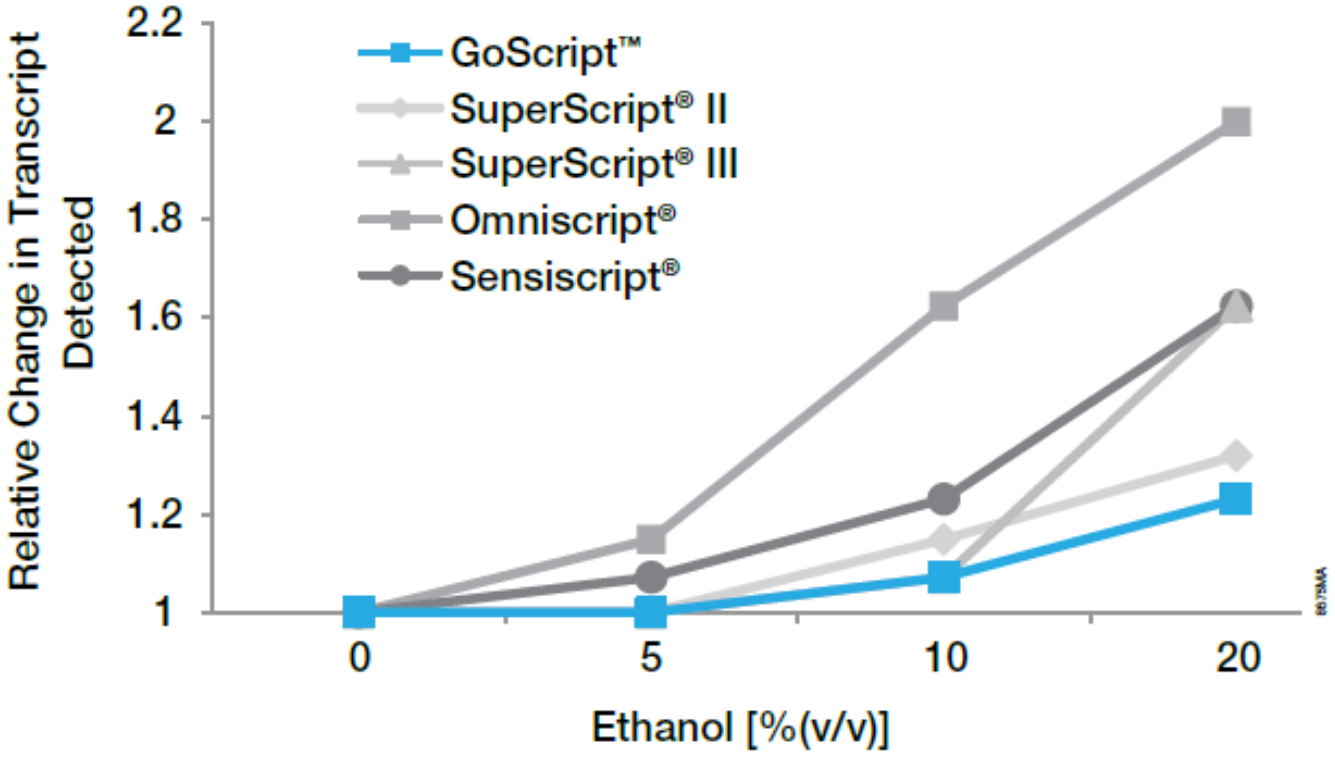
- Dimethyl sulphoxide, formamide, glycerol, non-ionic detergents, PEG, proteinase inhibitors, etc.

Reagent Choice Makes a Difference

Performance Examples

Performance with Common Inhibitors

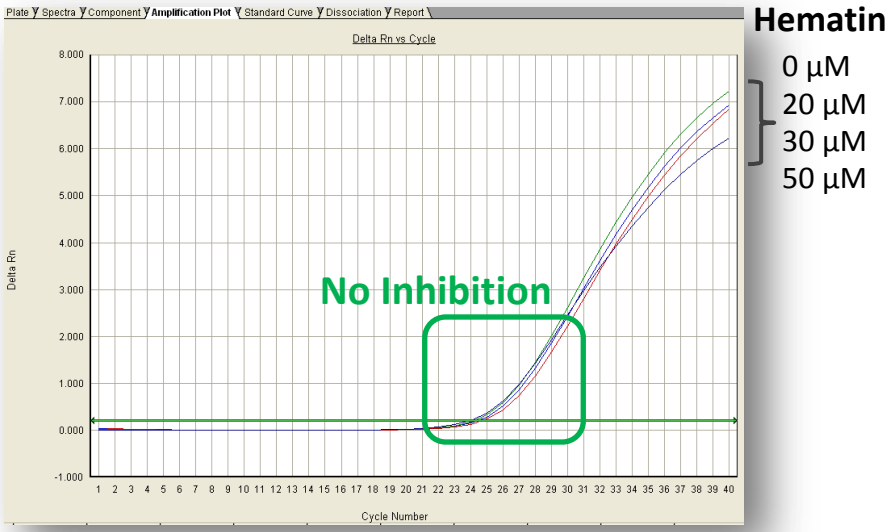
GoScript™ System Exhibits Resistance to Ethanol



Performance with Common Inhibitors

GoTaq® System Exhibits Resistance to Hematin

GoTaq® Probe qPCR (Promega)



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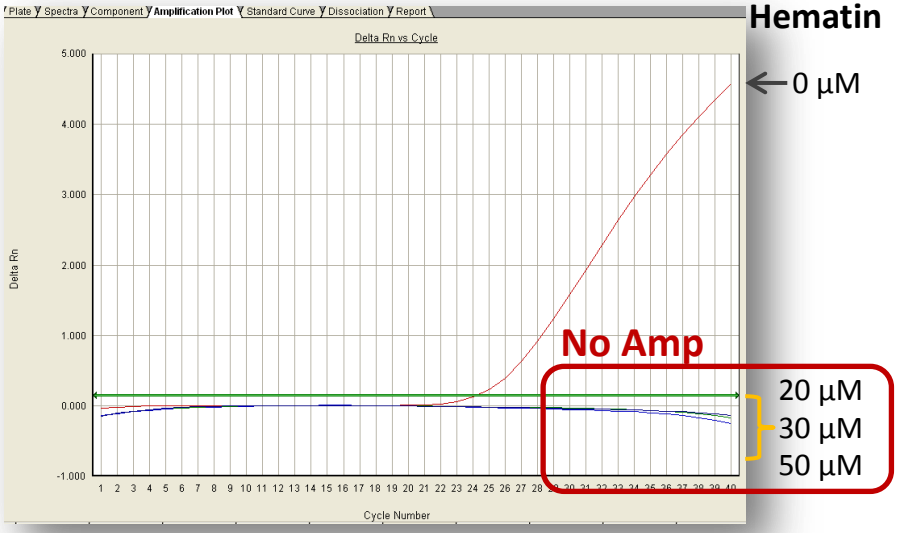
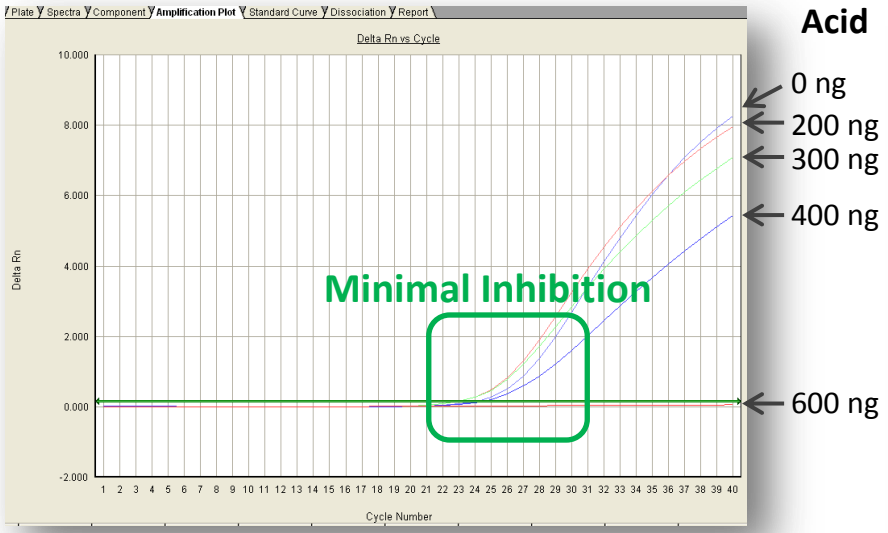


Performance with Common Inhibitors

GoTaq® System Exhibits Resistance to Humic Acid

GoTaq® Probe qPCR (Promega)



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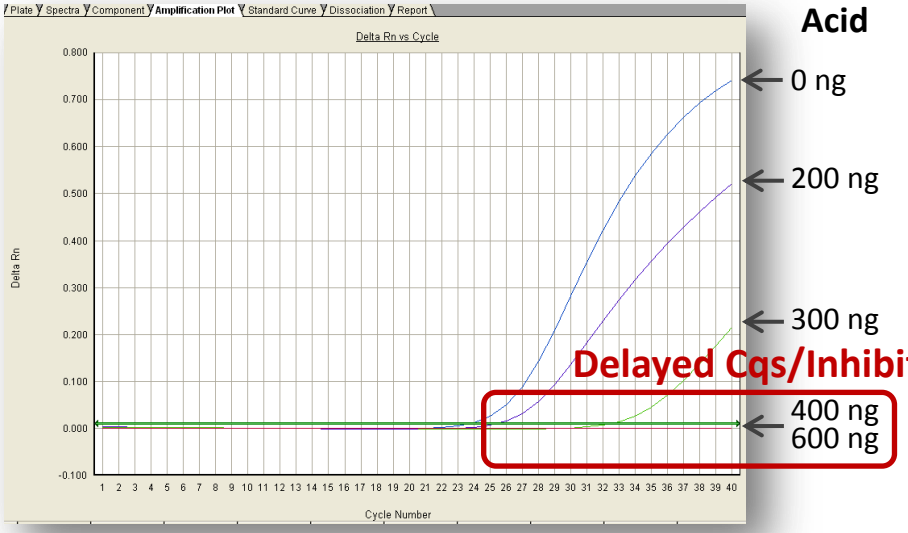
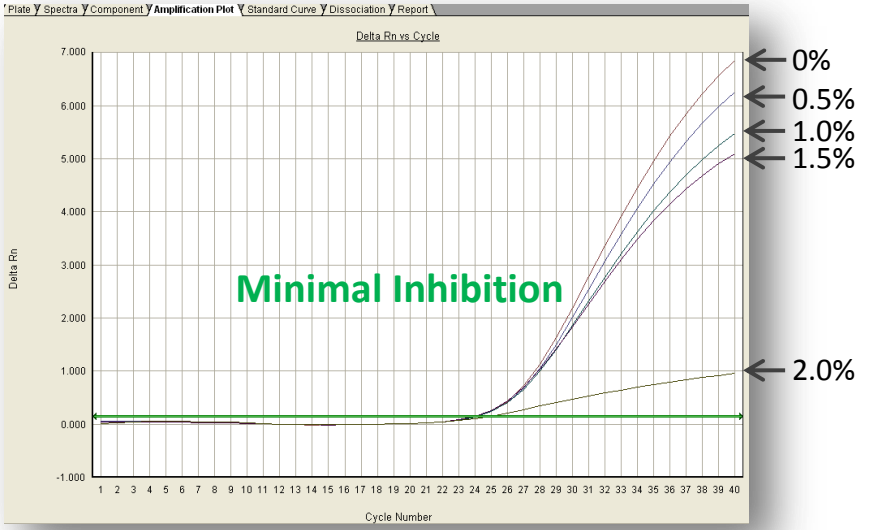


Performance with Common Inhibitors

GoTaq® System Exhibits Resistance to Phenol

GoTaq® Probe qPCR (Promega)



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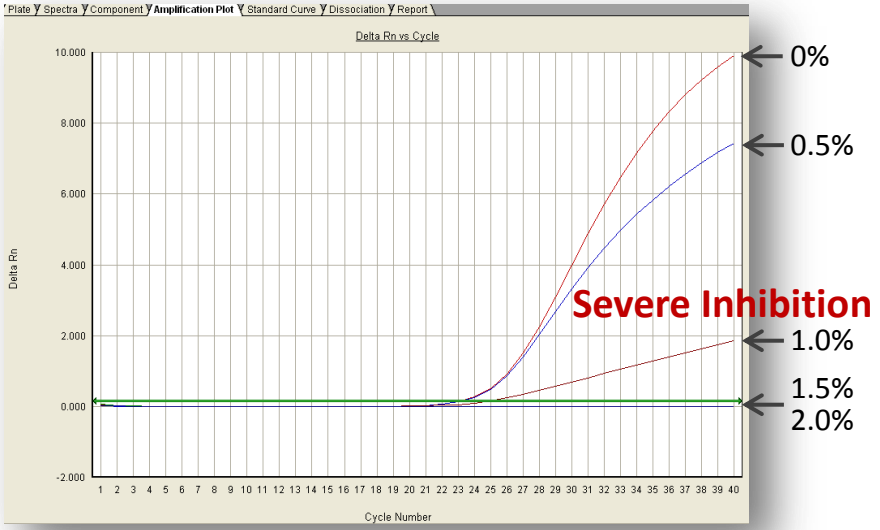


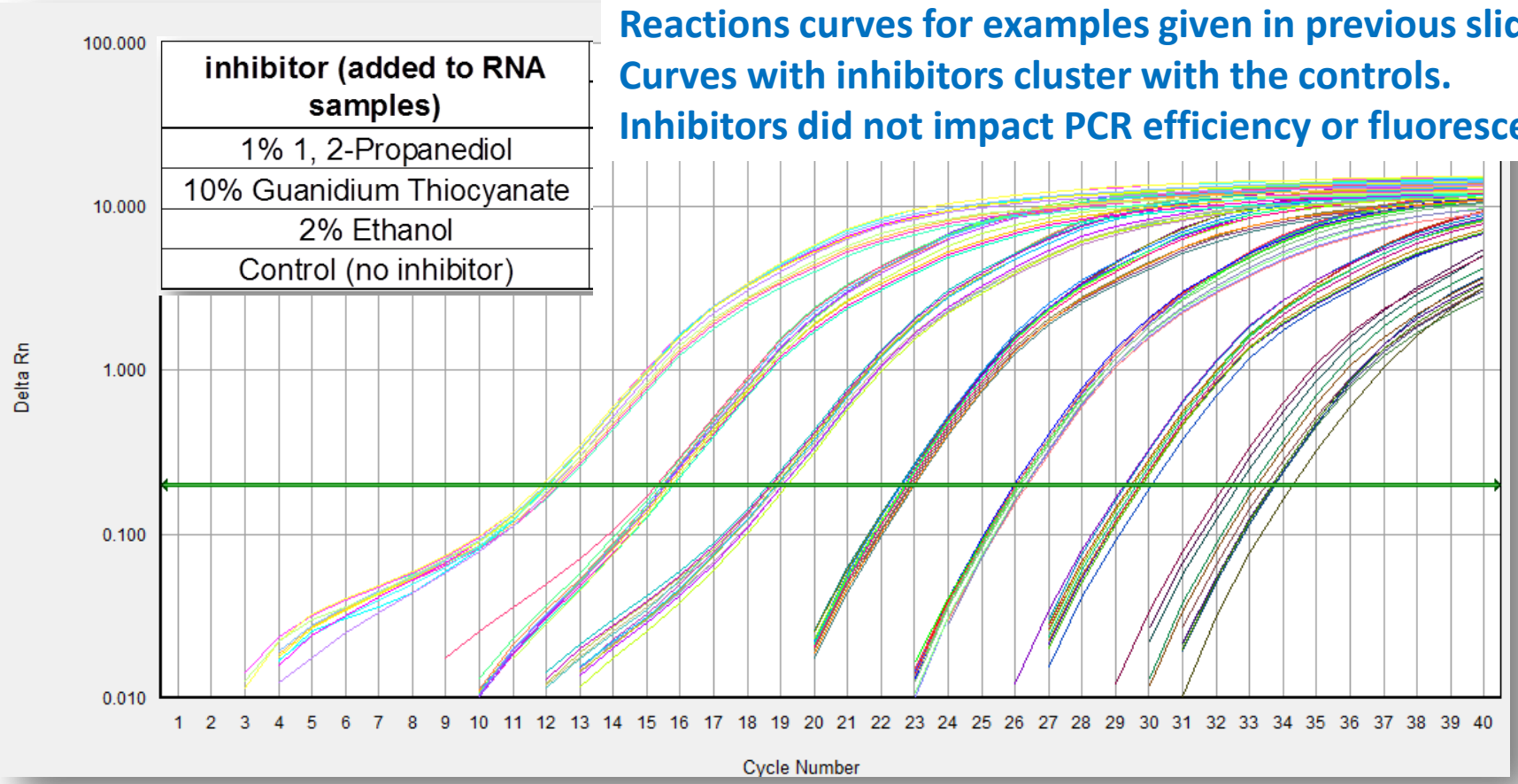
GoTaq® One Step RT qPCR System
Strong Resistance to Inhibitors

- Amplification of GAPDH mRNA
- Inhibitors were added to RNA samples prior to reaction set up
  - Selected inhibitors = compounds that could possibly carry over from the RNA isolation procedure
- Standard curve measurements show no difference in performance

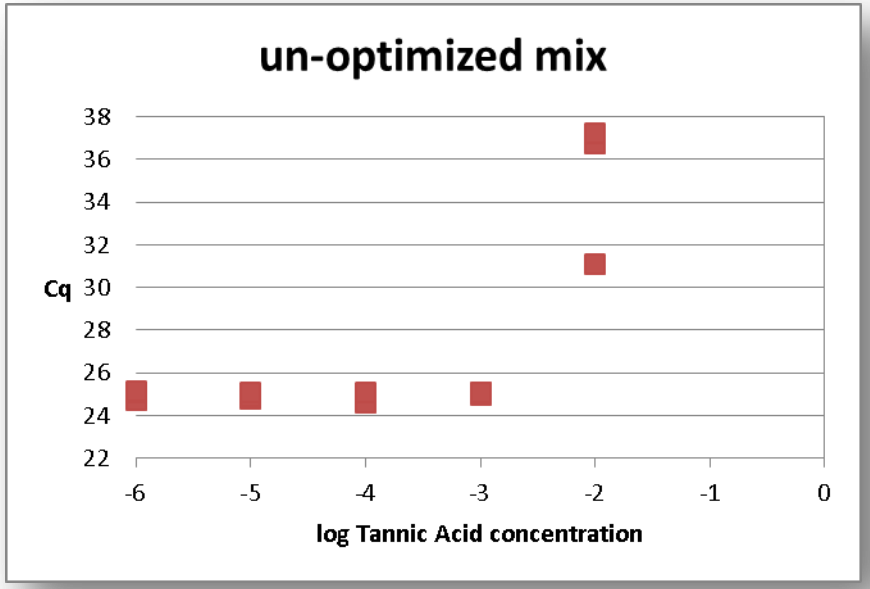
inhibitor (added to RNA samples)	Standard Curve		
	slope	intercept	R^2
1% 1, 2-Propanediol	-3.55	40.2	0.999
10% Guanidium Thiocyanate	-3.52	40.1	0.998
2% Ethanol	-3.54	40.5	0.999
Control (no inhibitor)	-3.50	40.1	0.999

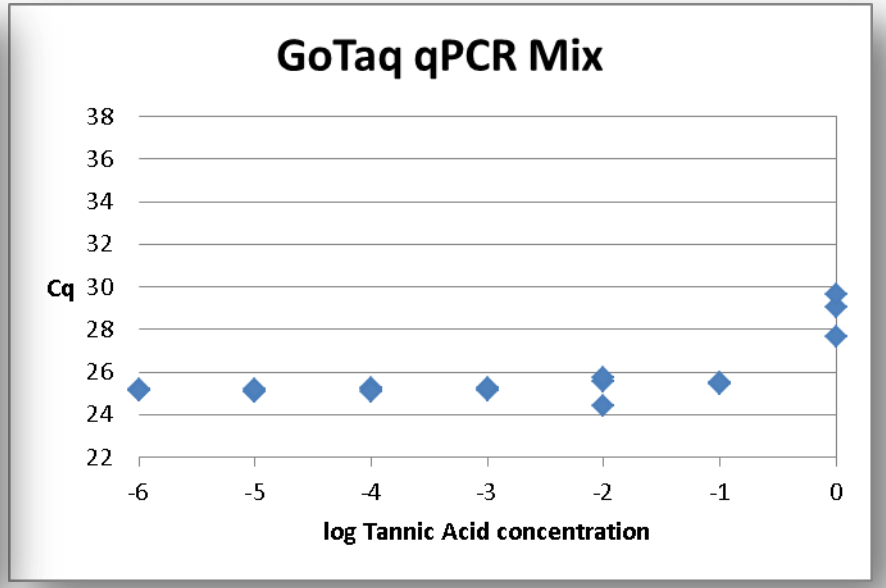
GoTaq® One Step RT qPCR System
Strong Resistance to Inhibitors

Reactions curves for examples given in previous slide.
Curves with inhibitors cluster with the controls.
Inhibitors did not impact PCR efficiency or fluorescence



GoTaq® qPCR System Exhibits Resistance to Inhibition by Tannic Acid





GoTaq® Probe qPCR System Resistance to Inhibitors

Hematin	Promega	AB Environmental
0uM	22.5	20.7
15uM	23.0	22.4
20uM	22.9	22.2
25uM	23.0	21.9
30uM	23.1	21.4
50uM	22.6	23.5

$CaCl_2$	Promega	AB Environmental
0mM	24.2	24.2
1mM	23.6	23.1
2mM	23.0	22.7
4mM	22.9	22.6
8mM		

GHCl	Promega	AB Environmental
0M	23.9	23.6
50mM	22.4	23.7
100mM	22.5	
200mM		
300mM		
400mM		

Humic Acid	Promega	AB Environmental
0uM	23.0	22.6
15uM	22.8	22.5
20uM	22.6	22.1
25uM	22.5	22.6
30uM	24.7	22.7
50uM		23.9

EtOH	Promega	AB Environmental
0%	24.1	23.7
0.50%	24.0	23.3
1%	24.0	22.9
2%	24.3	23.1
4%	24.3	22.7
8%		

GTC	Promega	AB Environmental
0M	23.6	23.1
50mM	21.8	
100mM		
200mM		
300mM		
400mM		

= not detected (no C_q/C_t)

GoTaq® qPCR System
Advantages of the Proprietary BRYT Green Dye

- SYBR® Green Dye
  - Used in many commercially available qPCR reaction mixes
  - Inhibitory to PCR at high concentrations
- GoTaq® qPCR and RT-qPCR Systems utilize BRYT Green® Dye.
  - Proprietary Promega dye with reduced inhibitory properties
  - Can be used at higher concentrations
- **Result**: GoTaq® qPCR and RT-qPCR Systems provide higher fluorescence which can improve PCR sensitivity in some situations

Final Comments
Wide Variety of Inhibitors Can Impact Your RT-qPCR

Best strategies

- **Know your sample matrix!**
  - Be aware of inhibitors typically associated with your sample type
- **Know your workflow!**
  - Be aware of potential inhibitor access points (sample handling, work environment, nucleic acid isolation method, gloves, etc.)
- **Be alert for signs of inhibition**
  - Flat amp curves
  - Cq differences between different sample dilutions that don't make sense, etc.

Final Comments
Wide Variety of Inhibitors Can Impact Your RT-qPCR

Best strategies

- **Consider running control experiments**
  - Spike known RNA/DNA quantities into your samples or reactions to verify they amp as expected
  - Internal PCR controls
- **Consider using PCR enhancers**

Final Comments
Choose Your Reagents and Kits Wisely

Best strategies

Carefully select your extraction kits and protocols!

- Some kits and protocols reduce the carryover of inhibitors in the purified DNA or RNA

Carefully select your amplification reagents!

- If inhibitors are unavoidable, use reagents with known robust performance with difficult/inhibitor containing samples
- Promega's GoTaq® and GoScript™ reagents provide solid performance with samples containing a wide range of inhibitors

PCR Inhibition and General References

Inhibition

Opel, et al. 20109. A Study of PCR Inhibition Mechanisms Using Real Time PCR. J. Forensic Sci. 55: 1-9.

Schrader, et al. 2012. PCR inhibitors-occurrence, properties and removal. Journal of Applied Microbiology 113: 1014-1026.

General

Gene Quantification: www.gene-quantification.info/

Promega.com: FAQ for RT-qPCR

A–Z of quantitative PCR (Editor: S.A. Bustin), Int. University Line, La Jolla, CA.

General qPCR Resources

Webinars

"Maximize Your Reverse Transcription-qPCR (RT-qPCR) Assays" -- Carl Strayer, PhD

"Optimizing RNA Expression Analysis from Start to Finish" -- Doug Wieczorek, PhD

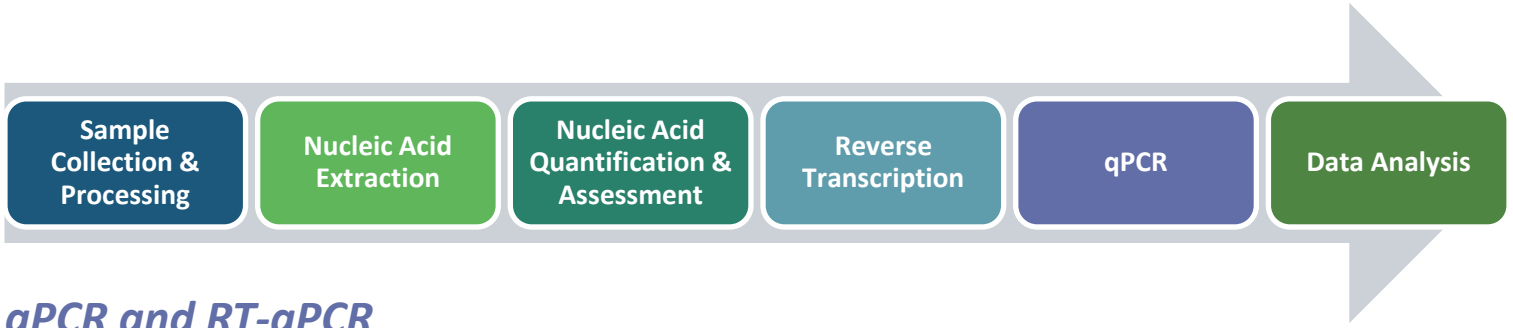
" Optimize Your qPCR and RT-qPCR Assays with Careful Planning and Design"

-- Nadine Nassif

Technical support

http://www.promega.com/support/customer-and-technical-support/

Promega Products for qPCR and RT-qPCR



qPCR and RT-qPCR

- GoTaq® qPCR and RT-qPCR Master Mixes – *dye-based detection with BRYT® Green*
- GoTaq® Probe qPCR and RT-qPCR Master Mixes – *for label-based detection*

Reverse Transcription

- GoScript™ Reverse Transcription System

Nucleic Acid Extraction / Purification

- ReliaPrep™ gDNA or Total RNA Purification Kits
  - Blood, tissue, cells, FFPE samples
- Maxwell® 16 Purification Kits – *for use with the Maxwell® 16 instrument*