Considerations for standardizing qPCR assays.

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Outline:

- Introduction
- Controls, references and standards in qPCR
- The importance of standard curves
- Confidence intervals and data comparison
- Uses for natural reference RNA
- Uses for synthetic reference RNA

Introduction

In order for a scientific result to be valid an independent person who is skilled in the art must be able to reproduce it.

A typical qRT-PCR protocol:

- Select cells or tissue of interest
- Extract total RNA
- Reverse transcribe RNA using random primers
- Determine RNA concentration, if possible
- Quantify each gene of interest relative to a standard curve using QPCR
- Express data normalized to input RNA and relative to a control sample

Control – Reference - Standard

Individual Reaction – Assay – Global Comparison

Variability in Determining Initial Target Concentration

Assuming that the same qRT-PCR reagent and platform used to amplify and detect the same RNA target on different days will give the same result might be wrong. There is an inherent variability in each of the necessary components, which are:

Instrumentation
Reagents
Template
Operator
Analysis

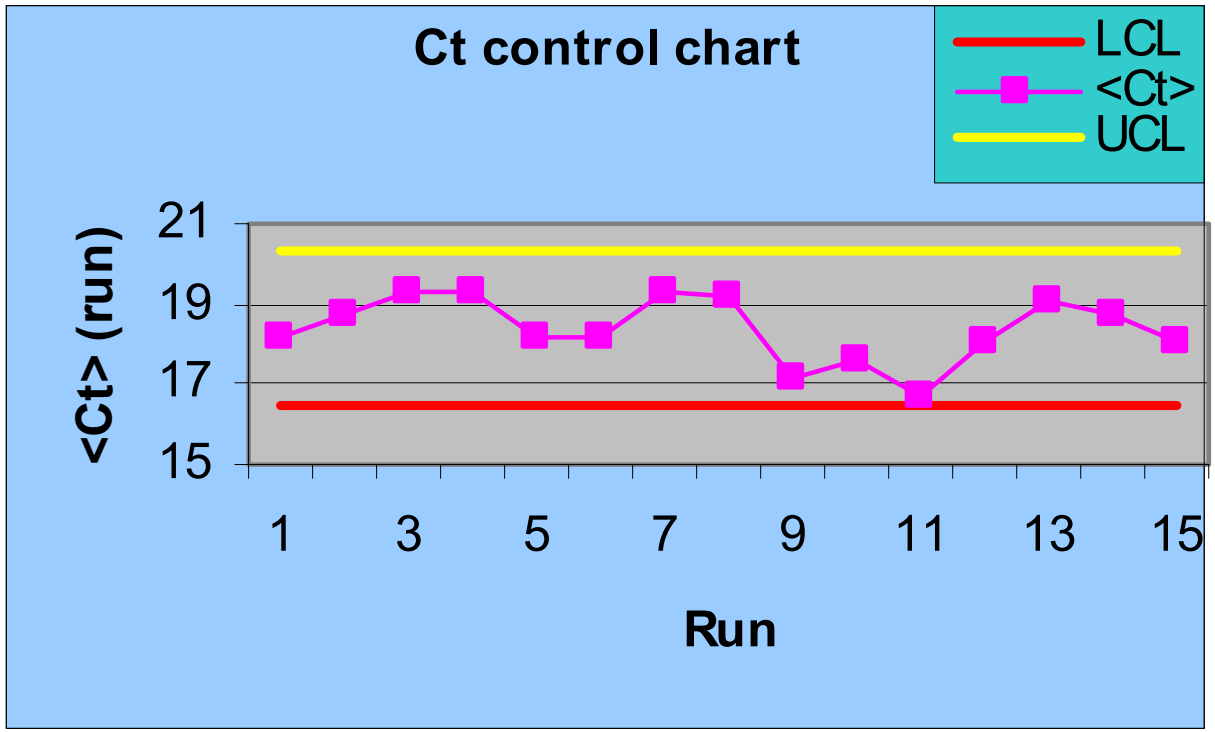
PCR Amplification

PCR: Correlation of amount of amplified DNA to amount of initial target DNA

$$Y = X(1 + E)^n$$

Y = PCR amplified quantity
X = target DNA quantity prior to PCR
E = amplification efficiency
n = number of cycles

Intra-Laboratory Variability



The lower confidence level (LCL) and the upper confidence level (UCL) were calculated for the measured Ct-The target is TBP and the template is 100 ng QPCR Reference Total RNA

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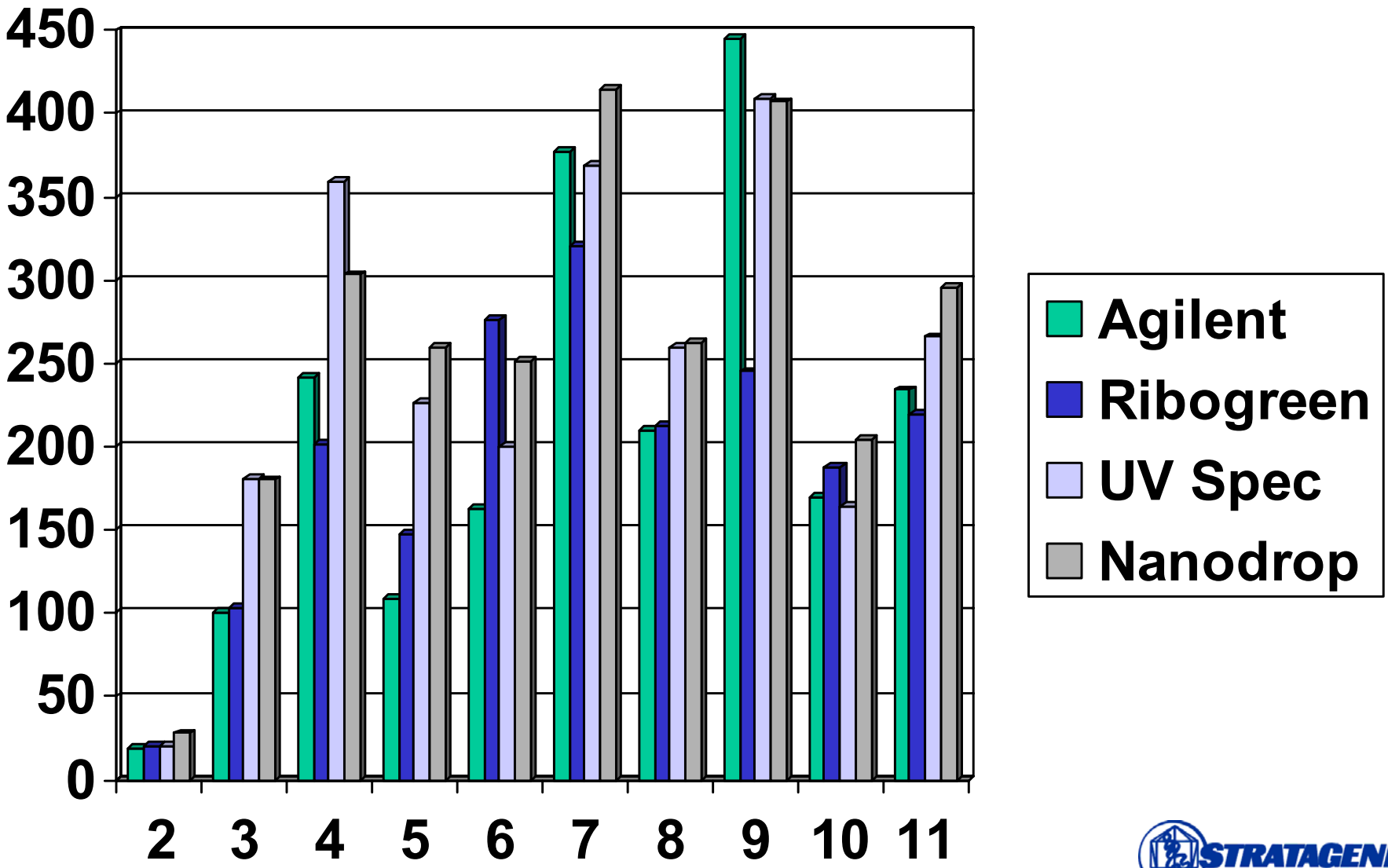
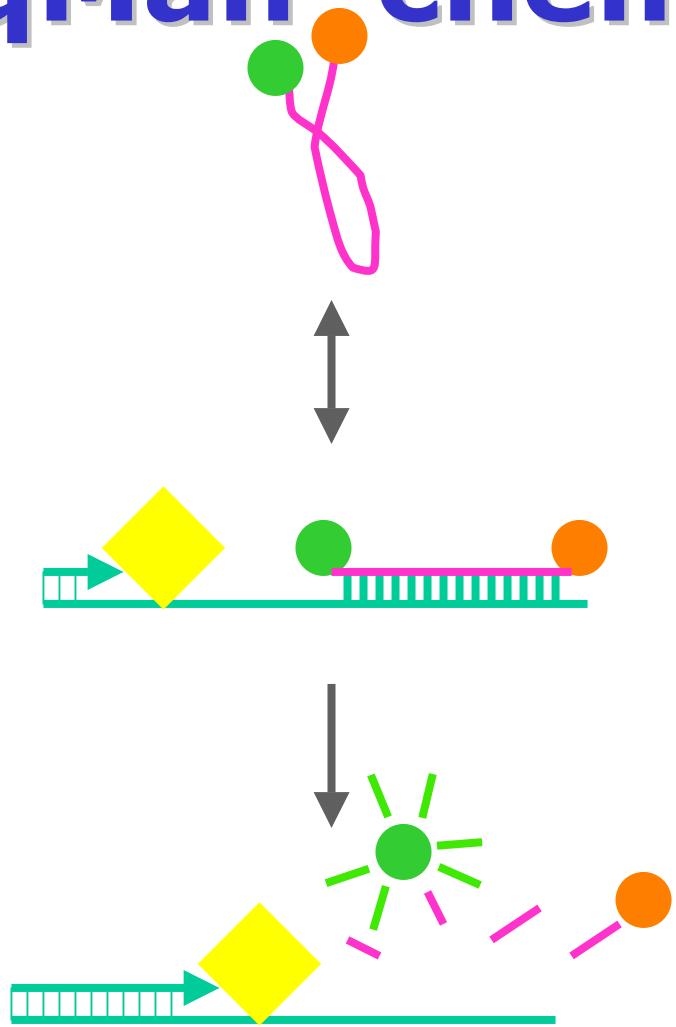
Determination of RNA Concentration

- A260/A280
- NanoDrop
- Agilent Bioanalyzer
- RiboGreen

RNA quantification



TaqMan® chemistry

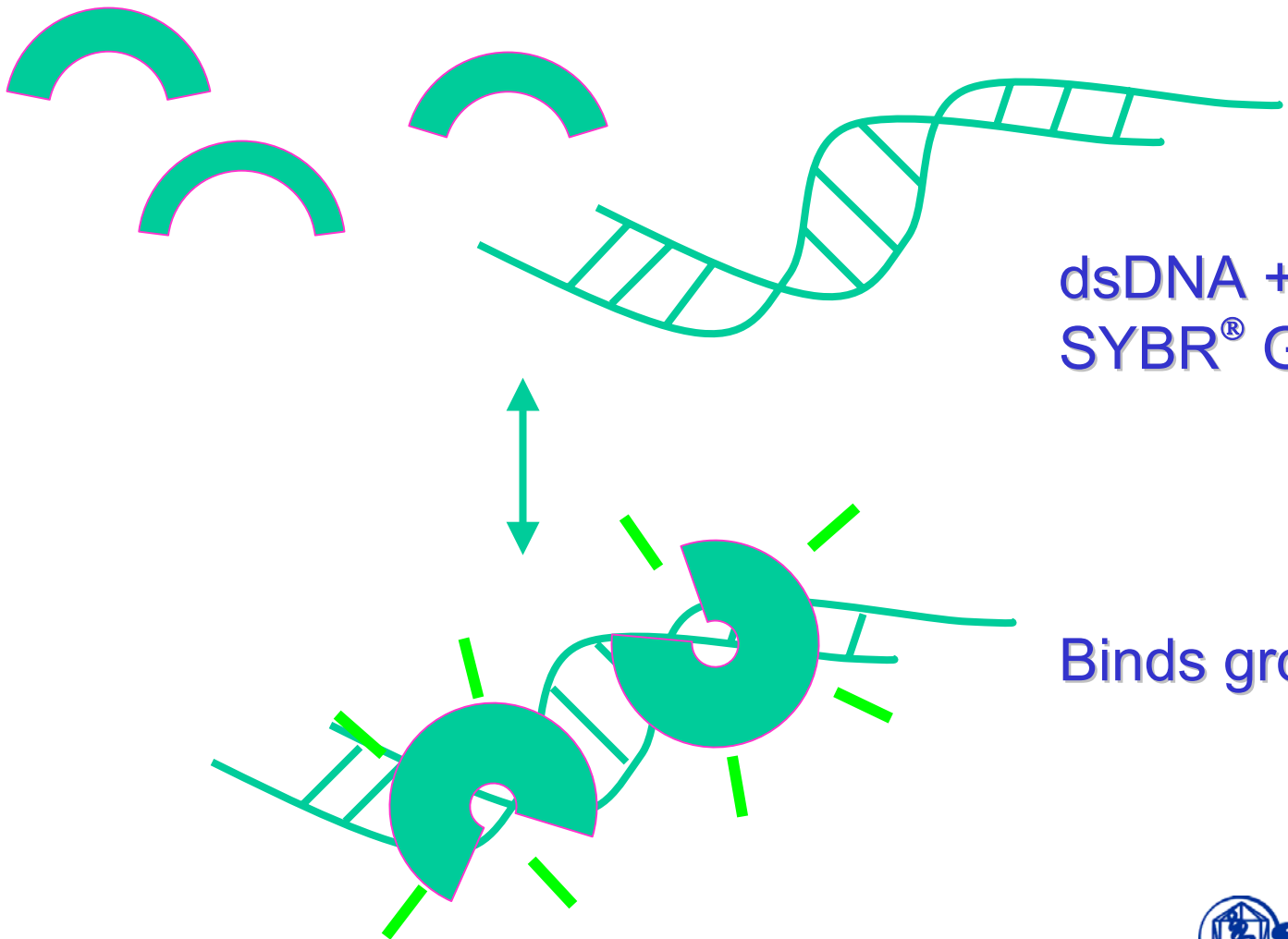


Probe

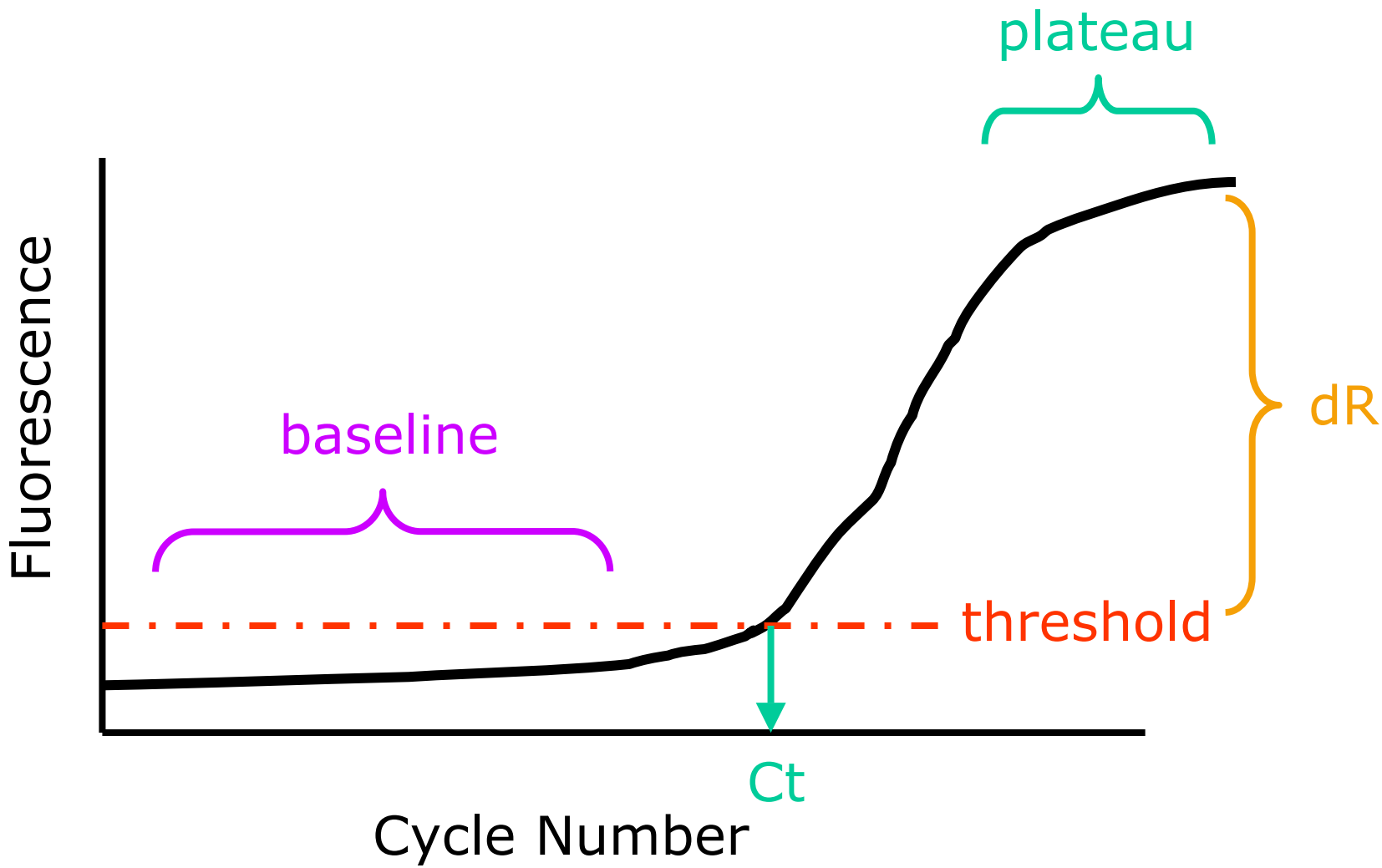
Target and enzyme

Hydrolysis

SYBR® Green I Chemistry

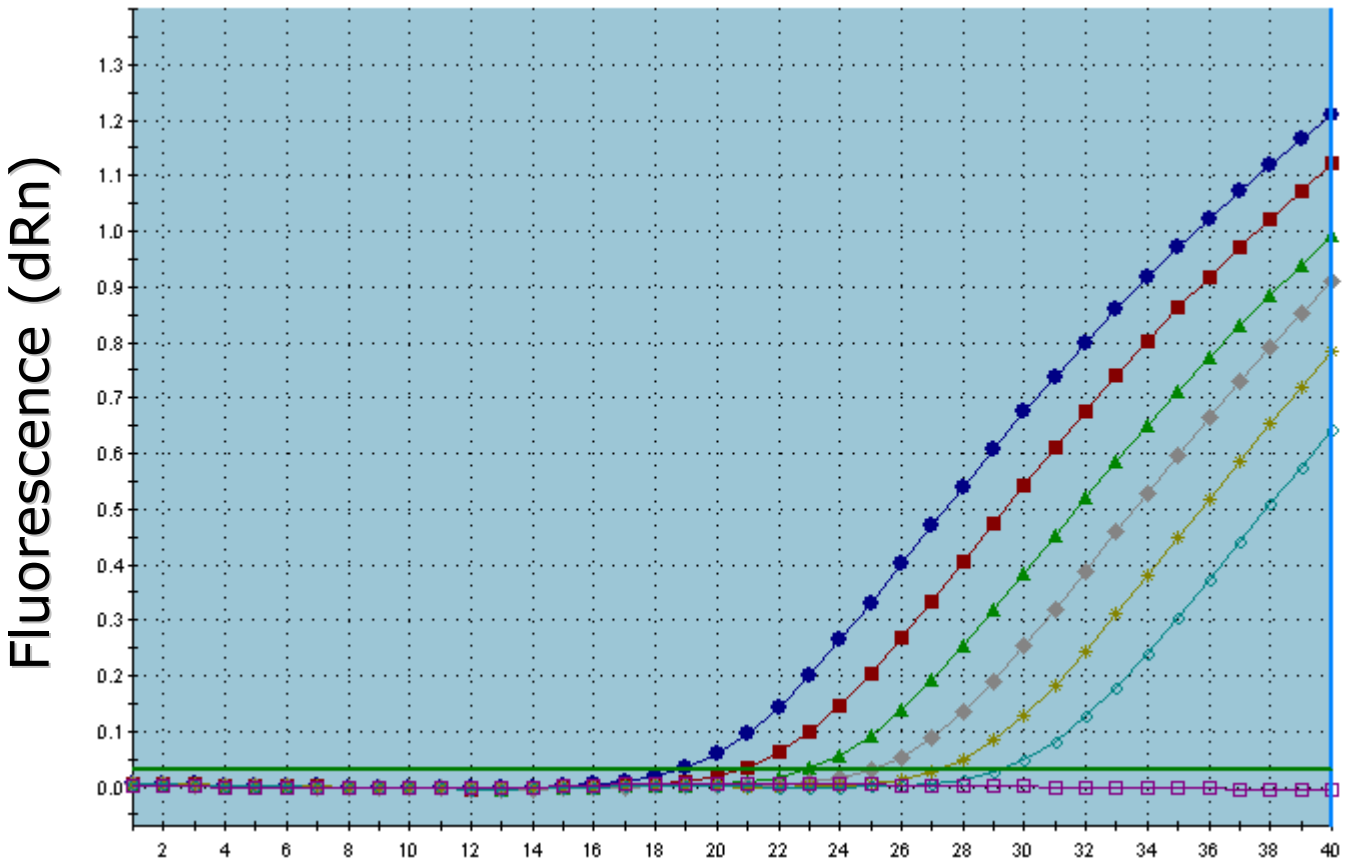


Amplification Plot Terms



Amplification Plot

GUS - 4x Dilution, starting at 1000 ng (linear/linear)



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Standard Curve Quantitation Versus Comparative Quantitation

Standard Curve Quantitation:

- Gene of interest (GOI)
- Normalizer
- Passive reference dye

Relative Comparison:

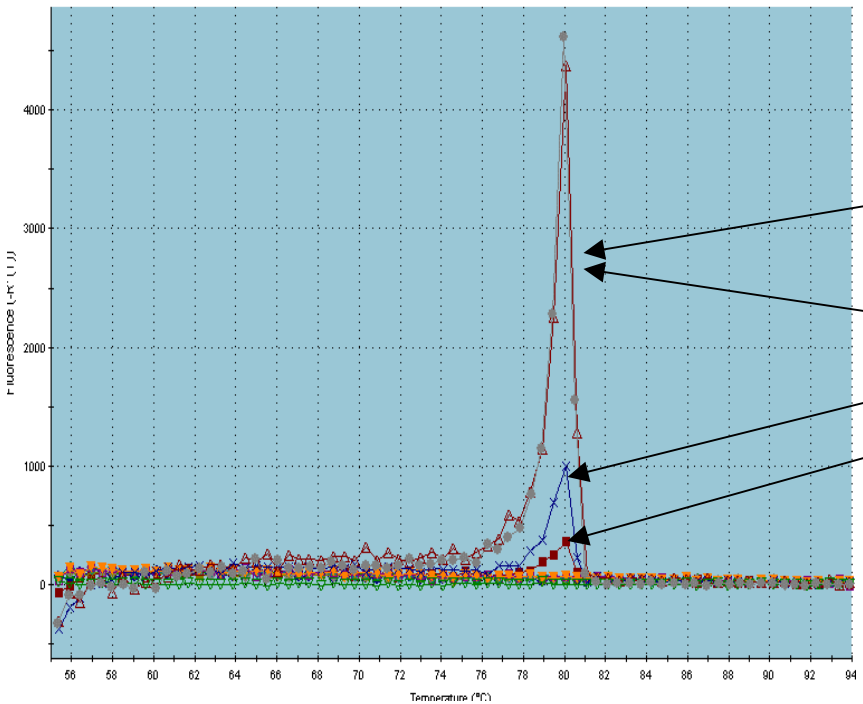
- Gene of interest
- Normalizer
- Calibrator
- Passive reference dye

Controls, references and standards in qRT-PCR

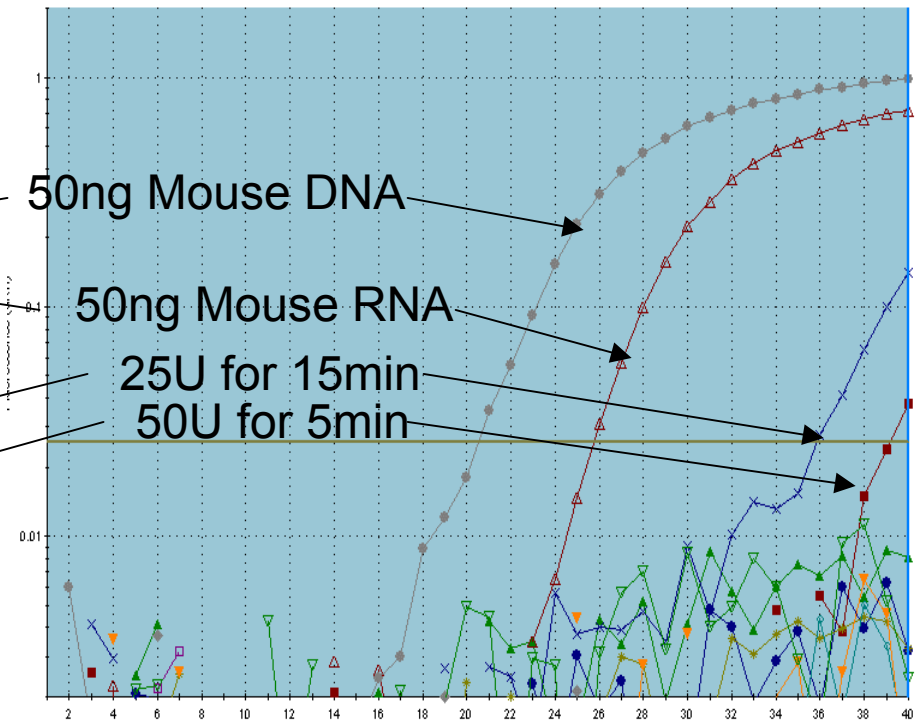
Controls for Quantification Using Comparative and Standard Curve Methods

- Reverse Transcription Controls
- PCR Controls
- Operator Controls
- Controls for Instrumentation

Testing of different DNase treated Mouse RNA for DNA contamination using TNFα Intron 1 primers.



SYBR Green I Dissociation Profile



SYBR Green I Amplification Plot

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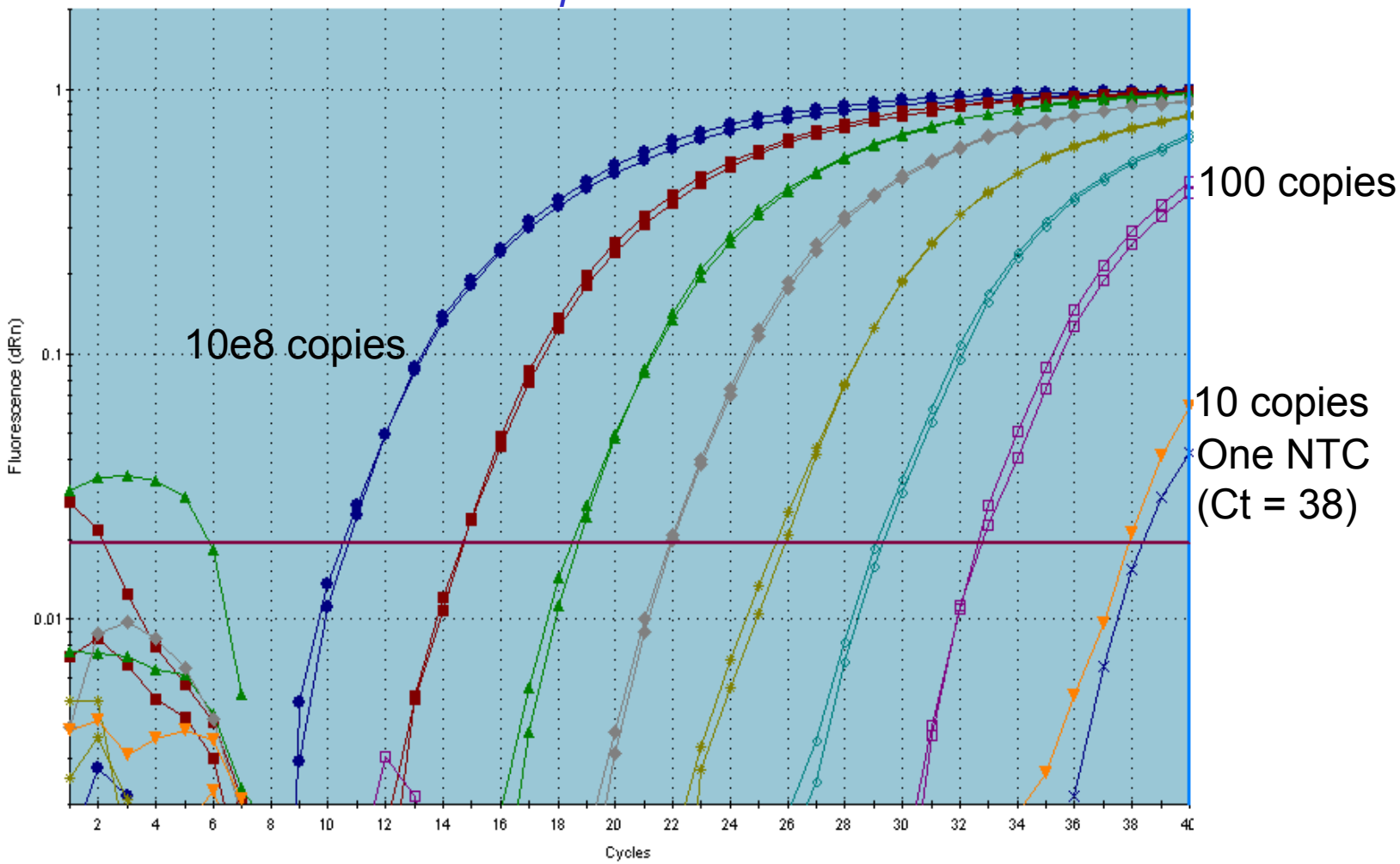
Standard curve: 100 - 10^8 copies

SYBR Green, Alien RNA Template; 50nM of each forward and reverse primer



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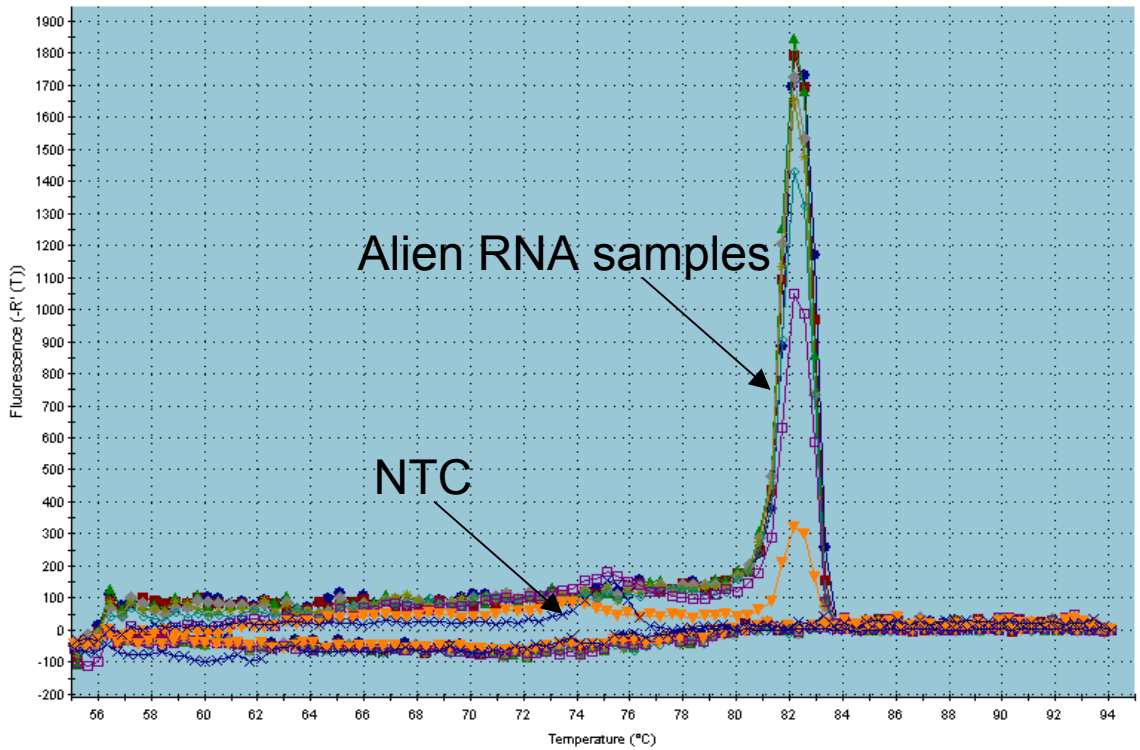
Dissociation Curve for Alien RNA Amplicon



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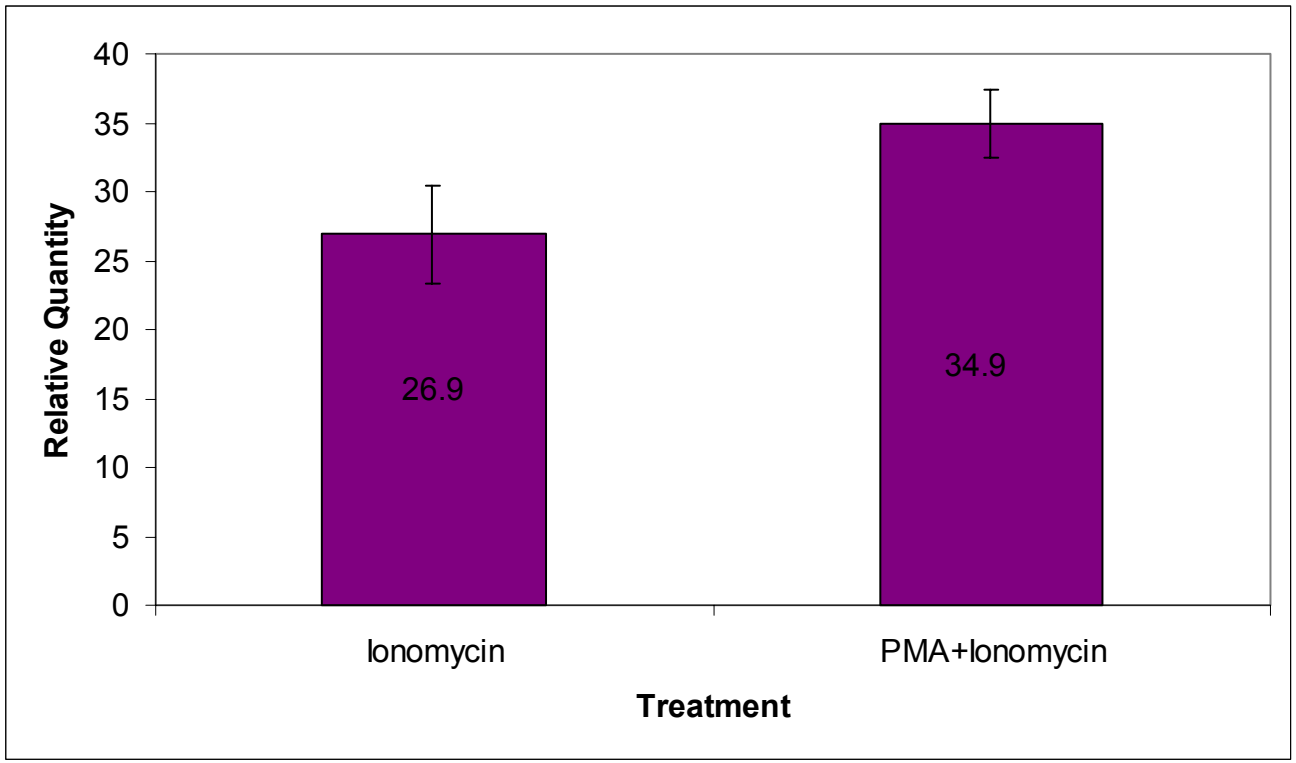
References for Quantification Using Comparative and Standard Curve Methods

- Endogenous Reference
- Exogenous Reference
- Calibrator

Relative Quantification of PMCA 1 mRNA



Target is PMCA 1; SYBR Green I detection;
Normalizer is β2-microglobulin;
Calibrator is total RNA from untreated Jurkat cells;
Experimental RNAs are from Jurkat cells treated with Ionomycin, or treated with Ionomycin/PMA.

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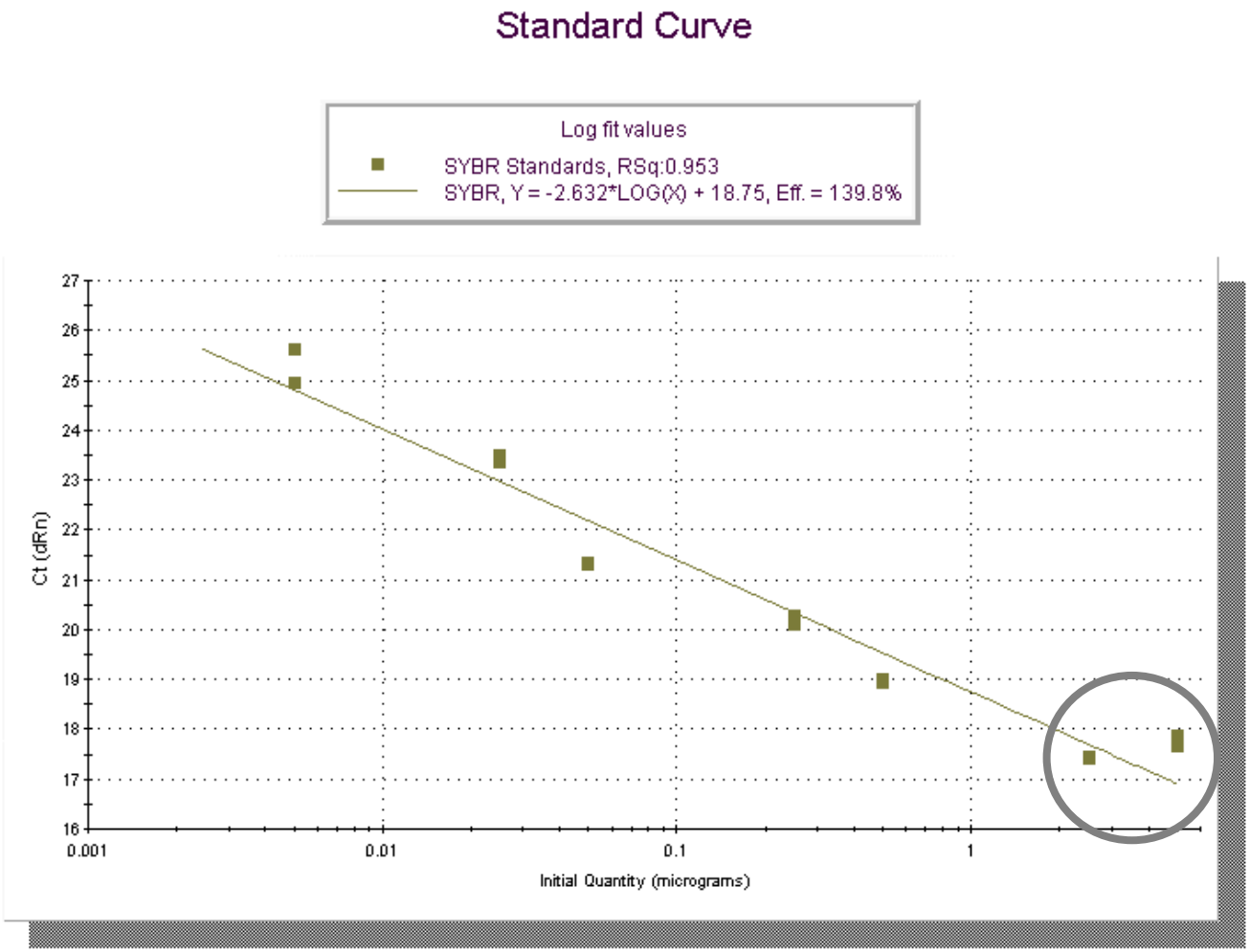
Standards for Quantification Using Comparative and Standard Curve Methods

- Standard Curves
- Natural Standards
- Un-natural Standards
- Standards for Instrumentation

Reverse transcribed RNA dilution series (β-actin)



Variability in qRT-PCR assays can be assessed with a constant RNA target

The Template in a Universal Reference (Or Standard) for QPCR can be...

- Total RNA
- Poly(A)$_n$-containing RNA
- Synthetic RNA
- cDNA

Quantitative PCR
Human Reference Total RNA

- Good gene representation
- Precise determination of RNA concentration
- Extensive quality control
- Production in large batches
- Convenient buffer

High, Medium and Low Abundant Targets Analyzed with TaqMan® Probes (PDARs)

β2-Microglobulin		
	Lot 1	Lot 2
100ng	19.3	19.1
10ng	22.2	22.0
1ng	25.9	25.3
0.1ng	29.2	29.0
NTC	no ct	no ct

TBP		
	Lot 1	Lot 2
100ng	23.7	23.4
10ng	26.7	26.5
1ng	30.2	29.5
0.1ng	34.5	34.5
NTC	no ct	no ct

GUS		
	Lot 1	Lot 2
100ng	24.6	24.3
10ng	26.9	26.5
1ng	30.5	29.8
0.1ng	34.6	33.4
NTC	no ct	no ct

IL-5		
	Lot 1	Lot 2
100ng	28.6	28.8
10ng	32.2	32.8
NTC	no ct	no ct

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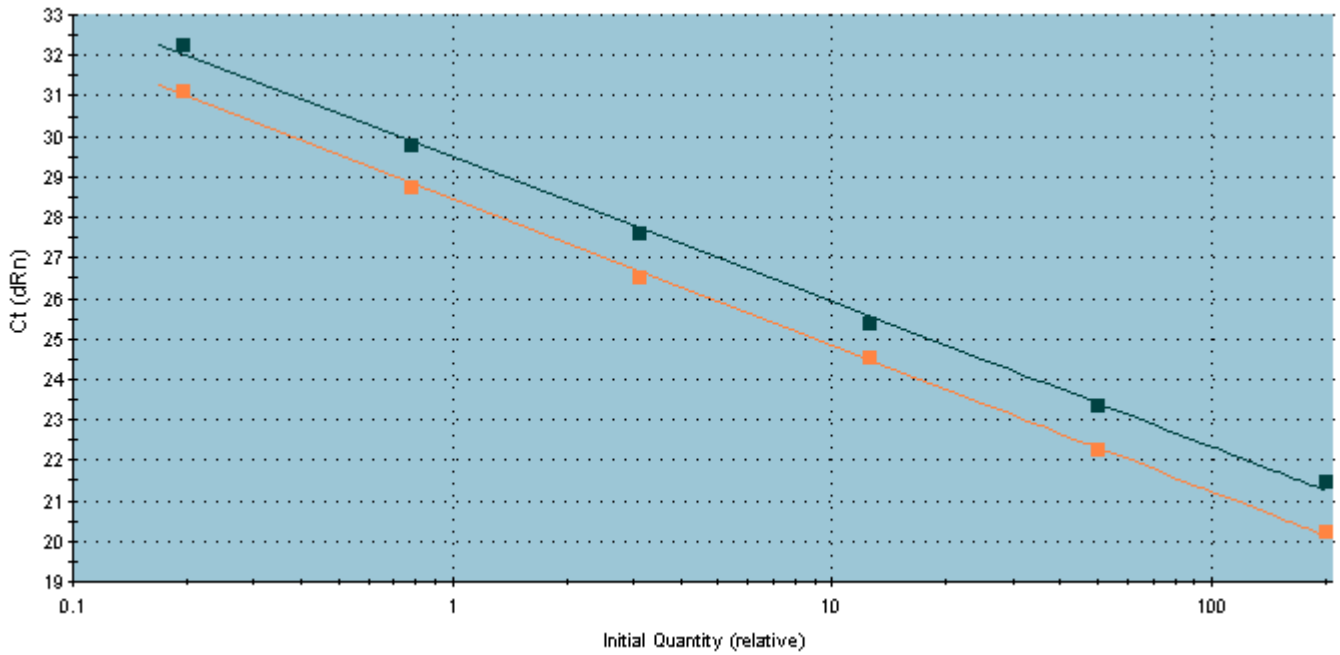
Assay Validation

Assay Validation



A SYBR Green I assay for PMCA 1 was validated using QPCR reference RNA. The reference gene was β2-microglobulin.

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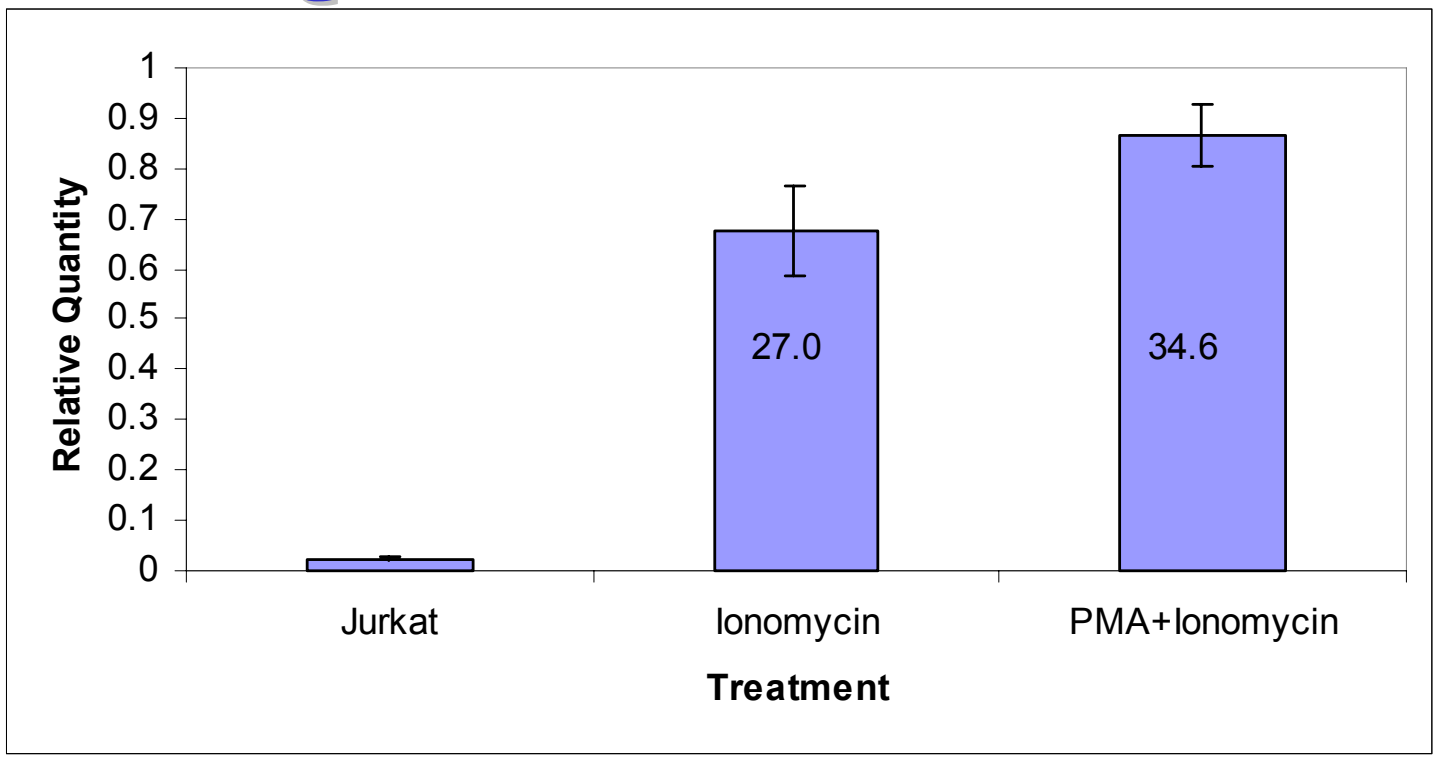
Assay Development

Relative Quantification with QPCR Reference RNA



Target is PMCA 1; SYBR Green I detection;
Normalizer is β2-microglobulin;
Calibrator is QPCR Reference Total RNA;
Total RNA from untreated Jurkat cells, treated with Ionomycin, or treated with Ionomycin/PMA.

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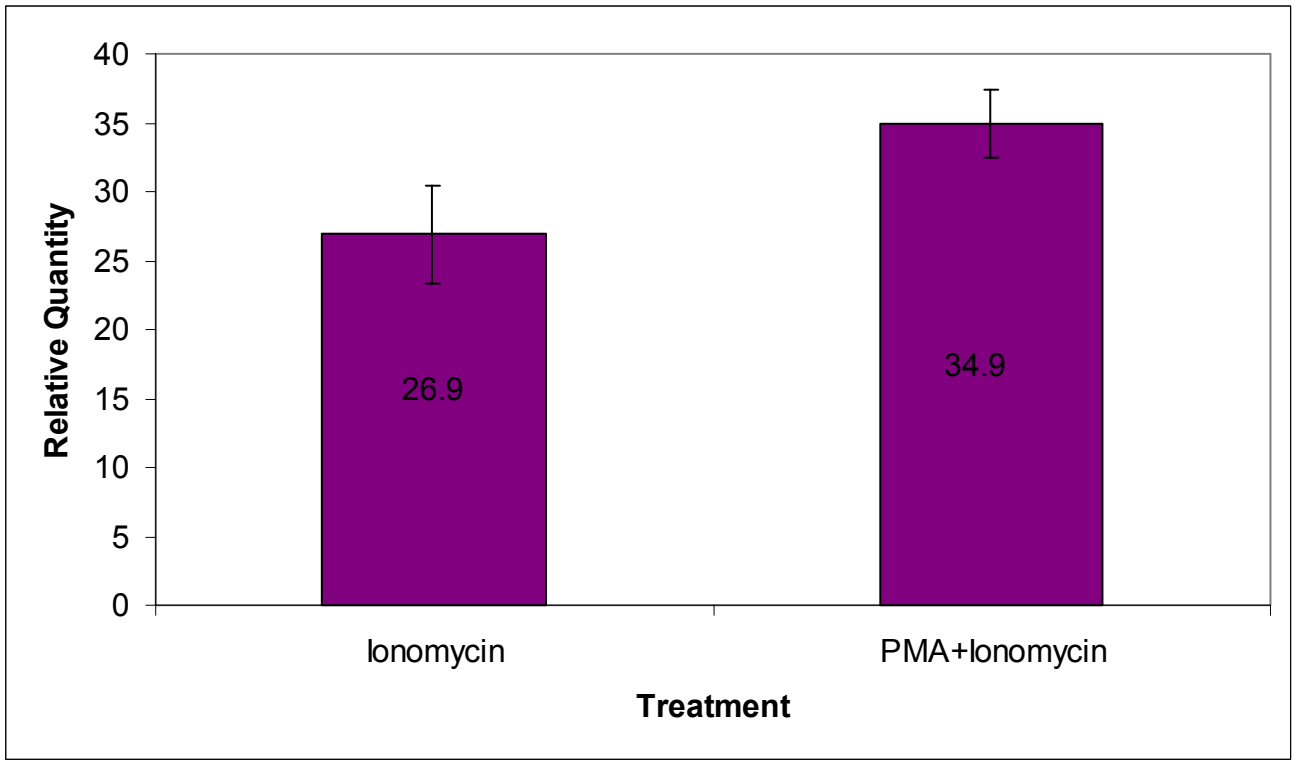
Relative Quantification of PMCA 1 mRNA



Target is PMCA 1; SYBR Green I detection;
Normalizer is β2-microglobulin;
Calibrator is total RNA from untreated Jurkat cells;
Experimental RNAs are from Jurkat cells treated with Ionomycin, or treated with Ionomycin/PMA.

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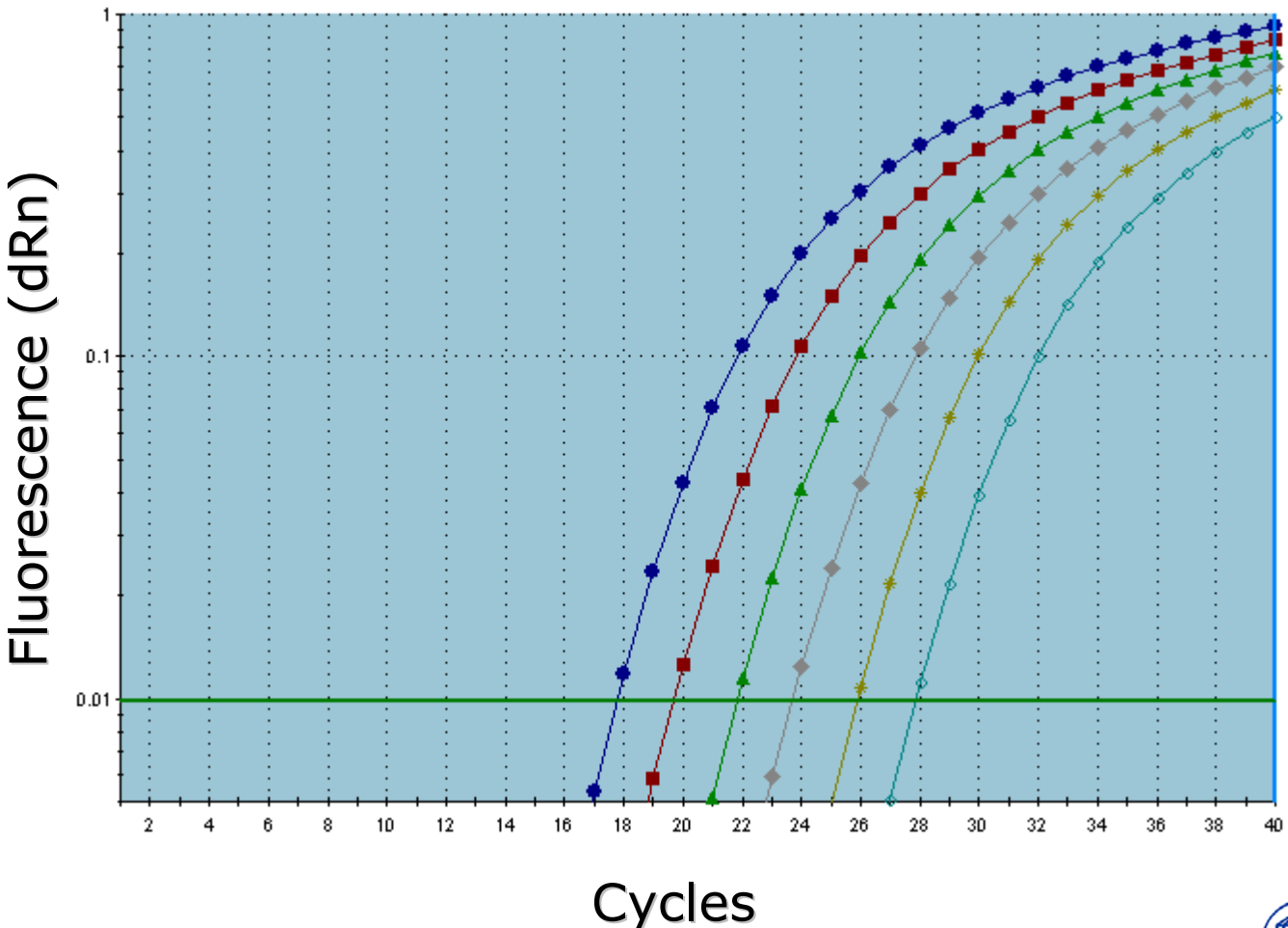
Assay Standardization

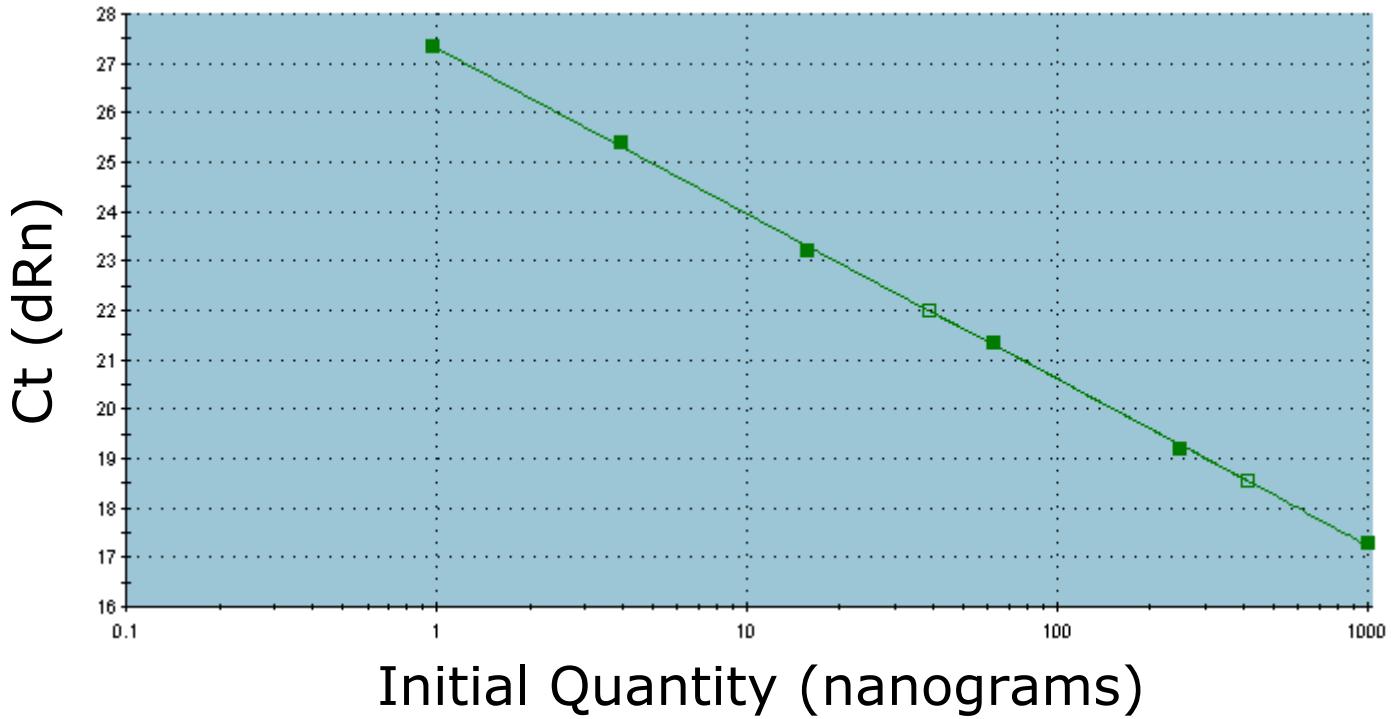
Amplification Plot

GUS - 4x Dilution, starting at 1000 ng (linear/log)



Standard Curve

GUS - 4x Dilution, starting with 1000 ng



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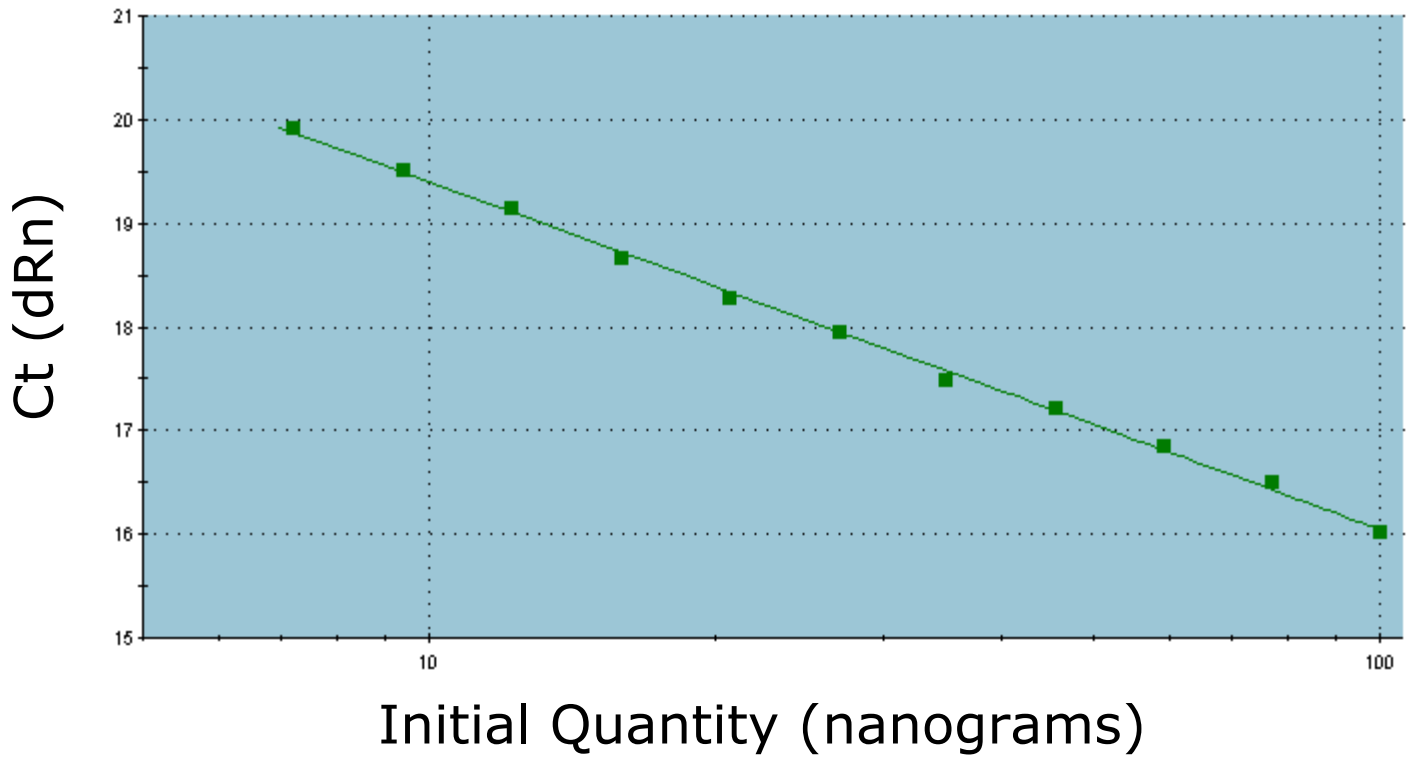
Standard Curve

Cyclophilin - 1.3x Dilution, starting with 100 ng



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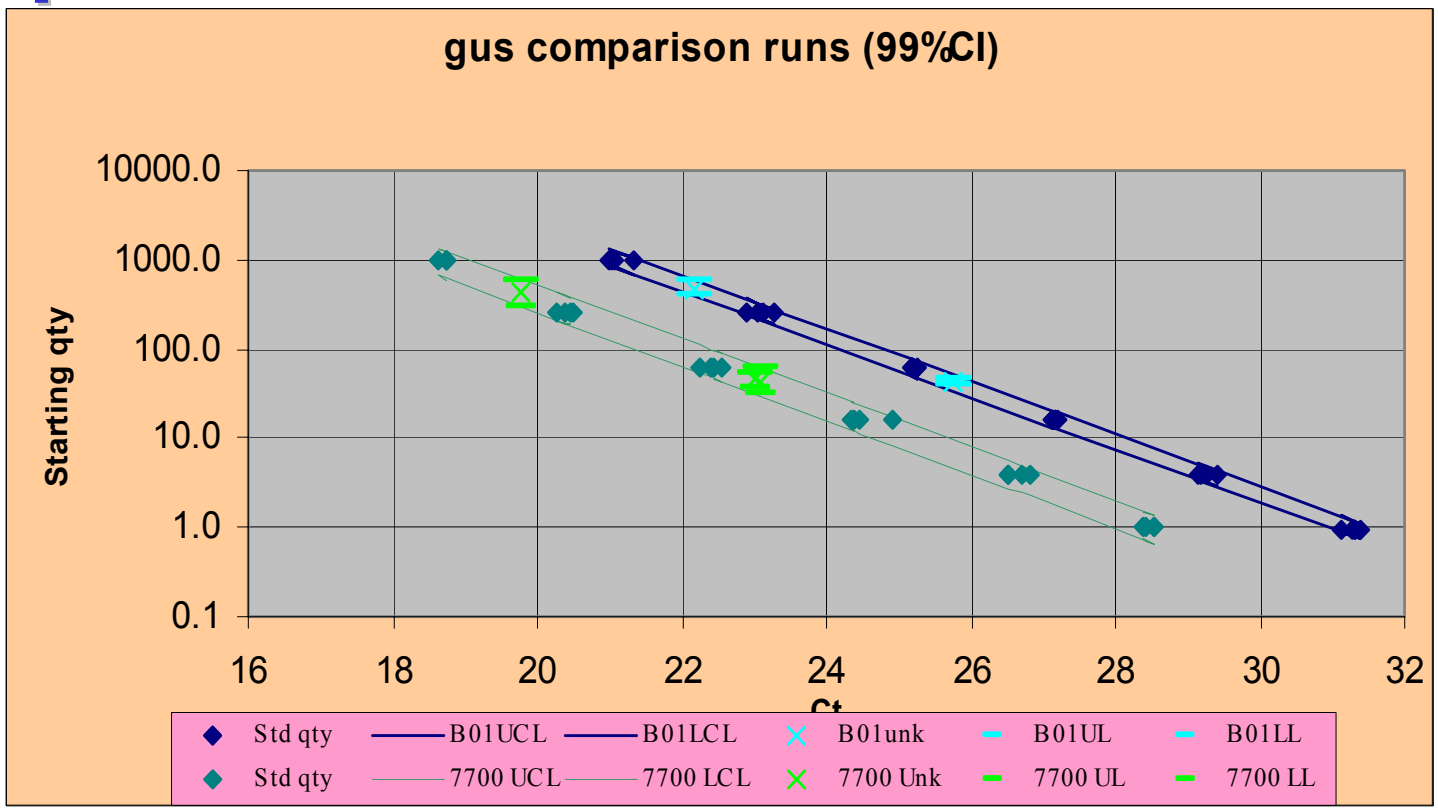
Describing a Standard Curve

1. Linear Range
2. Standard deviation of replicates and R^2-value
3. Confidence interval
4. Slope of the best-fit line
5. Y-axis intercept

Mathematical Model for Comparison of Two QRT-PCR Runs

- Decide on number of replicates.
- Generate a standard curve with QPCR Reference Total RNA.
- Calculate Confidence Intervals (CI).
- Compare quantities from runs on different days or on different platforms.
- Apply modified t-test.
- Decide if the two "Unknown" quantities were different.

Comparing Initial Target Concentration Acquired on Two Different Platforms



	7700			Mx4000 B01					
	Ct 1	n	St Qty	Ct 2	n	St Qty	ratio	1-(p)	Different?
unk1	19.78	1	426.80	22.17	1	476.95	1.12	54.0%	**Not sure**
unk2	23.07	1	43.16	25.73	1	42.37	1.02	10.2%	**Not sure**
unk1-2	19.61	1	480.44	25.70	1	43.24	11.11	100.0%	**YES!**

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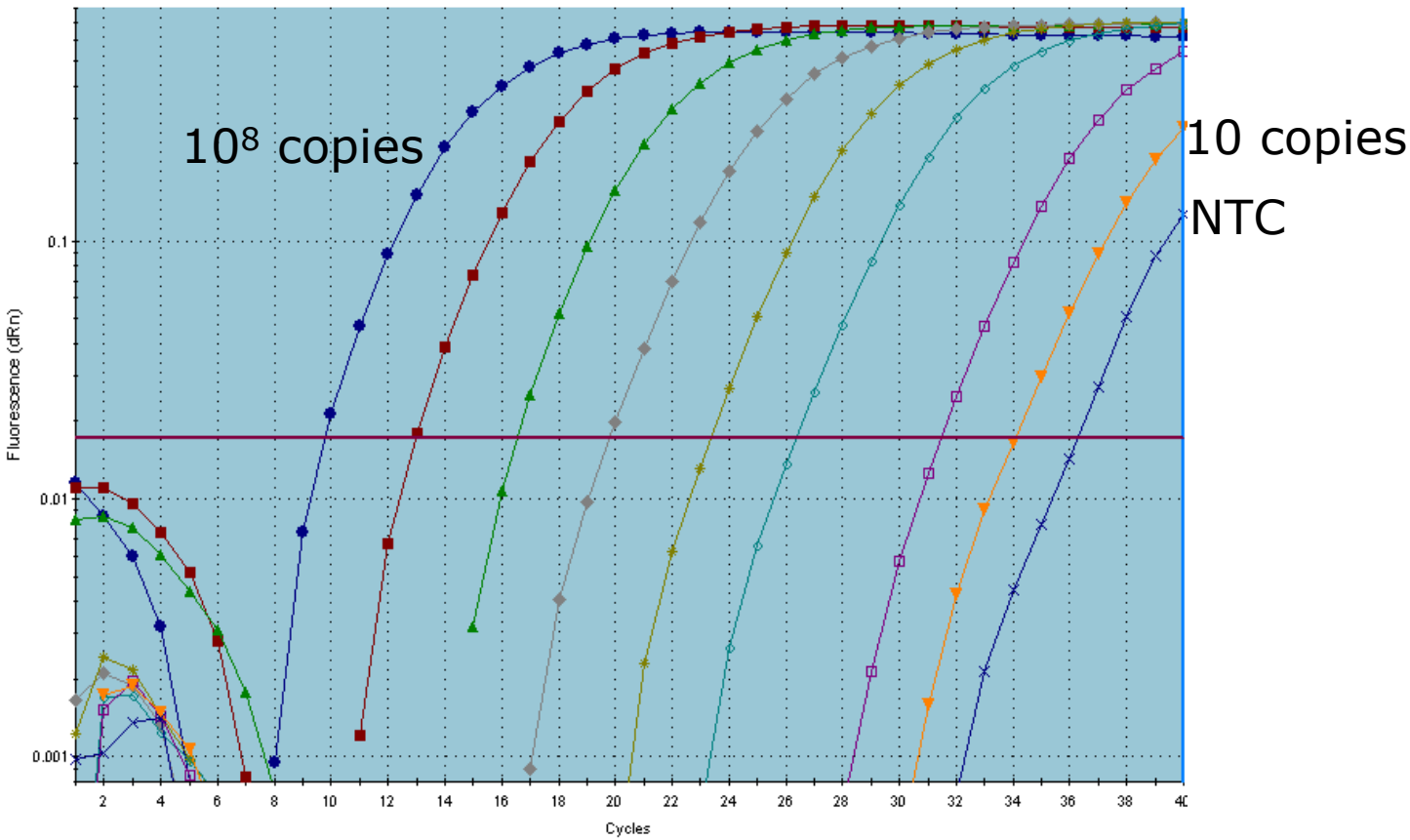
Synthetic reference RNA

Alien RNA standard curve (100-10^8 copies)

SYBR Green, 100nM of each forward and reverse primer



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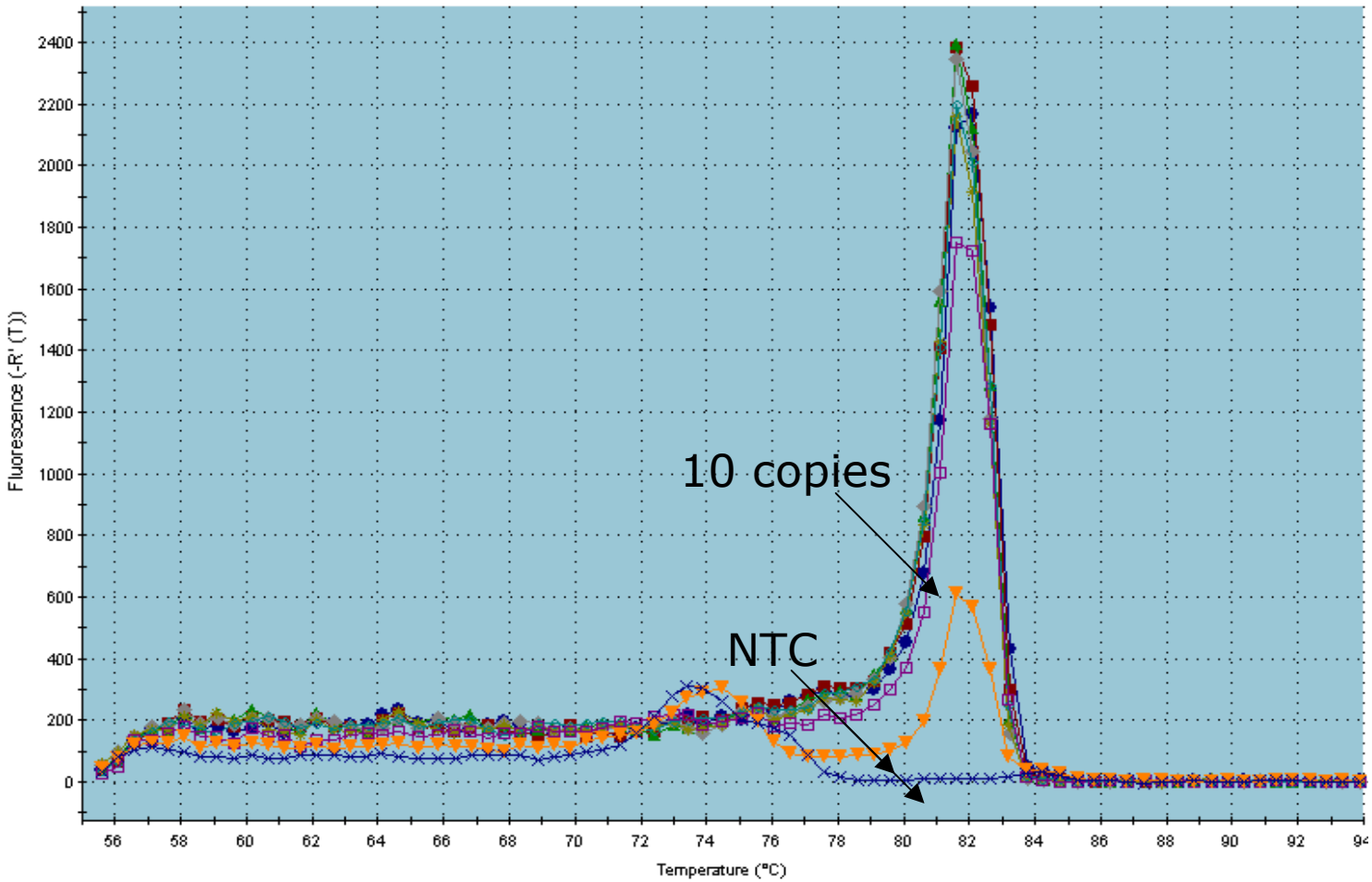
Dissociation Curve



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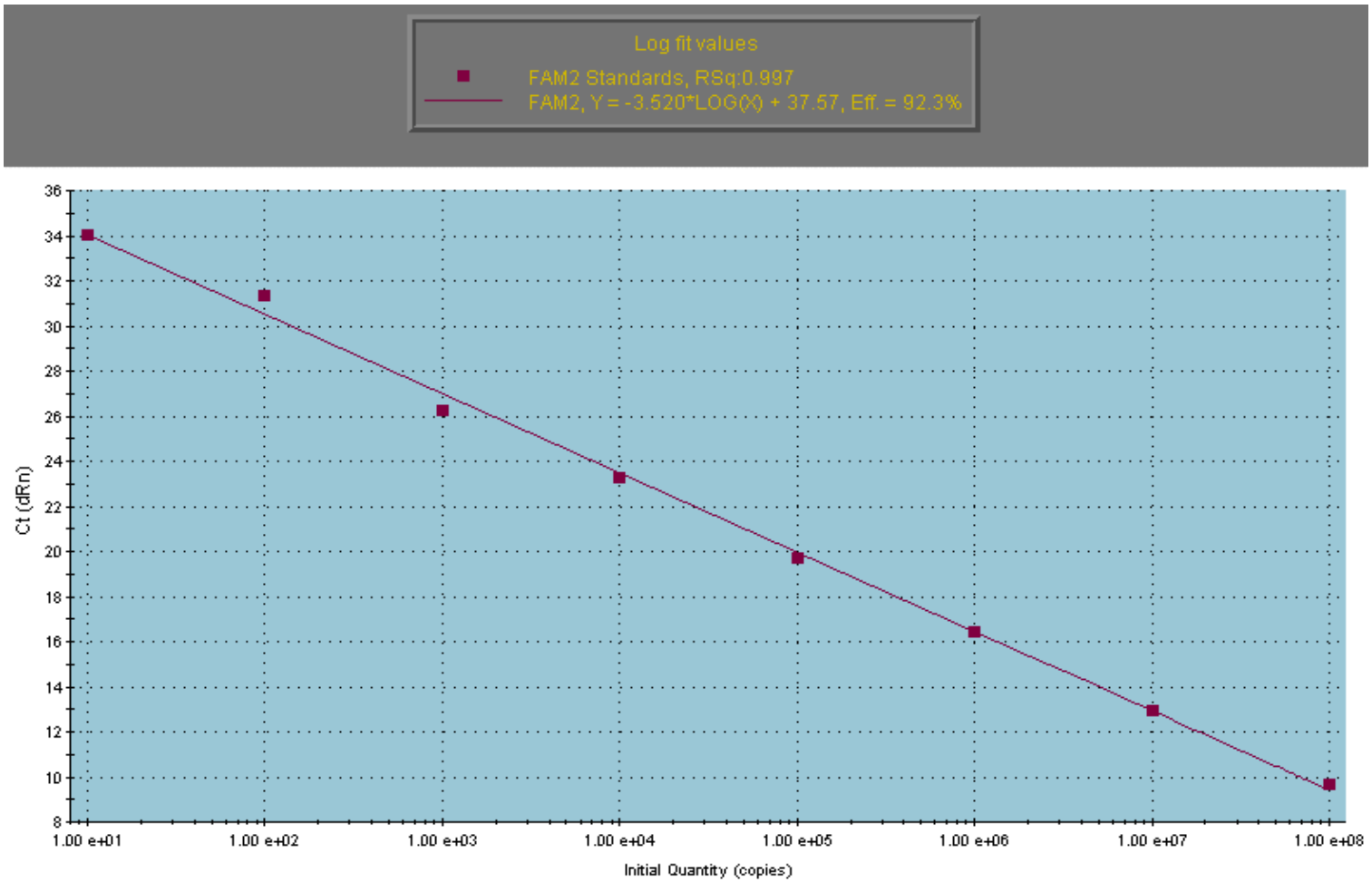
Standard Curve (10 - 10^8copies) of Alien RNA using 100nM of each primer



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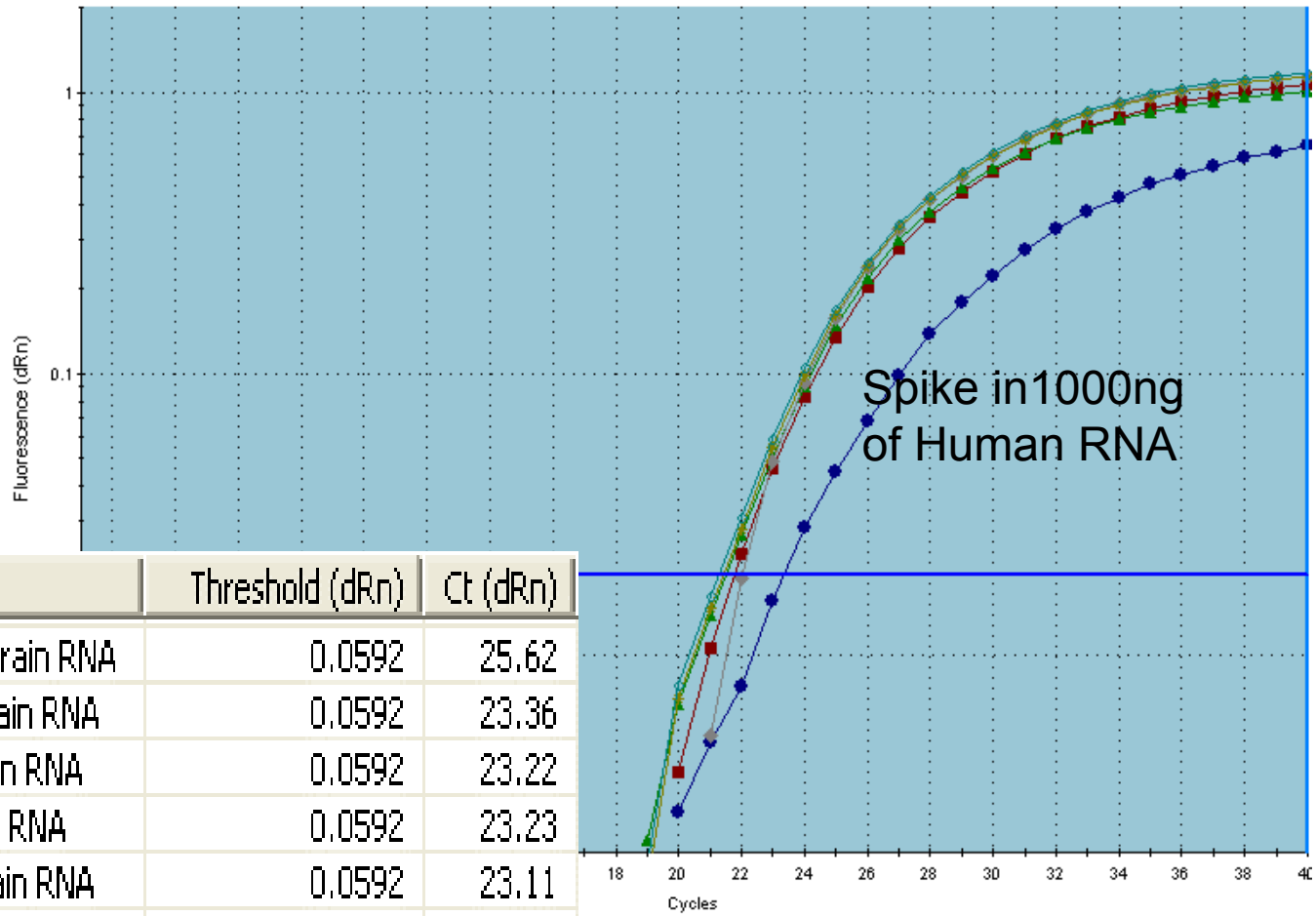
Spike in 0.1 – 1000ng Human Brain RNA into Alien RNA (10^5 copies)



Well Name	Threshold (dRn)	Ct (dRn)
1000 ng Human Brain RNA	0.0592	25.62
100 ng Human Brain RNA	0.0592	23.36
10 ng Human Brain RNA	0.0592	23.22
1 ng Human Brain RNA	0.0592	23.23
0.1 ng Human Brain RNA	0.0592	23.11
no Human Brain RNA	0.0592	23.01
NTC	0.0592	No Ct

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Summary

Acknowledgements

Gothami Padmabandu
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Tania Nolan